



# **Draft Assessment Report**

## **Evaluation of Active Substances**

Plant Protection Products

Prepared according to **Regulation (EC) 1107/2009**  
as it applies in Great Britain

### **Pydiflumetofen**

#### **Volume 3 – B.6 (AS) – part II**

#### **Toxicology & Metabolism Data**

Great Britain

June 2023

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## Version History

When	What
October 2022	Initial DAR
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SYN545974 was evaluated for potential to cause effects on reproductive and developmental toxicity in a multi-generation reproductive toxicity study in the rat and in pre-natal developmental toxicity studies in the rat and rabbit.

**B.6.6.1. Generational studies**

<b>Report:</b>	K-CA 5.6.1/01 [REDACTED] (2015). SYN545974: Oral (Dietary) Two-Generation Reproduction Toxicity Study in the Rat. [REDACTED] [REDACTED] Laboratory Report No. [REDACTED], 30 July 2015, Unpublished. Syngenta File No. SYN545974_10246.
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**Guidelines:** Two-generation reproduction study (rat) OECD 416 (2001); OPPTS 870.3800 (1998); 2004/73/EC B.35 (2004)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable. The analytical method for determination of pydiflumetofen in rodent diet has been assessed and validated (see DAR Vol. 3 B. 5.1.2.2).

**EXECUTIVE SUMMARY**

In a two-generation reproduction toxicity study, four groups of 24 male and 24 female rats of the [REDACTED]:WI(Han) strain were administered SYN545974 orally via the diet at fixed concentrations of 0, 150, 750 or 4500 ppm (males) or 0, 150, 450 or 1500 ppm (females) from 10 weeks before pairing and then until necropsy. Four groups of 24 males and 24 females were selected from the filial (F1) litters to form the F1 generation; formulated diets at the same concentrations were available to these animals from birth and then from weaning for approximately 10 weeks and then until necropsy. During the pairing periods, males received a lower dietary concentration as given to the pairing females.

All animals were examined for effects on general condition, body weight and food intake. The stage of the oestrous cycle was recorded for 21 days before the start of the pairing period for parental and F1 females, and during the pairing period vaginal smears were taken daily until sperm were found in the smear. The ano-genital distance was recorded for the F1 generation litters and the day of sexual development was recorded for all selected F1 animals.

The P and F1 parental males were subjected to a macroscopic necropsy once successful littering was completed. The testes and epididymides were removed and weighed and sperm evaluation was conducted. The P generation females were subjected to necropsy on Day 21 of lactation and the F1 generation females on Day 24 of lactation. A macroscopic necropsy was performed and the number of implantation scars was recorded. For both sexes, a selection of organs were weighed, fixed and microscopically examined for control and high dose animals. Unselected F1 pups and F2 pups were killed on Day 21 of age. A gross macroscopic necropsy was performed on all pups. For one male and one female pup per litter from the F1 and F2 generations, dead body weight was recorded and the brain, spleen and thymus were weighed and retained together with any gross lesions. Homogenisation resistant testicular spermatid counts were performed for the P and F1 parental males and ovarian follicle evaluation was performed for the F1 generation parental females.

There were no treatment-related deaths or clinical signs for either the P or F1 generation animals or their litters.

P and F1 generation males given 4500 ppm gained less weight than the controls during the initial weeks of their pre-pairing periods, with slight effects on food intake only seen during the F1 generation. There was no effect of treatment on female body weight gain at any concentration, in either generation.

There was no effect of treatment on oestrous cycling, fertility and mating performance or gestation length for either generation at any dietary concentration. All pregnant females gave birth to live litters with a similar number of pups born, and there was no effect of treatment on the postnatal survival of P or F1 generation litters to Day 21 of age.

Sexual maturation was slightly delayed for F1 generation males given 4500 ppm and females given 1500 ppm. Sexual maturation was slightly delayed for F1 generation males given 4500 ppm and females given 1500 ppm. The delay in sexual maturation in males was secondary to reduced body weight gain and not a direct effect of treatment with SYN545974. In female, the delay was not secondary to bodyweight effects. However, this can be considered questionable as there was no effect on related parameters such as oestrus cycling, mating performance or fertility and no effect on ano-genital distance of F1 generation pups. There was no effect of treatment on sperm parameters for males from either generation or on the quantification of the F1 generation ovarian follicles.

Body weight-related liver weights were increased for males given 750 and 4500 ppm in both generations, for the P generation females given 450 ppm and for the P and F1 generation females given 1500 ppm. Microscopic findings in the liver (diffuse hepatocyte hypertrophy) were only seen at 4500 ppm for P and F1 generation males and 1500 ppm for P generation females only. Microscopic findings in the thyroid (minimal follicular epithelial hypertrophy) were seen in males of both generations given 4500 ppm.

**In conclusion, when administered orally via the diet at concentrations up to 4500 ppm for males and 1500 ppm for females for two successive generations, SYN545974 was well tolerated.**

**There was a slight reduction in the body weight gain of males given 4500 ppm noted during the early pre-pairing period of both the P and F1 generations, which resulted in a slightly lower group mean body weight, compared with controls, for the remainder of the dosing period.**

**There were no adverse effects on reproductive performance, mating behaviour, conception or pup development. However, a slight but statistical significant delay in vaginal opening was observed in females given 1500 ppm. Microscopic changes were seen in the liver (diffuse hepatocyte hypertrophy) and thyroid (follicular epithelial hypertrophy) of high dose P and F1 generation males; the liver**

changes were also seen for high dose P generation females. The microscopic changes of the liver generally correlated with organ weight changes.

Based on these findings, the No-Observed-Adverse-Effect level (NOAEL) for parental toxicity was considered to be 750 ppm for P and F1 generation males (46.1 and 59.1 mg/kg/day, respectively) and 450 ppm for females in the P (36.1 mg/kg/day (pre-pairing)) and F1 generations (42.4 mg/kg/day (pre-pairing)).

The No-Observed-Adverse-Effect level (NOAEL) for reproductive toxicity was considered to be in excess of 4500 ppm for P and F1 generation males (276.6 and 363.8 mg/kg/day, respectively) and 1500 ppm for female in the P (116.2 mg/kg/day (pre-pairing)) and F1 generations (140.6 mg/kg/day (pre-pairing))

The No-Observed-Adverse-Effect level (NOAEL) for offspring toxicity was considered to be in excess of 4500 ppm for P and F1 generation males (276.6 and 363.8 mg/kg/day, respectively) and 450 ppm for female in the P (36.1 mg/kg/day (pre-pairing)) and F1 generations (42.4 mg/kg/day (pre-pairing))

## MATERIALS AND METHODS

### Materials:

Test Material:	SYN545974
Description:	White to off-white powder
Lot/Batch number:	SMU2EP12007
Purity:	98.5 % w/w
CAS#:	1228284-64-7
Stability of test compound:	30 June 2016

**Vehicle and/or positive control:** The test substance was administered in VRF1 diet, manufactured by [REDACTED], supplied by [REDACTED]

Test Animals:	
Species	Rat
Strain	[REDACTED]:WI(Han)
Age/weight at dosing	Approximately 6 weeks/ 185 g to 248 g (males) and 111 g to 160 g (females)
Source	[REDACTED]
Housing	P and selected F1 generation animals were housed in groups of four, by sex, until pairing and for males post-pairing. For pairing, one male and one female were housed together in grid floor cages suspended over paper-lined trays. One mated females were housed individually/with their litter, in solid-floor cages with appropriate bedding material
Acclimatisation period	11 days
Diet	<i>ad libitum</i>
Water	<i>ad libitum</i> (mains tap water)
Environmental conditions	Temperature: 19 °C to 21 °C Humidity: 40 % to 68 % Photoperiod: Alternating 12 hour light and dark cycles.

### Study Design and Methods:

**In-life dates:** Start: 08 May 2014 (animal arrival)  
End: 23 January 2015 (last necropsy)

**Mating procedure:** - One male was caged with one female as follows;

Male Group	Female Group
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On confirmation of mating via vaginal smears, the males were returned to the group cages and each pregnant female was housed individually in a cage with a solid floor and bedding where it was kept throughout gestation and lactation.

**Animal assignment:** Parental animals were randomly allocated to groups using a stratified body weight randomisation procedure based on individual body weights recorded on arrival.

	Dietary Concentration of SYN545974 (ppm)							
	Males				Females			
Generation	0	150	750	4500	0	150	450	1500
P	1-24	25-48	49-72	73-96	97-120	121-144	145-168	169-192
F1	201-224	225-248	249-272	273-296	297-320	321-344	345-368	369-392

The dose levels were selected following evaluation of subchronic studies with SYN545974 with [REDACTED]WI (Han) rats conducted previously.

**Diet/dosage preparation and analysis:** The formulated diets were prepared using powdered VRF1 diet, manufactured by [REDACTED], supplied by [REDACTED]

The diet was dispensed into labelled plastic containers and stored at room temperature.

During the study, samples were taken from each preparation. Samples taken from the first preparation and those taken approximately every two months thereafter were analysed for SYN545974, using a validated



method (BFI012LC), to confirm homogeneity and achieved concentrations. Basal diets given to control animals were analysed to confirm absence of test item.

**Concentration analysis results:** The mean concentrations for all batches of diet preparations analysed were typically within 10 % of the nominal concentration.

**Homogeneity results:** The homogeneity of SYN545974 in diet at concentrations of 150 ppm to 4500 ppm, for a batch size of up to 35 kg, was determined and considered satisfactory, percentage deviations from the overall mean were within 5 %.

### Observations:

**Parental animals:** Body weights, food consumption and clinical signs were recorded at regular intervals from the first day of dosing. For 21 days before the start of the pairing period vaginal smears were taken to determine the stage of the oestrous cycle. During the pairing period, vaginal smears were also taken until mating was confirmed by sperm being found in the smear.

All adult animals were individually assessed as follows:

Observation	Frequency
Mortality and morbidity:	Twice daily, individual record
Clinical signs:	Daily, individual record
Body weight:	Males - weekly using an electronic balance Females - Weekly until day of mating, Days 0, 7, 14 and 20 of gestation, Days 1, 4, 7, 14 and 21 of lactation and on the day of necropsy using an electronic balance
Food consumption:	Males - Weekly (except during cohabitation for mating) based on the food weighed in and the food weighed out; an electronic balance was used. Females - Weekly during the pre-pairing period (not recorded during cohabitation for mating), over Days 0 to 4, 4 to 7, 7 to 10, 10 to 14, 14 to 17 and 17 to 20 of gestation and over Days 1 to 4, 4 to 7, 7 to 10, 10 to 14, 14 to 17 and 17 to 21 of lactation based on the food weighed in and the food weighed out; an electronic balance was used.
Determination of the oestrous cycle stage:	Daily for 21 days pre-pairing and throughout cohabitation until evidence of positive mating and on the necropsy day

**Litter observations:** Body weights, food consumption and clinical signs were recorded at regular intervals from Day 1 of age.

Each pup (F<sub>1</sub> and F<sub>2</sub>) was individually assessed as follows:

Observation	Frequency (during lactation period)
Mortality:	Twice daily, individual record
Sex:	Determined by external examination on Day 1 of age
Clinical signs:	Recorded at least once daily to assess the presence of malformations and signs of toxicity
Body weight:	Measured individually on days 1, 4, 7, 14, and 21 of age using an electronic balance

On day 4 of age, litters were standardized on a total randomisation basis to a maximum of 8 pups/litter (4/sex/litter, as nearly as possible). Litters fewer than 8 were not altered. Culled pups were killed and discarded without necropsy.

With the exception of pups culled on Day 4 of lactation, a necropsy was conducted on all pups killed or found dead during lactation and all unselected pups on Day 21 of age. The pups were killed by an intraperitoneal injection of sodium pentobarbitone solution (for those up to the age of 14 days) or by exposure to carbon dioxide gas in a rising concentration (for older pups).

### Investigations post mortem:

**Parental animals:** All surviving parental males were killed following completion of the mating period once successful littering was completed. Parental generation females were killed on Day 21 of lactation at weaning of their litters and F1 generation females were killed on Day 24 of lactation. The remaining females, including non-mated, those apparently non-pregnant and those where all the litter had died before Day 21 of age, were killed on the same day of lactation as other animals from the same generation. These animals were subjected to examination post mortem as follows.

A dead body weight was recorded and the thoracic and abdominal cavities were opened by a ventral mid-line incision and the major organs and uterus were examined. The number of implantation scars/sites for each female was recorded and the uterus of any apparently non-pregnant female was assessed visually under magnification to confirm pregnancy status. Organs or tissues showing any macroscopic abnormalities were recorded and retained. The following organs were weighed after trimming of fat and other contiguous tissue (contralateral organs were weighed together):

adrenals	pituitary
brain	prostate & seminal vesicles (incl. coagulating gland)
epididymides	spleen
kidneys	testes <sup>1</sup>
liver	thyroids (including parathyroids) <sup>2</sup>
ovaries	uterus (incl. uterine cervix and oviducts)

<sup>1</sup> weighed separately

<sup>2</sup> weighed after fixation

From all animals surviving to scheduled termination, the following organs were retained in fixative:

adrenal glands	prostate & seminal vesicles (incl. coagulating gland)
brain (7 levels)	spleen
epididymides	testes
kidneys	thyroids (incl. parathyroids)
liver	uterus (incl. uterine cervix and oviducts)
ovaries	vagina
pituitary	all gross lesions

**Microscopic examination:** The epididymides, liver, ovaries, prostate and seminal vesicles, testes, thyroids, uterus and vagina were processed and examined by light microscopy.

**Offspring:** The F1 generation pups not selected as parental animals and F2 generation pups (with exception to those culled on Day 4 of lactation) were killed on Day 21 of age. These animals were subjected to examination *post-mortem*.

A dead body weight was recorded for pups where organ weights were to be determined and for one male and one female pup per litter, the brain, thymus and spleen were weighed and fixed. Organs or tissues showing any macroscopic abnormalities were removed and preserved for all animals.

#### Data analyses:

**Statistics:** Data were processed to give group mean values and standard deviations, where appropriate. Where the data allowed, the following methods were used for statistical analysis comparing Groups 2, 4 and 6 against Group 1 (females) and Groups 3, 5 and 7 against Group 1 (males).

**General Approach:** All statistical tests were two-sided with minimum significance levels of 5 % and 1 %. Non-parametric statistics were not routinely conducted. When used, Dunnett's test was conducted regardless of the outcome of the analysis of variance (ANOVA) or analysis of covariance (ANCOVA).

Data were examined for unusually high or low values which could influence the statistical analysis and interpretation (possible outliers).

*For Quantitative Data:* Body weight, food intake, cumulative body weight gain from the start of dosing (throughout gestation and lactation), pup body weights, cumulative pup body weight gain, litter size, gestation length, total litter weight, mean cycle length and mean number of cycles per female during the pre-mating vaginal smears, the day of balano-preputial separation and vaginal opening, sperm velocities (VSL and VAP), total sperm number in the right testis, the number of sperm per g right testis and the number of primordial follicles were analysed using ANOVA.

Parental and pup organ weights were analysed using ANOVA for the absolute weights and by analysis of covariance (ANCOVA) using terminal kill body weight as covariate. Group summaries (mean, standard deviation and number of observations) and individual values were presented for organ weights as a percentage of body weight, but these were not evaluated for statistical significance.

*For Percentages:* Pup survival, pup sex ratios, litter based mean percentages, sperm motility and sperm abnormalities were analysed using a parametric ANOVA, following a double arcsine transformation (Freeman and Tukey, 1950).

*Outliers:* Exclusion of outlier values were considered where this was deemed appropriate. If a particular value was excluded from statistical analysis of a group because of known mitigating circumstances (e.g. missing value, instrument malfunction), the reason for exclusion was clearly stated in the final report data tables. For outliers that were biologically plausible, statistical results were presented with and without influential values if they affected the interpretation of the parameter. In these rare cases, the results were presented including all values and a secondary table provided after excluding the outlier. The interpretation discussed the influence of the outlier.

*Dunnett's test:* For all of the parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test was used to compare the control and treated groups, based on the error mean square in the ANOVA or ANCOVA. The Dunnett's test was performed for all continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant, and statistical flags were presented in the tables of results in the final report.

### Indices:

*Reproductive indices:* The following reproductive indices were calculated from breeding and parturition records of animals in the study.

Female copulation index (%)	=	$\frac{\text{no. of females mated}}{\text{no. of females paired}}$	x 100
Male copulation index (%)	=	$\frac{\text{no. of males mated}}{\text{no. of males paired}}$	x 100
Female fertility index (%)	=	$\frac{\text{no. of pregnant females}}{\text{no. of females paired}}$	x 100
Male fertility index (%)	=	$\frac{\text{no. of males siring a pregnancy}}{\text{no. of males paired}}$	x 100
Gestation index (%)	=	$\frac{\text{no. of pregnant females with live pups born}}{\text{no. of pregnant females}}$	x 100
Post-Implantation Loss (%)	=	$\frac{(\text{no. of implantation scars} - \text{no. pups born})}{\text{no. of implantation scars}}$	x 100

*Offspring viability indices:* The following viability indices were calculated from lactation records of litters in the study:

Live birth index (%)	=	$\frac{\text{no. pups born alive}}{\text{total no. pups born}}$	x 100
Viability index 1 (%)	=	$\frac{\text{no. pups alive on Day 4 of age before culling}}{\text{no. pups born alive}}$	x 100
Viability index 2 (%)	=	$\frac{\text{no. pups alive on Day 7 of age}}{\text{no. pups alive on Day 4 of age after culling}}$	x 100
Viability index 3 (%)	=	$\frac{\text{no. pups alive on Day 14 of age}}{\text{no. pups alive on Day 7 of age}}$	x 100
Viability index 4 (%)	=	$\frac{\text{no. pups alive on Day 21 of age}}{\text{no. pups alive on Day 14 of age}}$	x 100
Lactation index (%)	=	$\frac{\text{no. pups alive on Day 21 of age}}{\text{no. pups on Day 4 of age after culling}}$	x 100
Cumulative survival index (%) =			
$\frac{\text{no. pups alive Day 21}}{\text{no. pups alive Day 4 after culling}}$	x	$\frac{\text{no. pups alive Day 4 before culling}}{\text{no. pups born}}$	x 100

## RESULTS

### Parental animals:

#### Mortality and clinical signs:

**P:** There were no deaths and no treatment-related clinical signs.

On Day 19 of the dosing period, one female given 450 ppm showed slow and laboured breathing, decreased activity and unsteady gait. As these findings were observed on a single occasion in one animal only, they were considered not to be related to treatment.

**F1:** There were no deaths or clinical signs that were considered to be related to treatment.

One female given 1500 ppm and one control male were found dead on Days 7 and 98, respectively; no clinical signs had previously been recorded for these animals.

One control female was euthanased on Day 43 of the pre-pairing period following swelling and loss of use of the front limbs. Macroscopic and microscopic findings confirmed a recent fracture of the left forelimb of this female.

#### Body weight and food consumption:

**P:** There was a statistically significant reduction in cumulative body weight gain in males at 4500 ppm, up to Week 5 of dosing ( $p < 0.05$  to  $p < 0.01$ ), which resulted in a slightly lower group mean body weight, compared with controls, for the remainder of the dosing period. There was no effect of SYN545974 administration on the body weight or body weight gain of males at any other dose level.

There were no effects of SYN545974 administration on body weight or body weight gain of the females during the pre-pairing period, gestation or lactation.

**F1:** At the start of the dosing period, the group mean body weights of males given 4500 ppm were statistically significantly lower than controls ( $p<0.01$ ). Cumulative body weight gain was statistically significantly lower thereafter ( $p<0.01$ ) and resulted in a reduction in overall cumulative body weight gain ( $p<0.01$ ).

At 1500 ppm, female group mean body weight was significantly ( $p<0.05$  to  $p<0.01$ ) lower than controls at the start of the dosing period; however body weight gains thereafter (pre-pairing, gestation and lactation periods) were similar to controls with no statistical significance achieved.

There was no effect of treatment on body weight or body weight gain at 150 ppm or 450/750 ppm SYN545974.

Reported body weight results are summarised in Table 6.6.1-1.

**Table 6.6.1-1: Intergroup comparison of group mean body weight gains (g)**

		Dietary Concentration of SYN545974 (ppm)							
		Males				Females			
Generation	Days	0	150	750	4500	0	150	450	1500
P	0 to 17 weeks	214.0	223.2	215.0	<b>192.7*</b>	-	-	-	-
	0 to 10 weeks (pre-pairing)	-	-	-	-	96.2	87.4*	91.8	91.3
	0 to 20 (gestation)	-	-	-	-	107.3	112.1	106.5	103.2
	1 to 21 (lactation)	-	-	-	-	31.1	30.6	32.2	36.3
F1	0 to 17 weeks	364.3	367.2	354.5	<b>328.6**</b>	-	-	-	-
	0 to 10 weeks (pre-pairing)	-	-	-	-	157.7	159.8	161.8	157.4
	0 to 20 (gestation)	-	-	-	-	101.4	99.7	105.5	99.0
	1 to 21 (lactation)	-	-	-	-	33.3	30.7	33.9	36.6

\* Statistically significant difference from control group mean,  $p<0.05$  (Dunnett's test 2-Sided)

\*\* Statistically significant difference from control group mean,  $p<0.01$  (Dunnett's test 2-Sided)

**P:** There was no effect of SYN5475974 administration on the food intake of males or females during the pre-pairing and lactation periods. For females given 1500 ppm, mean food intake was slightly lower than controls, achieving statistical significance over Days 0 to 4 ( $p<0.01$ ) and Days 4 to 10 of gestation ( $p<0.05$ ).

**F1:** Food intake was slightly, but statistically significantly ( $p<0.05$  to  $p<0.01$ ), lower than controls for males given 4500 ppm for the majority of the dosing period and for males given 750 ppm from Week 9 onwards ( $p<0.05$ ). There was no effect on the food intake of females during the pre-pairing, gestation or lactation periods.

Selected food consumption results are summarised in Table 6.6.1-2.

**Table 6.6.1-2: Intergroup comparison of group mean food consumption (g/animal/day)**

		Dietary Concentration of SYN545974 (ppm)							
Generation	Days	Males				Females			
		0	150	750	4500	0	150	450	1500
P	0 to 17 weeks	21.33	21.20	21.16	20.53	-	-	-	-
	0 to 10 weeks (pre-pairing)	-	-	-	-	14.70	14.44	14.60	14.15
	0 to 20 (gestation)	-	-	-	-	21.01	21.65	20.98	19.41
	1 to 21 (lactation)	-	-	-	-	55.08	56.39	53.99	53.22
F1	0 to 17 weeks	21.27	21.28	20.55	19.53**	-	-	-	-
	0 to 10 weeks (pre-pairing)	-	-	-	-	15.02	14.95	15.02	14.79
	0 to 20 (gestation)	-	-	-	-	25.0	25.9	22.5	23.8
	1 to 21 (lactation)	-	-	-	-	47.5	48.8	47.9	49.5

\*\* Statistically significant difference from control group mean,  $p < 0.01$  (Dunnett's test 2-Sided)

**Dose received:** Dose levels (based on nominal dietary levels of SYN545974) were calculated in terms of mg SYN545974/kg body weight/day. Mean values are shown below:

**Table 6.6.1-3: P Generation mean dose received (mg/kg/day)**

Dose Level				
ppm	(mg/kg/day)			
	Males	Females		
		Pre-pairing	Gestation	Lactation
	Mean	Mean	Mean	Mean
150	9.1	11.9	12.6	30.0
450		36.1	36.8	87.0
750	46.1			
1500		116.2	115.1	290.8
4500	276.6			

**Table 6.6.1-4: F1 Generation mean dose received (mg/kg/day)**

Dose Level				
ppm	(mg/kg/day)			
	Males	Females		
		Pre-pairing	Gestation	Lactation
	Mean	Mean	Mean	Mean
150	11.9	14.1	15.1	26.3
450		42.4	39.5	77.8
750	59.1			
1500		140.6	141.4	267.3
4500	363.8			

**Reproductive function:**

**Oestrous cycle length and periodicity:**

**P:** Females given 1500 ppm SYN545974 had very slightly longer and therefore, fewer oestrous cycles during the 21 days before the pairing period, compared with the controls, with the former achieving statistical significance ( $p < 0.05$ ). However, cycling parameters for all females were within the normal range (see Table 6.6.1-5 and 6.6.1-6); In addition, this effect was driven by 2 females which presents longer mean length cycles (5 and 4.5 days) and was not reproduced in the F1 generation. Therefore, any differences are biologically insignificant and unrelated to treatment.

**Table 6.6.1-5: P Generation Oestrous cycle length and periodicity**

Observation		Dose Group (ppm)			
		0	150	450	1500
No. of oestrous cycles (over 21 days)	Mean	4.8	4.7	4.6	4.5
	SD	0.4	0.5	0.5	0.5
	N	24	24	24	24
Oestrous cycle length (days)	Mean	3.93	3.95	3.95	4.05*
	SD	0.10	0.13	0.11	0.24
	N	24	24	24	24

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett's test 2-Sided)

**Table 6.6.1-6: Historical Control Data (2008 to 2014) - P Generation Oestrous cycle length and periodicity**

Observation		Study					
		1	2	3	4	5	6
		Jan. 2008	Mar. 2009	Jan. 2009	Jan. 2014	Feb. 2014	Feb. 2014
No. of oestrous cycles (over 21 days)	Mean	5.0	4.8	4.4	4.7	4.3	4.5
	Range	5 - 5	3 - 5	4 - 5	4 - 5	3 - 5	3 - 5
	N	30	30	25	24	23	24
Oestrous cycle length (days)	Mean	-	-	4.0	4.0	4.3	4.1
	Range	-	-	4 - 4	3.8 - 4.5	4.0 - 6.0	4.0 - 6.3
	N	30	30	25	24	23	24

**F1:** There was no effect of treatment on the mean number of oestrous cycles or mean cycle length.

**Table 6.6.1-7: F1 Generation Oestrous cycle length and periodicity**

Observation		Dose Group (ppm)			
		0	150	450	1500
No. of oestrous cycles (over 21 days)	Mean	4.6	4.6	4.7	4.5
	SD	0.7	0.5	0.5	0.6
	N	23	24	24	23
Oestrous cycle length (days)	Mean	4.13	4.14	3.98	4.15
	SD	0.5	0.38	0.15	0.39
	N	23	24	24	23

#### Sperm measures:

**P:** There was no effect of treatment on sperm parameters at any dietary concentration of SYN545974, when compared with controls.

**F1:** There was no effect of treatment on sperm parameters at any dietary concentration, when compared with controls.

#### Reproductive performance:

**P:** All females mated within four days. There was no effect of treatment on pre-coital interval or the copulation index at any dose level. There were no effects of treatment on the fertility of males or females.

There was no effect of treatment on gestation length with all animals littering within the concurrent control range of Days 21.5 to 23 of gestation. All pregnant females gave birth to live litters. There was no treatment related effect on post-natal survival of pups with 24, 23, 24 and 23 females in the groups given 0, 150, 450 and 1500 ppm respectively, rearing their pups to Day 21 of age.

**Table 6.6.1-8: P Generation reproductive performance**

Males				
Observation	Dose Group (ppm)			
	0	150	750	4500
Males placed with females	24	24	24	24
Mated	24	24	24	24
Male mating index	100 %	100 %	100 %	100 %
Males with females pregnant	24	24	24	23
Male fertility index	100.0 %	100.0 %	100.0 %	95.8 %
Females				
	Dose Group (ppm)			
	0	150	450	1500
Mean pre-coital interval (days)	2.3	2.5	2.6	2.4
Females placed with males	24	24	24	24
Number inseminated	24	24	24	24
Female mating index	100 %	100 %	100 %	100 %
Pregnant	24	24	24	23
Female fertility index	100 %	100 %	100 %	95.8 %
Mean gestation interval (days)	22.29	22.13	22.08	22.04
Number of litters	24	24	24	23
Gestation index	100 %	100 %	100 %	100 %
Live birth index	100 %	100 %	100 %	100 %

**F1:** There was no effect of treatment on pre-coital interval or on the copulation index at any dose level. There were no effects of treatment on mean gestation length and all animals littered between Day 21.0 and 23.5 of gestation. All females that were pregnant gave birth to live litters. There was no effect of SYN545974 on post-natal survival of the pups with 17, 18, 21 and 16 females in the groups given 0, 150, 450 and 1500 ppm respectively, rearing their pups to Day 21 of age.

**Table 6.6.1-9: F1 Generation reproductive performance**

Males				
Observation	Dose Group (ppm)			
	0	150	750	4500
Males placed with females	23	24	24	23
Mated	22	23	24	22
Male mating index	95.7 %	95.8 %	100 %	95.7 %
Males with females pregnant	21	22	22	21
Male fertility index	91.3 %	91.7 %	91.7 %	91.3 %
Females				
	Dose Group (ppm)			
	0	150	450	1500
Mean pre-coital interval (days)	3.5	2.1*	2.3	3.1
Females placed with males	23	24	24	23
Number inseminated	22	23	24	22
Female mating index	95.7 %	95.8 %	100 %	95.7 %
Pregnant	21	22	22	21
Female fertility index	91.3 %	91.7 %	91.7 %	91.3 %



Mean gestation interval (days)	22.36	22.27	22.11	22.00*
Number of litters	21	22	22	21
Gestation index	100 %	100 %	100 %	100 %
Live birth index	100 %	100 %	93.36 %	100 %

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett's test 2-Sided)

### Necropsy and pathology:

### Parental animals:

### Organ weights:

In males given 750 or 4500 ppm, absolute, adjusted (calculated from a covariance analysis (ANCOVA) using terminal kill body weight as covariate) and body weight related liver weights were higher than controls with statistical significance achieved for absolute liver weight at 4500 ppm ( $p < 0.01$ ) and adjusted liver weight at both dose levels ( $p < 0.01$ ). In females given 450 or 1500 ppm absolute, adjusted and body weight related liver weights were higher than controls with adjusted liver weights achieving statistical significance at both dose levels ( $p < 0.05$  and  $p < 0.01$  at 450 or 1500 ppm respectively) and absolute liver weight achieving significance at 1500 ppm ( $p < 0.01$ ). The increase in liver weight was associated with microscopic findings in the liver of both sexes at the high dose only.

Absolute and adjusted thyroid weights of the males were statistically significantly higher at 4500 ppm compared with controls ( $p < 0.01$  to  $p < 0.05$ ) but were significantly lower for females given 1500 ppm ( $p < 0.05$ ); however, thyroid weights were generally within the normal range (0.013 to 0.035g for males and 0.009 to 0.030g for females<sup>1</sup>) and therefore were considered not to be treatment related. Although microscopic findings were observed in the thyroids of the males, the applicant considered that there was no direct link between a higher thyroid weight and microscopic findings.

Other statistically significant increases ( $p < 0.05$  to  $p < 0.01$ ) compared with the controls were seen in adjusted adrenal weights (males at 4500 and 750 ppm and females at 1500 ppm) and adjusted kidney weights (males at 4500 ppm); however, in the absence of associated macroscopic or microscopic findings, the changes in organ weight are considered incidental and not related to administration of SYN545974.

**Table 6.6.1-10: Intergroup comparison of selected organ weights in parental animals**

		Dietary Concentration (ppm)							
		Males				Females			
		0	250	750	4500	0	150	450	1500
Liver	Absolute	13.10	13.62	14.23 (+9%)	<b>17.19**</b> (+31%)	13.35	13.44	13.96 (+5%)	<b>15.28**</b> (+14%)
	Adjusted	12.96	13.12	<b>14.18**</b> (+9%)	<b>17.88**</b> (+38%)	13.24	13.38	<b>14.05*</b> (+6%)	<b>15.36**</b> (+16%)
	Relative†	3.09	3.14	3.37 (+9%)	4.27 (+38%)	4.89	4.94	5.20 (+6%)	5.67 (+16%)
Kidney	Absolute	2.63	2.62	2.62	2.72	2.07	2.08	2.10	2.14
	Adjusted	2.61	2.55	2.61	<b>2.82**</b> (+8%)	2.05	2.07	2.12	2.15
	Relative†	0.62	0.61	0.62	0.68	0.76	0.76	0.78	0.80
Thyroid	Absolute	0.022	0.024	0.024	<b>0.025*</b> (+14%)	0.017	0.016	0.017	<b>0.015*</b> (-12%)
	Adjusted	0.022	0.023	0.024	<b>0.026**</b> (+18%)	0.017	0.016	0.017	<b>0.015*</b> (-12%)
	Relative†	0.005	0.005	0.006	0.006	0.006	0.006	0.006	0.006

<sup>1</sup> [REDACTED] background control data from 2-generation OECD416 studies on CR Han Wistar rat: 6 studies performed from January 2008 to February 2014 with a total of 157 males and 149 females for the P generation and 147 (males) and 137 females for the F1 generation (source: study report K-CA 5.6.1/01 [REDACTED] 2015).

Adrenals	Absolute	0.051	0.055	0.055	0.055	0.080	0.079	0.085	0.086
	Adjusted	0.051	0.053	0.055	<b>0.057**</b> (+12%)	0.080	0.079	0.085	<b>0.087*</b> (+9%)
	Relative†	0.012	0.013	0.013	0.014	0.029	0.029	0.032	0.032

\* Statistically significant difference from control group mean, p<0.05

\*\* Statistically significant difference from control group mean, p<0.01

† no statistical analysis was performed

## F1 animals

**Organ weights:** In males given 750 or 4500 ppm, absolute, adjusted and body weight related liver weights were higher than controls with statistical significance achieved for absolute liver weight at 4500 ppm (p<0.01) and adjusted liver weight at both dose levels (p<0.01). This was associated with microscopic findings in the liver for males given 4500 ppm, only. In females given 450 or 1500 ppm, absolute, adjusted and body weight related liver weights were higher than controls with absolute and adjusted liver weights achieving statistical significance at 1500 ppm (p<0.05 and p<0.01 for absolute and adjusted values respectively). However, there were no associated microscopic findings in the livers of females.

Male absolute (p<0.05 at 4500 ppm) and adjusted thyroid weights (p<0.05 at 150 and 750 ppm and p<0.01 at 4500 ppm) were higher than controls at all dose levels. However, thyroid weights were within normal range (0.012 to 0.032 g<sup>2</sup>) with exception of one male at 750 ppm. Although microscopic findings were observed in the thyroids of the males at the top dose level of 4500 ppm, the applicant considered that there was no direct link between a higher thyroid weight and microscopic findings. At 150 ppm and 750 ppm there were no macroscopic or microscopic findings. Therefore, any difference in thyroid weight at any dose level was considered incidental to treatment.

Other statistical increases were seen in adjusted adrenal (p<0.05) and kidney (p<0.01) weights (males at 4500 ppm); however, there were no macroscopic or microscopic findings to confirm an association with treatment.

**Table 6.6.1-11: Intergroup comparison of selected organ weights in F1 animals**

		Dietary Concentration (ppm)							
		Males				Females			
		0	250	750	4500	0	150	450	1500
Liver	Absolute	13.06	13.39	13.93	<b>16.47**</b> (+26%)	11.30	11.09	12.01	<b>13.24**</b> (+17%)
	Adjusted	12.43	12.82	<b>13.93**</b> (+12%)	<b>17.63**</b> (+42%)	11.26	11.09	12.05	<b>13.23**</b> (+17%)
	Relative†	3.02	3.10	3.35 (+11%)	4.27 (+41%)	4.47	4.43	4.83	5.30 (+18%)
Kidney	Absolute	2.59	2.54	2.56	2.60	2.12	2.06	2.09	2.15
	Ajusted	2.52	2.47	2.56	<b>2.74**</b> (+9%)	2.11	2.06	2.10	2.15
	Relative†	0.60	0.59	0.62	0.68	0.84	0.82	0.84	0.86
Thyroid	Absolute	0.020	0.022	0.022	<b>0.023*</b> (+15%)	0.019	0.020	0.019	0.019
	Adjusted	0.019	<b>0.022*</b> (+16%)	<b>0.022*</b> (+16%)	<b>0.024**</b> (+26%)	0.018	0.020	0.019	0.019
	Relative†	0.005	0.005	0.005	0.006	0.007	0.008	0.008	0.008

<sup>2</sup> [REDACTED] background control data from 2-generation OECD416 studies on CR Han Wistar rat: 6 studies performed from January 2008 to February 2014 with a total of 157 males and 149 females for the P generation and 147 (males) and 137 females for the F1 generation (source: study report K-CA 5.6.1/01 [REDACTED] 2015).

Adrenals	Absolute	0.54	0.060	0.055	0.058	0.079	0.081	0.080	0.077
	Adjusted	0.053	0.059	0.055	<b>0.060*</b>	0.079	0.081	0.080	0.077
	Relative†	0.013	0.014	0.013	<b>(+13%)</b> 0.015	0.031	0.032	0.032	0.031

\* Statistically significant difference from control group mean, p<0.05

\*\* Statistically significant difference from control group mean, p<0.01

† no statistical analysis was performed

### Macroscopic findings:

**P:** There were no treatment-related macroscopic changes.

The spectrum of macroscopic findings was consistent with changes encountered in rats of this age kept under laboratory conditions.

**F1:** There were no treatment-related macroscopic changes.

The spectrum of macroscopic findings was consistent with changes encountered in rats of this age kept under laboratory conditions.

### Microscopic findings:

**P:** Treatment-related changes were found in the liver and thyroids of males given 4500 ppm and in the liver of females given 1500 ppm.

### Liver

Hepatocyte hypertrophy (diffuse) was found in the liver of males given 4500 ppm and in females given 1500 ppm. This change was not found in males or females from the intermediate or low dose groups. The incidence and severity of the findings are shown in Table 6.6.1-12.

**Table 6.6.1-12: Histopathology findings in the liver in P-Generation animals**

P Generation		Males				Females			
Group		1	3	5	7	1	2	4	6
Treatment (ppm)		0	150	750	4500	0	150	450	1500
Hepatocyte hypertrophy	Minimal	0	0	0	0	0	0	0	8
	Slight	0	0	0	19	0	0	0	0
	Total	0	0	0	<b>19</b>	0	0	0	<b>8</b>
Number of rats examined		24	24	24	24	24	24	24	24

The hepatocyte hypertrophy is associated with the increase in liver weights for the parental generation males given 4500 ppm and females given 1500 ppm SYN545974.

### Thyroid

There was an increased incidence of follicular epithelial hypertrophy (minimal) in males given 4500 ppm when compared with the controls. The incidence of this finding in males given 750 or 150 ppm was similar to the controls. There was no histological evidence of a treatment-related effect in females. The incidence and severity of the findings in males are shown in Table 6.6.1-13.

**Table 6.6.1-13: Histopathology findings in the thyroid of P-Generation males**

P Generation		Males			
Group		1	3	5	7
Treatment (ppm)		0	150	750	4500
Hypertrophy, follicular	Minimal	1	1	2	7
Number of rats examined		24	24	24	24

### Other Findings

The spectrum of all other microscopic findings was consistent with changes encountered in rats of this age kept under laboratory conditions.

**F1:** Treatment-related changes were found in the liver and thyroids of males given 4500 ppm.

### Liver

Hepatocyte hypertrophy (diffuse) was found in the liver of males given 4500 ppm. This change was not found in females or in males from the intermediate or low dose groups. The incidence and severity of the findings are shown in Table 6.6.1-14.

**Table 6.6.1-14: Histopathology findings in the liver of F1-Generation males**

F1 Generation		Males			
Group		1	3	5	7
Treatment (ppm)		0	150	750	4500
Hepatocyte hypertrophy	Slight	0	0	0	18
Number of rats examined		24	24	24	24

The hepatocyte hypertrophy is associated with the significant increase in liver weights for the F1 generation parental males given SYN545974 at 4500 ppm.

### Thyroid

There was an increased incidence of follicular epithelial hypertrophy (minimal) in males given 4500 ppm when compared with the controls. The incidence of this finding in males given 750 ppm was similar to the controls. There was no histological evidence of a treatment-related effect in females. The incidence and severity of the findings in males are shown in Table 6.6.1-15.

**Table 6.6.1-15: Histopathology findings in the thyroid of F1-Generation males**

F1 Generation		Males			
Group		1	3	5	7
Treatment (ppm)		0	150	750	4500
Hypertrophy, follicular	Minimal	2	0	1	7
Number of rats examined		24	24	24	24

### Other Findings

The spectrum of all other microscopic findings was consistent with changes encountered in rats of this age kept under laboratory conditions.

### Offspring:

#### Viability and clinical signs:

**F1 Generation Pups:** There were no pup deaths or clinical signs that were considered to be related to treatment.

**F2 Generation Pups:** There were no deaths or clinical signs that were considered to be related to treatment. The anogenital distance of males and females was similar between all groups, including controls.

Mean litter size and viability results from pups during lactation are summarized in Table 6.6.1-16:

Table 6.6.1-16: Litter parameters for F1 and F2

Observation	Dose Group (ppm)			
	0	150	450	1500
<b>F1 Generation</b>				
Mean Implantation Sites	12.3	12.1	11.5	11.7
Number born	283	276	271	262
Number found dead/killed prematurely	3	12	2	7
Missing (presumed cannibalised)	3	3	1	1
Sex Ratio Day 0 (% ♂)	45.87	51.77	54.72	54.62
# Deaths Days 1-4	1	7	2	7
# Deaths Days 5-7	1	5	1	4
# Deaths Days 8-14	1	2	0	0
# Deaths Days 15-21	0	0	0	0
Mean litter size Day 0	11.8	11.5	11.3	11.4
Day 4 <sup>a</sup>	11.6	11.2	11.2	11.0
Day 7	7.9	7.8	7.8	7.6
Day 14	7.8	7.7	7.8	7.6
Day 21	7.8	7.7	7.8	7.6
Live birth index	100 %	100 %	100 %	100 %
Viability index 1	98.72 %	97.28 %	99.33 %	95.84 %
Lactation index	98.78 %	95.83 %	99.40 %	97.67 %
<b>F2 Generation</b>				
Mean Implantation Sites	12.3	10.7	11.6	11.2
Number born	239	222	238	212
Number found dead/killed prematurely	33	29	24	32
Missing (presumed cannibalised)	84	67	42	53
Sex Ratio Day 0 (% ♂)	51.50	49.74	48.31	51.57
# Deaths Days 1-4	64	43	17	53
# Deaths Days 5-7	18	31	25	19
# Deaths Days 8-14	21	16	22	14
# Deaths Days 15-21	0	1	0	0
Mean litter size Day 0	11.4	9.9	10.8	10.6
Day 4 <sup>a</sup>	7.7	7.9	10.0	8.0
Day 7	5.2	5.2	6.5	5.5
Day 14	4.2	4.5	5.5	4.8
Day 21	4.2	4.4	5.5	4.8
Live birth index	100 %	97.66 %*	100 %	100 %
Viability index 1	68.09 %	81.71 %	93.36 %	76.02 %
Lactation index	64.58 %	59.49 %	72.38 %	65.00 %

<sup>a</sup> Before standardisation (culling)

\* Statistically different from control, p&lt;0.05

**Body weight (Table 6.6.1-17):**

**F1 Generation Pups:** Group mean absolute pup weight was similar among all groups, including controls, on Day 1 of age; however body weight gains at 1500 ppm were slightly, but statistically significantly, lower than controls over Days 1 to 21 of age. The study director considered this finding not to be a direct effect of treatment as the reduced pup weight was not repeated in the F1 generation litters and the group mean was within the historical control range (Table 6.6.1-18), with the concurrent control pups being at the higher end of the historical control means.

**F2 Generation Pups:** Group mean pup weight was similar among all groups, including controls, on Day 1 of age, and there was no difference in body weight gains over Days 1 to 21 of age.

**Table 6.6.1-17: F1 and F2 intergroup comparison of body weight (g)**

Lactation Day	Dose Group (ppm)							
	0	150	450	1500	0	150	450	1500
	F1 Pups - male & female				F2 Pups - male & female			
1	6.69	6.64	6.72	6.45	6.23	6.45	6.60	6.35
4a	10.17	9.98	10.30	9.27	8.16	7.81	8.76	8.05
7	16.83	15.99	16.50	14.89**	12.60	12.32	13.47	12.95
14	33.97	32.75	33.11	29.91**	30.32	29.58	30.93	29.33
21	51.63	50.63	50.28	46.29**	48.94	47.27	49.60	47.32

a - Before standardisation (culling)

\*\* - Statistically different from control,  $p < 0.01$ **Table 6.6.1-18: Historical Control Data (2008 to 2014) - F1 and F2 pup body weights (g)**

Lactation Day		Study											
		1	2	3	4	5	6	1	2	3	4	5	6
		Jan. 2008	Mar. 2009	Jan. 2009	Jan. 2014	Feb. 2014	Feb. 2014	Jan. 2008	Mar. 2009	Jan. 2009	Jan. 2014	Feb. 2014	Feb. 2014
		F1 Pups - male & female						F2 Pups - male & female					
1	Mean	6.35	6.00	6.05	5.59	6.70	5.71	6.05	5.85	6.30	6.61	6.72	6.83
	N	29	29	24	23	22	19	24	24	22	24	21	22
4a	Mean	9.25	8.40	7.80	8.42	10.40	9.25	8.30	8.20	9.00	10.42	10.47	10.70
	N	29	29	24	22	22	15	24	24	22	24	21	22
7	Mean	13.75	12.30	11.60	13.06	16.73	14.56	12.45	12.30	13.05	16.55	17.12	16.76
	N	29	29	24	22	22	15	23	24	22	24	21	22
14	Mean	26.50	25.70	25.30	26.46	32.77	29.26	25.50	24.90	25.85	32.95	34.32	32.94
	N	29	29	24	22	22	15	23	24	22	24	21	22
21	Mean	41.35	39.95	40.95	43.03	53.09	46.47	40.45	38.65	40.75	53.38	54.25	53.65
	N	29	29	24	22	22	15	23	24	22	24	21	22

a - Before standardisation (culling)

**Sexual maturation:**

**F1 Generation Pups:** Sexual maturation was slightly delayed with statistical significance for males given 4500 ppm (43.0 days for control and 45.9 days for 4500 ppm; males given 4500 ppm had consistently lower body weights compared with controls in the period leading up to the point of sexual maturation and were lighter than controls at the time of maturation (group mean body weight of 191.9 g and 183.9 g for control and 4500 ppm males respectively), indicating the delay in sexual maturation of males was secondary to reduced body weight gain and not a direct effect of treatment with SYN545974 (Tables 6.6.1-19 and 20).

Sexual maturation was slightly delayed with statistical significance ( $p < 0.01$ ) in females given 1500 ppm (30.3 and 33.0 days for control and 1500 ppm females respectively). The delay in sexual maturation of female was not secondary to reduced body weight gain as females given 1500 ppm had no consistently lower body weights compared with controls at the time of maturation. However, this finding was considered by the study director to be unrelated to treatment with SYN545974 as there was no effect on related parameters such as oestrous cycling (in the F1 generation), mating performance or fertility, and there was no effect on ano-genital distance of F1 generation pups (Tables 6.6.1-19 and 20).

Although HSE has agreed that no adverse effects were observed on other reproductive or developmental performances in F1 females, the vaginal opening is delayed by around 3 days compared to controls and even if this variation is within the historical control data range, the delay increase is statistically significant at 1500 ppm ( $p < 0.01$ ). The age of vaginal opening is dependent on both bodyweight at weaning and bodyweight the day at vaginal opening. Bodyweight at vaginal opening is dependent upon both weight at weaning and time from weaning to vaginal opening, with weight at vaginal opening increasing with weight at weaning and increasing time between weaning and vaginal opening (Edwards and Kay, 1985<sup>3</sup>).

<sup>3</sup> Edwards D.F., and Kay R. (1985): Weight and vaginal opening in the albino rat. *J. Reprod. Fert.* **73**, 1-8

In this study (see above table 6.6.1-21), the mean female pup bodyweight, as close as possible from weaning (day “-1” in the tables and annexes of the study report) was on average 4.6 grams heavier in the control group than in the 1500 ppm group. As well, control females reached the age of puberty (vaginal opening) 2.6 days before the high dose treated F1 females. However, the speed of growth between weaning and puberty was similar between control and high dose group (4.8 and 4.9 g/day respectively). These values indicate that the delay in vaginal opening was in a large part due to the delay in development during the lactation period and that, despite a growth velocity similar between control and high dose groups, this specific endpoint of development was indicative of a general developmental delay. Therefore, an effect of treatment cannot be excluded.

**Table 6.6.1-19: F1 generation pups sexual maturation and body weights (g)**

Males						
Observation			Dose Group (ppm)			
			0	150	750	4500
Preputial Separation	Day of age	Mean	43.0	43.2	44.1	45.9**
		SD	2.4	2.7	2.3	3.5
		N	24	24	24	24
	Body weight	Mean	191.9	189.8	193.3	183.9
		SD	18.3	16.3	15.5	12.7
		N	24	24	24	24
Females						
Observation			Dose Group (ppm)			
			0	150	450	1500
Vaginal Opening	Day of age	Mean	30.3	31.3	31.8	33.0**
		SD	2.1	2.6	2.1	2.5
		N	24	24	24	24
	Body weight	Mean	97.2	100.3	103.7	105.7
		SD	11.1	16.5	12.4	11.8
		N	24	24	24	24

\*\* - Statistically different from control,  $p < 0.01$

**Table 6.6.1-20: Historical Control Data (2008 to 2014) - F1 generation pups sexual maturation and body weights (g)**

Males								
Observation			Study					
			1	2	3	4	5	6
			Jan. 2008	Mar. 2009	Jan. 2009	Jan. 2014	Feb. 2014	Feb. 2014
Preputial Separation	Day of age	Mean	45.2	45.0	45.3	43.0	43.5	43.3
		Range	42 - 52	42 - 50	41 - 51	40 - 46	38 - 49	40 - 47
		N	25	25	25	24	24	24
	Body weight	Mean	184.8	177.8	193.4	172.3	183.4	179.6
		Range	156 - 222	152 - 207	150 - 213	149 - 200	132 - 222	153 - 221
		N	25	25	25	24	24	24
Females								
Observation			Study					
			1	2	3	4	5	6
			Jan. 2008	Mar. 2009	Jan. 2009	Jan. 2014	Feb. 2014	Feb. 2014
Vaginal Opening	Day of age	Mean	32.7	34.1	31.8	31.3	29.3	29.9
		Range	29 - 38	31 - 39	27 - 36	29 - 35	25 - 33	27 - 34
		N	25	25	25	24	24	24
	Body weight	Mean	92.3	98.1	89.3	88.1	89.4	85.7
		Range	75 - 114	77 - 124	56 - 115	78 - 105	72 - 117	62 - 110
		N	25	25	25	24	24	24

		N	25	25	25	24	24	24
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**Table 6.6.1-21: Relationship between age at/around weaning and vaginal opening in females F1 (controls and 1500ppm treatment group)**

0 ppm					1500 ppm				
Animal number	Bodyweight “-1” (g)	Age of vaginal opening (days)	Time to vaginal opening	BW gain D-1 to vaginal opening (g)	Animal number	Bodyweight “-1” (g)	Age of vaginal opening (days)	Time to vaginal opening	BW gain D-1 to vaginal opening (g)
297	50	30	9	48	369	45	30	9	41
298	44	32	11	53	370	48	33	12	59
299	53	29	9	32	371	51	31	10	48
300	59	29	9	44	372	51	29	8	40
301	55	28	7	29	373	51	29	8	44
302	52	31	10	49	374	48	33	12	61
303	52	33	12	72	375	39	32	11	58
304	42	33	12	58	376	50	33	12	55
305	49	30	9	43	377	50	34	13	67
306	54	29	8	38	378	54	33	12	60
307	44	33	12	49	379	51	33	12	56
308	55	33	12	60	380	36	36	15	64
309	49	34	13	59	381	46	35	14	60
310	48	31	10	47	382	39	32	11	55
311	50	28	7	40	383	49	32	11	54
312	52	32	11	55	384	45	35	14	74
313	48	30	9	42	385	47	35	14	65
314	57	32	11	53	386	46	35	14	72
315	54	29	8	34	387	44	37	16	77
316	55	28	7	35	388	48	29	8	39
317	49	29	8	37	389	48	38	17	85
318	60	27	6	29	390	48	35	14	70
319	54	31	10	57	391	44	32	11	61
320	51	27	6	30	392	47	31	10	47
<b>Means</b>	<b>51.5</b>	<b>30.3</b>	<b>9.4</b>	<b>45.6</b>		<b>46.9</b>	<b>33</b>	<b>12</b>	<b>58.8</b>

#### Offspring *postmortem* results:

#### Organ weights:

**F1 Generation Pups:** Statistically significant decreases ( $p < 0.05$ ) in absolute thymus and spleen weights were seen for males at 1500 ppm; however, the differences from control were minimal and there was no difference from control in body weight-adjusted thymus and spleen weights and similar changes were not observed for female pups or pups of the F1 generation litters. Therefore, these changes were considered to be unrelated to treatment.

**F2 Generation Pups:** There was a slight, statistically significant increase ( $p < 0.05$ ) in adjusted brain weight for male and female pups from the group given 1500 ppm; however, the differences from control were minimal and since there was no differences in the absolute brain weights, this was considered to be unrelated to treatment.

#### Pathology:

#### Macroscopic examination:

**F1 Generation:** At necropsy, there were no findings that were considered to be related to treatment.

**F2 Generation Pups:** At necropsy, there were no findings that were considered to be related to treatment.



## CONCLUSION:

Pydiflumetofen was evaluated in a GLP and guideline two-generation reproduction study in the Wistar (Han) rat at dietary levels of 0, 150, 450 and 1500 ppm (females, equivalent to 0, 11.9, 36.1 and 116.2 mg/kg bw/d) or 0, 150, 750 and 4500 ppm (males, equivalent to 0, 11.9, 59.1 and 276.6 mg/kg bw/d). The dose levels were selected based on non-proportionality of the kinetics with respect to dose due to dose limited absorption of pydiflumetofen (see ADME section).

### Reproductive toxicity

There were no effects on fertility and mating performance or gestation length for either generation at any dietary concentration. Sperm parameters were unaffected. All pregnant females gave birth to live litters with a similar number of pups born, and there was no effect of treatment on the postnatal survival of P or F1 generation litters to Day 21 of age.

The mean length of the oestrous cycle was statistically significantly increased at the top dose (4.05 d vs 3.93 d in controls) in the P generation only. The increase was driven by 2 females with longer cycle length (5 and 4.5 d) and was well within the laboratory historical control data (HCD) mean range (4.0 – 4.3 d) from four studies conducted between 2009 and 2014. Therefore, the effect was considered unrelated to treatment.

A delay in sexual maturation was noted in both sexes at the top dose in the F1 generation. Mean age at preputial separation (PS) was statistically significantly increased by approx. 3 days (45.9 d vs 43.0 in controls). However, when excluding from the analysis a clear outlier, with an age at PS of 57 days, the top dose mean was 45.4 d. This increase was at the upper bound of the laboratory HCD mean range (43.0 – 45.3 d; mean = 44.2 d) from 6 studies conducted between 2008 and 2014. However, when excluding from the HCD 3 studies from 2008 and 2009 (because > 5 years from study's year), the increase (45.4 d) was clearly above the more time-restricted and more relevant HCD mean range (43.0 – 43.5 d). In addition, it is unclear whether the outlier was a spontaneous aberration or was caused by the test substance. It is therefore uncertain whether the very high PS value in one top-dose animal should have been excluded from the analysis.

Mean age at vaginal opening (VO) was statistically significantly increased by approx. 3 days (33.0 d vs 30.3 d in controls). This increase was well within the laboratory HCD mean range (29.3 – 34.1 d; mean = 31.5 d) from 6 studies conducted between 2008 and 2014. However, when excluding from the HCD 3 studies from 2008 and 2009 (because > 5 years from study's year), the increase (33.0 d) was clearly above the more time-restricted and more relevant HCD mean range (29.3 – 31.3 d).

Therefore the delay in PS and VO at the top dose was considered to be treatment related. Pup body weights were statistically significantly reduced at the top dose compared with controls in both sexes of the F1 generation from day 7 to day 21 of lactation (by 10-12%). However, the reduction was within the laboratory HCD range from 6 studies conducted between 2008 and 2014, and more importantly was not replicated in the F2 generation. Therefore, the decrease in pup body weight was unconvincing and hence the delay in sexual maturation could not be considered the secondary unspecific consequence of the reduced pup body weight development.

In agreement with RAC, HSE concludes that although these pups went on to mate and reproduce successfully and despite the absence of endocrine effects and the lack of effects on other developmental landmarks, ano-genital distance and other reproductive parameters and organs, the delay in puberty onset at the top dose was seen in both sexes, was clear (statistically significant and outside time-relevant HCD) and specific (i.e. independent of reductions in pup body weight development). Based on this analysis, HSE agrees that classification of pydiflumetofen for adverse effects on fertility and sexual function in category 2 (H361f) is warranted. For further information on classification, please see the [GB MCL Technical Report](#). A NOAEL for reproductive toxicity was therefore set at 450/750 ppm in females/males (equivalent to **36.1 mg/kg bw/d in females and 59.1 mg/kg bw/d in males**) based

on a delay in sexual maturation at the top dose of 1500/4500 ppm in females/males (equivalent to 116.2 mg/kg bw/d in females and 276.6 mg/kg bw/d in males).

#### Parental toxicity

In males, there were decreases in body weight gains (10%) in both generations, reductions in food consumption (8%) in the F1 generation and statistically significant increases in liver and thyroid weights with associated hypertrophy in both generations at the top dose. In top dose females, there was a statistically significant increase in liver weight with associated hypertrophy in both generations. Therefore the **NOAEL for parental toxicity** was set at 450/750 ppm in females/males (equivalent to **36.1 mg/kg bw/d in females and 59.1 mg/kg bw/d in males**).

#### Offspring toxicity

There were no effects on offspring up to the top dose of 1500/4500 ppm in males/females. Therefore the **NOAEL for offspring toxicity** was set at 1500/4500 ppm (equivalent to **116.2 mg/kg bw/d in females and 276.6 mg/kg bw/d in males**).

#### Adequacy of top dose

RAC concluded that only minimal parental toxicity was evident at the top dose, especially in females. Therefore RAC agreed that the top dose was inadequate and that the study had not fully investigated the reproductive toxicity potential of pydiflumetofen. HSE considers that the top dose was adequate in males, with decreases in body weight gains (10%) in both generations, reductions in food consumption (8%) in the F1 generation and statistically significant increases in liver and thyroid weights with associated hypertrophy in both generations. The top dose should have been higher in females; however, the Agency notes that the OECD TG (No. 416, 2001) states '*the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering*'. Attainment of the MTD is not specified in the OECD TG. In top dose females, there was a statistically significant increase in liver weight with associated hypertrophy in both generations, which is the most sensitive effect of the toxicity profile of the substance. Hence, target organ toxicity in the liver was induced at the top dose in both generations in females. Therefore, HSE concludes that the requirements of the OECD TG for selection of the top dose in females was also met, as 'toxicity' was induced.

(██████████, 2015)

### **B.6.6.2. Developmental toxicity studies**

The developmental toxicity of pydiflumetofen was investigated in the (SD) rat and (NZW) rabbit in GLP and guideline compliant studies with preliminary range-finding studies for both.

#### ***B.6.6.2.1. Rat studies***

#### **Range-finding study**

<b>Report:</b>	K-CA 5.6.2/01 ██████████ (2011). SYN545974, SYN546022: Preliminary Oral (Gavage) Prenatal Developmental Toxicity Dose Range Finding Study in the Rat. ██████████ ██████████ Laboratory Report No. ██████████, 26 October 2011. Unpublished. Syngenta File No. SYN545974_10003.
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<b>Report:</b>	K-CA 5.6.2/02 ██████████, (2016) SYN545974 - Historical Control Data for SYN545974_10003, ██████████. Syngenta File No. SYN545974_10454.
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**Guidelines:** This is a preliminary study with no applicable guidelines.

**GLP:** This study has not been subjected to any study specific Quality Assurance procedures. This study was conducted in a Good Laboratory Practice (GLP) compliant facility to a standard based on GLP, however the study does not claim compliance with GLP.

This study was conducted on 2 Active Ingredients, only data on SYN545974 are presented.

Following a request of the EU RMS, additional Historical Control Data for some fetal parameters have been provided by the applicant and the information incorporated into the study summary below.

## EXECUTIVE SUMMARY

The study was designed to investigate the effects of the test item, SYN545974 on the pregnant rat and on embryonic and fetal development when administered from implantation and throughout gestation (Days 6 to 19 of gestation), in order to select dose levels for a subsequent developmental toxicity study.

Fifty four time-mated female rats of the Sprague-Dawley strain were allocated to study, (6 females were assigned to a control group and 24 females were assigned to groups administered SYN545974, remaining animals were assigned to groups with another active ingredient). SYN545974 was administered daily, from Days 6 to 19 of gestation, at dose levels of 100, 200, 500 or 1000 mg/kg/day (Groups 2 to 5). Group 1 was dosed with the vehicle, 1 % carboxymethylcellulose alone, following the same dosing regimen and served as controls.

Body weights, food consumption and clinical observations were recorded at regular intervals during the study. Blood samples for toxicokinetic evaluation were taken from 2 animals per group on Day 19 of gestation at 4, 8 and 24 hours after dosing. The animals were killed by carbon dioxide asphyxiation on Day 20 of gestation, a necropsy performed and internal organs examined for gross abnormalities. Any macroscopic abnormalities were recorded and retained. Pregnancy parameters were recorded and the fetuses were removed from the uterus, weighed, sexed and examined for external and visceral abnormalities. Placental and gravid uterus weights were also recorded.

There was no treatment-related mortality or clinical signs following administration with SYN545974.

Treatment with SYN545974 at 1000 mg/kg/day showed a transient reduction in body weight gain. There was no effect on maternal body weight at any other dose level of SYN545974 tested.

All pregnancy parameters and fetal data, including the incidence of fetal abnormalities, were comparable with controls.

**Oral administration of SYN545974 to the pregnant Sprague-Dawley rat, from Day 6 to Day 19 of gestation, at dose levels of 100, 200, 500 or 1000 mg/kg/day, was well tolerated. A reduction in body weight gain for females given 500 mg/kg/day and a body weight loss for females given 1000 mg/kg/day, were observed between Days 6 to 7 of gestation. There was no indication of fetal toxicity. A dose level of 1000 mg/kg/day is considered a suitable high dose level for administration in a subsequent developmental toxicity main study with this test item.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	<b>SYN545974</b>
<b>Description:</b>	White powder
<b>Lot/Batch number:</b>	2491-DC/110
<b>Purity:</b>	98.6 % w/w

<b>Test Material:</b>	SYN545974
<b>CAS#:</b>	Not reported
<b>Stability of test compound:</b>	Stable until 31 October 2011 (stored at room temperature in the dark)

**Vehicle:** 1 % carboxymethylcellulose (500mPa.s).

<b>Test Animals:</b>	
<b>Species</b>	Rat
<b>Strain</b>	CD (SD)
<b>Age/weight at dosing</b>	Females were approximately 9 to 10 weeks of age, time-mated and delivered to CD by Day 2 of gestation. On the first day of dosing females weighed between 223g and 289g.
<b>Source</b>	
<b>Housing</b>	Individually in grid-floor cages over paper lined trays.
<b>Acclimatisation period</b>	At least 3 days
<b>Diet</b>	Pelleted rodent diet ad libitum. VRF1 (manufactured by ) supplied by
<b>Water</b>	Mains tap water ad libitum
<b>Environmental conditions</b>	Temperature: 20-23°C (target range 21± 2 ° C) Humidity: 31-60% (target range 55± 15 %). Air changes: Not reported Photoperiod: 12 hours light, 12 hours dark

### Study Design and Methods:

In-life dates: Start: 28 January 2011, End: 16 February 2011

**Mating procedure:** Mating was conducted at the supplier's premises and the day on which sperm was observed was designated Day 0 of gestation. Each female was paired with a sexually mature male of the same strain.

**Animal assignment:** Animals were allocated to groups using a stratified randomisation procedure based on individual body weights recorded during the acclimatisation period (making sure that females mated with the same male were spread across the groups) as shown in the following table.

### Animal numbers and treatment groups

Group	Number of females	Treatment	Dose level (mg/kg bw/day)	Dose volume (mL/kg bw)
1	6	Vehicle	0	10
2	6	SYN545974	100	10
3	6		200	10
4	6		500	10
5	6		1000	10

**Dose selection rationale:** The dose levels were selected by the Sponsor after examining existing toxicity data and on the basis of a tolerability study performed at .

**Dose preparation and analysis:** The test item was formulated for dosing as suspensions in the vehicle (1 % carboxymethylcellulose). The weighed quantity of test item was suspended in the appropriate quantity of vehicle. Separate formulations were prepared for each dose level. Formulations were prepared on the morning of each day of dosing. Test item formulations were stirred for at least 5 minutes prior to use and throughout the dosing period. There was no assessment of dose formulation stability or achieved concentration. A visual check to assess homogeneity of the formulation at each dose level was performed prior to dosing.

**Dose administration:** All animals were dosed once daily, by oral gavage, on Days 6 to 19 of gestation, inclusive, at a dose volume of 10 mL/kg body weight, using a rubber catheter and disposable syringe. Individual doses were adjusted according to the most recent body weight. Control animals received the vehicle only, following the same regimen as the other groups.

### Observations:

*Maternal observations:* Animals were examined twice daily for mortality and morbidity. All females were examined daily from the beginning of the treatment period until necropsy for clinical signs of toxicity and/or changes in behaviour and appearance. During the treatment period, each female was routinely checked pre-dose and immediately after the completion of dosing for that group. Additional observations were made at least twice daily.

Body weights were recorded on Day 0 of gestation at the supplier's, on the day of arrival at [REDACTED] and then daily from Days 6 to 20 of gestation inclusive. The amount of food consumed by each animal was recorded over Days 6 to 9, 9 to 12, 12 to 15, 15 to 18 and 18 to 20 of gestation.

*Toxicokinetics:* Blood samples were collected from the tail vein on Day 19 of gestation at 4, 8 and 24 hours post dose from 2 rats/group and submitted for toxicokinetic analysis. The results are reported under [REDACTED]  
[REDACTED] Study Number: 312692.

*Post mortem observations:* The females were killed on Day 20 of gestation by exposure to carbon dioxide. All females were weighed, the thoracic and abdominal cavities were opened by a ventral mid-line incision and the major organs were examined. Organs or tissues which displayed macroscopic abnormalities were removed and fixed in neutral buffered formaldehyde. The uterus of any apparently non-pregnant female was stained with ammonium sulphide to confirm pregnancy status.

For pregnant females, the following data were recorded:

- 1) Pregnancy status
- 2) Gravid uterus weight
- 3) Number of corpora lutea
- 4) Number and distribution of implantation sites. The implantations were classified as early resorptions, late resorptions, dead fetuses or live fetuses.

Fetal observations: The fetuses were examined and the following observations made:

- 1) Fetal weights (live fetuses)
- 2) Fetal sexes (live fetuses)
- 3) External abnormalities of fetuses
- 4) Placental weights

Fetuses were killed by cooling before immersion in cold fixative of 70% IDA (Industrial Denatured Alcohol) and were subsequently subjected to a visceral examination.

Abnormalities were assessed as follows; structural congenital abnormalities that impair, or potentially impair, the survival or constitution of the fetus were regarded as major abnormalities. Other defects were classified as minor abnormalities. Common variations in the extent of renal pelvic cavitation and ureter dilation were recorded as variants.

*Indices:* The following indices were calculated:

Pre-implantation loss (%) =  $\frac{(\text{number of corpora lutea} - \text{number of implantation sites})}{\text{number of corpora lutea}} \times 100$

Post-implantation loss (%) =  $\frac{(\text{number of implantation sites} - \text{number of live fetuses})}{\text{number of implantation sites}} \times 100$

Statistical analyses: Body weights and cumulative body weight gains, body weight gains corrected for gravid uterus weight, food consumption, number of corpora lutea, number of implants, number of live fetuses, gravid uterus weight and total litter weight were analysed using analysis of variance and Dunnett's test. Pre-implantation loss, post-implantation loss, early intra-uterine deaths, late intra-uterine deaths and percentage of male fetuses were analysed using analysis of variance following double arcsine transformation and Dunnett's test. Fetal abnormalities were analysed using Fisher's Exact test.

## RESULTS

**Maternal toxicity:** Mortality and clinical signs: There were no clinical observations or mortality considered to be related to treatment.

**Body weight:** There was a slight reduction in body weight gain for females given 500 mg/kg/day and a slight body weight loss for females given 1000 mg/kg/day, between Days 6 to 7 of gestation when compared with the controls, attaining statistical significance  $p < 0.05$  and  $p < 0.001$ , respectively. However, these lower body weight gains or body weight losses were recouped over the duration of the treatment period, so that overall body weight gains were similar to the controls. There was no effect of treatment at 100 or 200 mg/kg/day on body weight or body weight gains when compared with the controls.

**Table 6.6.2-1: Intergroup comparison of body weight gain (g)**

Days	Dose level (mg/kg bw/day)				
	Control	SYN545974			
	0	100	200	500	1000
0-6	34.5	34.5	23.7	25.0	28.0
6-7	6.0	3.2	1.3	0.2*	-3.7***
6-8	10.0	10.7	8.5	7.6	7.2
6-9	16.2	15.7	14.0	14.6	14.5
6-12	37.5	35.7	33.7	29.0	32.0
6-19	111.3	102.5	109.5	106.2	106.2
6-20	124.5±25	117.7±22	124.8±24	122.4±16	122±5
6-20†	46.5±10	50.8±6	46±13	47.1±8	43.7±8
* Statistically significant difference from control group mean, $p < 0.05$ (Student's t-test, 2-sided) ** Statistically significant difference from control group mean, $p < 0.01$ (Student's t-test, 2-sided) *** Statistically significant difference from control group mean, $p < 0.001$ (Student's t-test, 2-sided) † Gain 6-20 corrected for gravid uterus weight					

**Food consumption:** There were no treatment-related effects for either test item.

**Sacrifice and pathology:** Gross pathology maternal: There were no treatment-related effects for either test item.

**Caesarean section data:** There was no effect of treatment on the mean number of corpora lutea, mean number of implantations, the extent of pre- or post-implantation losses or on the mean number of live fetuses. All pregnant females had litters with live fetuses at scheduled necropsy. There was no effect of

treatment on mean fetal weights, mean litter weights or the percentage of male fetuses. Mean placental and gravid uterus weights were similar across the groups.

**Table 6.6.2-2: Caesarean section observations**

Observation	Dose level (mg/kg bw/day)				
	0 (control)	100	200	500	1000
# Animals Assigned (Mated)	6	6	6	6	6
# Animals Pregnant	6	6	6	5	6
# Non pregnant	0	0	0	1	0
# Intercurrent deaths	0	0	0	0	0
# With live fetuses at scheduled kill	6	6	6	5	6
Corpora Lutea/Dam	15.0	13.5	13.8	15.0	14.0
Implantations/Dam	14.7	12.0	13.7	13.8	13.7
Mean % pre-implantation loss	2.0	15.3	1.2	8.1	2.2
# Early embryo/fetal deaths	8	4	1	3	2
# Late embryo/fetal deaths	0	0	0	0	0
# Dead fetuses	0	0	0	0	0
Mean % post-implantation loss	9.4	4.7	1.2	4.3	2.4
# Live Fetuses/Dam	13.3	11.3	13.5	13.2	13.3
Mean % implantations	90.6	95.3	98.8	95.7	97.6
Mean Litter Weight (g)	52.9	45.0	53.7	49.8	53.2
Mean Fetal Weight (g)	3.95	4.04	3.96	3.77	3.98
Males (g)	4.07	4.14	4.06	3.90	4.10
Females (g)	3.84	3.83	3.89	3.68	3.89
Sex Ratio (% Males)	47.2	64.3	42.7	47.6	46.2
Mean placental weight (g)	0.52	0.55	0.53	0.59	0.53
Mean gravid uterus weight (g)	78.0	66.9	78.8	75.3	78.4
No statistically significant differences					

**Fetal abnormalities:** There were no major fetal abnormalities.

The minor fetal abnormalities recorded were; absent innominate artery in 2 fetuses at 200 mg/kg/day, haemorrhage of the occipital region in 2 fetuses at 500 mg/kg/day and 1 fetus at 1000 mg/kg/day, and enlarged and haemorrhagic uni or bilateral thyroid in 1 fetus at 1000 mg/kg/day. None of the findings achieved statistical significance nor showed a dose-related response. Historical controls data (HCD) from the conducting laboratory have been provided by the applicant. They came from either the original study report of this range-finding study (K-CA 5.6.2/01 [REDACTED] (2011) or from an additional report including additional HCD from the conducting laboratory (K-CA 5.6.2/02 [REDACTED], (2016)) which have been submitted later by the applicant.

Minor and variant abnormalities and corresponding historical control data are summarized in Table 6.6.2-3. The variant findings seen at fresh visceral examination were similar in incidence across all dose groups and were within the foetal and litter incidences reported in the historical control, therefore, no findings were considered to be related to treatment with SYN545974.

**Table 6.6.2-3: Summary of minor fetal abnormalities and variants (fetal incidence/group mean % and litter incidence/group mean %)**

Observations			Doses (mg/kg/day)					Historical Control Data (2008-2013) ‡
Findings	Type		0 (control)	100	200	500	1000	
<u>Head:</u> occipital region - haemorrhage	Minor	foetus	0/80 (0%)	0/68 (0%)	0/81 (0%)	2/66 (3.2%)	1/80 (1.2%)	-
		litter	0/6 (0%)	0/6 (0%)	0/6 (0%)	2/5 (40%)	1/6 (16.7%)	-
<u>Thyroid:</u>  Uni or bilateral enlargement  Uni or bilateral haemorrhagic	Minor	foetus	0/80 (0%)	0/68 (0%)	0/81 (0%)	0/66 (0%)	1/80 (1.4%)	-
		litter	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/5 (0%)	1/6 (16.7%)	-
		foetus	0/80 (0%)	0/68 (0%)	0/81 (0%)	0/66 (0%)	1/80 (1.4%)	-
		litter	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/5 (0%)	1/6 (16.7%)	-
<u>Thoracic cavity:</u> innominate artery absent	Minor	foetus	0/80 (0%)	0/68 (0%)	2/81 (2.6%)	0/66 (0%)	0/80 (0%)	0-1 (mean: 0.08%) Range: 0-0.7%
		litter	0/6 (0%)	0/6 (0%)	1/6 (16.7%)	0/5 (0%)	0/6 (0%)	0-1 (mean: 1.1%) Range: 0-11.1%
<u>Kidney:</u> uni or bilateral pelvic cavitation	Variant	foetus	0/80 (0%)	0/68 (0%)	0/81 (0%)	2/66 (3.2%)	2/80 (2.4%)	0-1 (Mean: 0.5%) Range: 0-3.3%
		litter	0/6 (0%)	0/6 (0%)	0/6 (0%)	2/5 (40%)	1/6 (16.7%)	0-1 (Mean: 6.4%) Range: 0-20%
<u>Ureter:</u> uni or bilateral dilation	Variant	foetus	10/80 (12%)	0/68 (0%)	3/81 (3.6%)	6/66 (10.1%)	2/80 (2.4%)	1-11 (Mean : 4.8%) Range : 0.9-16.7%
		litter	5/6 (83.3%)	0/6 (0%)*	2/6 (33.3%)	3/5 (60%)	1/6 (16.7%)	1-5 (Mean : 32%) Range : 11-80%
Umbilical artery: left sided	Variant	foetus	2/80 (2.4%)	0/68 (0%)	0/81 (0%)	1/66 (1.4%)	1/80 (1.2%)	0-5 (Mean: 1.2%) Range: 0-4.5%
		litter	2/6 (33.3%)	0/6 (0%)	0/6 (0%)	1/5 (20%)	1/6 (16.7%)	0-3 (Mean: 14.9%) Range: 0-33.3%

‡ [REDACTED] background Data from Preliminary Prenatal Developmental Toxicity Studies on the Sprague-Dawley Rat [REDACTED]: 13 studies performed from February 2008 to April 2013 with a total of 1149 foetuses and 94 litters (sources: study report K-CA 5.6.2/01 [REDACTED] (2011) and report K-CA 5.6.2/02 [REDACTED] 2016).

## CONCLUSION:

In a PNDT range finding study in SD rats, in which groups of 6 pregnant females were given gavage doses of 0, 100, 200, 500 or 1000 mg/kg bw/d pydiflumetofen from day 6 to 19 of gestation, maternal toxicity was observed from 500 mg/kg bw/d as a reduction in body weight gain. At 1000 mg/kg bw/d, body weight loss was noted. No developmental toxicity was seen. A top dose level of 1000 mg/kg bw/d was proposed for the main study, but a top dose of 100 mg/kg bw/d was used.

([REDACTED], 2011)

## Main study

After the commenting period, EFSA has requested additional informations regarding the rat developmental study.

### EFSA Request for additional information (February 2018), Question 13:

Applicant to indicate the mean BWG, statistical analysis of the data and the difference (%) in BWG in females group given 100 mg/kg bw/d compared with controls over the first days of dosing (day 6 to 10) in the rat developmental study.

### EFSA Request for additional information (February 2018), Question 19:

Applicant to submit a report amendment regarding the HCD provided in the study report of the developmental toxicity study in the rats, and the additional HCD referred to during the commenting period.



An intergroup comparison of body weight to controls has been added to a table within the summary of [REDACTED] (2015). The applicant submitted additional HCD which have been included as part of the report amendment.

<b>Report:</b>	K-CA 5.6.2/03 [REDACTED] (2015). SYN545974: Oral (Gavage) Prenatal Developmental Toxicity Study in the Rat. [REDACTED] [REDACTED] Laboratory Report No. [REDACTED], 29 May 2015. Report Amendment 1, 13 February 2018. Unpublished. Syngenta File No. SYN545974_10190.
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<b>Report:</b>	K-CA 5.6.2/04 [REDACTED], (2016) SYN545974 - Historical Control Data for SYN545974_10190, [REDACTED]. Syngenta File No. SYN545974_10453.
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**Guidelines:** Prenatal Developmental Study (rat) OECD 414 (2001): OPPTS 870.3700 (1998): JMAFF 8147 (2000).

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

Following a request of the EU RMS, additional Historical Control Data for some fetal parameters have been provided by the applicant and the information incorporated into the study summary below.

## EXECUTIVE SUMMARY

In a prenatal developmental toxicity study, three groups of 24 time-mated, young adult, female [REDACTED]CD (SD) rats were dosed by oral gavage from Day 6 to Day 19 of gestation with SYN545974 (purity 98.5%) in the vehicle, 1 % carboxymethylcellulose, at dose levels of 10, 30 or 100 mg/kg/day (Groups 2 to 4, respectively). A fourth group of 24 similar animals (Group 1) was dosed with the vehicle only and served as controls. The dose volume used was 10 mL/kg body weight and was calculated from the most recently recorded body weight. Clinical observations, body weight and food intake were recorded throughout the treatment period. Animals were killed and subjected to gross necropsy on Day 20 of gestation. The progress and outcome of pregnancy was assessed and gravid uterus and placenta weights were recorded. Fetuses were removed from the uterus and weighed, their sex was determined and they were examined for external, visceral and skeletal abnormalities.

There were two unscheduled deaths on the study but neither of these deaths was related to treatment with SYN545974. One female given 100 mg/kg/day showed clinical signs of decreased activity, slow breathing, unsteady and abnormal gait, hunched posture, piloerection, partially closed eyes and was cold to the touch on the first day of dosing (Day 6 of gestation) and was sent for necropsy. There were no macroscopic changes to determine the poor physical condition of this animal and, in isolation, this finding was considered not to be related to treatment. Slow and laboured breathing and piloerection were observed for one female given 10 mg/kg/day on Day 15 of gestation, which was sent for necropsy; macroscopic findings determined that the clinical signs were due to dosing trauma. There were no further treatment-related clinical signs.

Although there was an initial reduction in body weight and food intake between Days 6 to 9 of gestation at 100 mg/kg/day, this was resolved and there were no overall adverse effects on these parameters.

There were 24, 21, 24 and 23 females in the groups given 0, 10, 30 and 100 mg/kg/day, respectively, with live fetuses on Day 20 of gestation.

Pregnancy rates were similar in all groups. There were no significant differences in terminal body weight, gravid uterus weight or body weight adjusted for the weight of the gravid uterus for treated animals, when compared with controls. The uterine/implantation data were unaffected by treatment with SYN545974. Fetal and placenta weights were similar in all groups.

The incidences and intergroup distribution of the major, minor and variant fetal abnormalities were considered not to be related to administration of SYN545974.

**Administration of SYN545974 once daily, by oral gavage, to pregnant [REDACTED] CD (SD) rats from Day 6 until Day 19 of gestation inclusive, at dose levels of 10, 30 and 100 mg/kg/day was well tolerated with no clinical signs and no effect on pregnancy or fetal parameters. At 100 mg/kg/day, an initial reduction in body weight gain was observed between the first days of dosing (Day 6 to day 10) in females. Based on the above findings, the No Observed Adverse Effect Level (NOAEL) for maternal was considered to be 30 mg/kg/day and the NOAEL for embryo-fetal development was considered to be the highest tested dose of 100 mg/kg/day.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545974
<b>Description:</b>	Technical, off white powder
<b>Lot/Batch number:</b>	SMU2EP12007
<b>Purity:</b>	98.5 %
<b>CAS#:</b>	1228284-64-7
<b>Stability of test compound:</b>	Retest date 30 June 2016 (stored at <30°C)

**Vehicle and/or positive control:** The test item was formulated as a suspension in 1% (w/v) aqueous carboxymethylcellulose.

<b>Test Animals:</b>	
<b>Species</b>	Rat
<b>Strain</b>	[REDACTED] CD(SD)
<b>Age/weight at dosing</b>	Approximately 9-10 weeks / 224-298 g
<b>Source</b>	[REDACTED]
<b>Housing</b>	Individually in grid-floor cages over paper
<b>Acclimatisation period</b>	At least 2 days
<b>Diet</b>	Pelleted rodent diet, VRF1 (manufactured by [REDACTED]) supplied by [REDACTED] <i>ad libitum</i>
<b>Water</b>	Mains tap water <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 19-23°C Humidity: 40-70% Air changes: Not reported Photoperiod: 12 hours light, 12 hours dark

### Study Design and Methods:

**Experimental dates:** Start: 10 October 2013, End: 20 December 2013

**Mating procedure:** Time-mated females were obtained from the supplier. The day on which mating was detected was designated Day 0 of gestation.

**Animal assignment:** The animals were examined on arrival and were found to be outwardly healthy. They were then acclimatised within the study room for at least two days after arrival after which time they were re-examined to confirm their suitability for use. Allocation to groups was performed using a stratified randomisation procedure based on individual body weights recorded during the acclimatisation period. The cages were positioned in the battery using a specific cage-plan.

#### Animal numbers and treatment groups

Group	Number of animals	Dose level (mg/kg/day)	Dose concentration (mg/mL)
1	24	0 (control)	0
2	24	10	1
3	24	30	3
4	24	100	10

**Dose selection rationale:** An evaluation of the toxicological and kinetic data following oral (gavage and dietary) administration of SYN545974 to rats for up to 90 days, demonstrated a clear, non-linear, exposure in males and females. The non-proportional kinetics of SYN545974 with increasing dose is due to dose limited absorption, therefore, as the external dose is increased the internal exposure does not increase and neither does the exposure to metabolites. Statistical analysis to assess the proportional relationship between pharmacokinetic parameters and dose demonstrated exposure/dose proportionality to be between 5 and 100 mg/kg/day for females. Therefore, 100 mg/kg/day was considered to be an adequate high dose level for this study.

Following a request of the EU RMS, the applicant provided further explanation in order to justify that it was not necessary to test doses up to 1000 mg/kg/day in the developmental rat study. HSE noted that even if the relationship between pharmacokinetic parameters and dose is not proportional, there appears to be an increase of toxicity between 200 and 1000 mg/kg/d regarding the body weight gains. The applicant argued that there was no evidence of toxicity on the dam in any doses (100, 200, 500 or 1000 mg/kg/day) in the preliminary range finding study in the rat as only a transient reduction in body weight gain (gestation day 6-7) was observed at the onset of dosing at 1000 mg/kg/day. As the effect was minimal in magnitude and limited to a single observation, the applicant considered it as a non-specific effect of SYN545974. In addition, toxicokinetic data in the rat was evaluated from a number of studies to identify the point of non-linearity and the applicant considered that the high dose was selected slightly above the inflection point for transition to non-linear toxicokinetic behaviour (see section 6.1). HSE considers this further explanation acceptable. However, it would have been interesting to have toxicokinetics parameters of SYN545974 in pregnant rats derived from this developmental rat study, as it has been done for the rabbit developmental study (see report K-CA 5.6.2/06 [REDACTED] (2015b)).

**Dose preparation and analysis:** The test item was formulated weekly as a suspension in the vehicle, 1% (w/v) aqueous carboxymethylcellulose, separately for each dose level. A weighed quantity of test item was mixed using a pestle with a small quantity of the vehicle in a mortar to form a paste. By progressive additions of vehicle and mixing, this was taken up to near final volume. The mortar was rinsed with vehicle and the rinsings were added to the formulation. Once at final volume, the formulation was mixed using a laboratory homogeniser (this did not apply to Groups 2 and 3 on the first preparation) and then dispensed into aliquots for each day of dosing. These were stored refrigerated (at approximately 4 °C) until the day of use when they were removed from the refrigerator and stirred for at least 15 minutes before the start of dosing and throughout the dosing procedure.

Formulations prepared at concentrations of 1, 20 and 200 mg/mL, spanning the concentrations used in this study (1 to 10 mg/mL), had previously been shown to be stable for at least three days at room temperature,

at least 10 days when refrigerated and at least 32 days when stored frozen at -18 °C, so confirming that the formulations prepared for this study were stable under their conditions of use.

All formulations were sampled and were analysed for SYN545974. Homogeneity was confirmed for the first preparation and therefore subsequent formulations were assessed for achieved concentration only. Samples from the vehicle used to dose the control group were assessed for the absence of test item.

*Analysis results:* Achieved concentrations of the test item formulations used to dose animals on the first and last days of dosing were within 9 % of nominal, with coefficients of variation no greater than 1.8 %, which fulfilled the acceptance criteria ( $\pm 10$  % and  $\leq 5$  % for accuracy and homogeneity, respectively). No SYN545974 was detected in the vehicle used to dose control animals. Test item formulations were, therefore, considered to have been accurately prepared.

**Dosage administration:** Animals were dosed using a rubber catheter and disposable syringe once daily, from Day 6 of gestation to Day 19 of gestation inclusive, at a constant dose volume of 10 mL/kg body weight, adjusted to the most recent body weight.

#### **Observations:**

**Maternal observations:** Animals were examined twice daily for mortality and morbidity. All animals were examined daily for clinical signs of toxicity or changes in behaviour and appearance from the start of treatment.

Day 0 of gestation body weight was recorded by the supplier and body weights were recorded for all females daily from Day 6 to Day 20 of gestation, inclusive. The amount of food consumed by each animal was recorded over Days 6 to 9, 9 to 12, 12 to 15, 15 to 18 and 18 to 20 of gestation.

**Proof of Absorption:** Surviving animals were bled at scheduled necropsy on day 20 of gestation, via the vena cava, immediately after each animal was killed. Samples (0.1 mL) were collected using a hypodermic needle into tubes containing K<sub>2</sub>EDTA anticoagulant. Immediately following collection, 0.05 mL of whole blood was mixed with exactly 0.05 mL of deionised water. The resultant blood:water samples were stored frozen ( $\leq -70$  °C). The blood:water samples, from animals given the test item, were analysed for SYN545974 by LC-MS/MS.

**Necropsy:** Females killed prematurely and surviving females on Day 20 of gestation were killed by exposure to carbon dioxide gas in a rising concentration. At scheduled necropsy on Day 20 of gestation, dead body weight, gravid uterus and placenta weights were recorded. For all females the thoracic and abdominal cavities were opened by a ventral mid-line incision and the major organs examined. Organs or tissues showing any macroscopic abnormalities were removed and retained in fixative.

The uterus of any apparently non-pregnant female was stained with ammonium sulphide to confirm pregnancy status.

For pregnant females the following observations were made: Number of corpora lutea; Number and distribution of implantations in each uterine horn (classified as early intrauterine deaths, late intrauterine deaths, dead fetuses or live fetuses). The live fetuses and their placentae were removed and the uterus and ovaries were retained in neutral buffered formaldehyde.

**Fetal observations:** The following observations were made for live fetuses on Day 20 of gestation: fetal weights; fetal sexes; external abnormalities. Live fetuses were killed by rapid cooling followed by immersion in fixative.

Approximately 50 % of the live fetuses were fixed in Bouin's fluid for subsequent examination of visceral abnormalities using a combined sectioning/dissection technique. The remaining fetuses were briefly fixed in 70 % alcohol and subjected to micro-dissection. The viscera were examined, then the fetuses eviscerated. The carcasses were subsequently cleared in potassium hydroxide, stained with Alizarin red S and Alcian

blue to visualise the ossified skeleton and cartilage and then examined for skeletal variants and abnormalities.

Structural congenital abnormalities that impair the survival or constitution of the fetus were classified as major abnormalities. Other defects were classified as minor abnormalities. Commonly observed variations in the degree of ossification from that expected of a Day 20 gestation fetus together with common variations in the extent of renal pelvic cavitation and ureter dilation were recorded as variants.

**Indices:** The following indices were calculated:

Pre-implantation loss (%) =  $\frac{\text{number of corpora lutea} - \text{number of implantation sites}}{\text{number of corpora lutea}} \times 100$

Post-implantation loss (%) =  $\frac{\text{number of implantation sites} - \text{number of live fetuses}}{\text{number of implantation sites}} \times 100$

**Statistical analyses:** All statistical tests were two-sided with minimum significance levels of 5 % and 1 %. Non-parametric statistics were not routinely conducted. The litter, rather than the fetus, was considered as the experimental unit. When used, Dunnett's test was conducted regardless of the outcome of the analysis of variance (ANOVA) or analysis of covariance (ANCOVA).

Data were examined for unusually high or low values which may influence the statistical analysis and interpretation (possible outliers). After examining for any outliers, if the variances were clearly heterogeneous, transformations (e.g. log, double arcsine or square root) were used in an attempt to stabilise the variances. If the transformations failed, the data set was examined and a decision taken on further action.

Body weight, cumulative body weight gain from the start of dosing, food intake, numbers of corpora lutea, implants, live fetuses, gravid uterus weight, total litter weight and mean fetal weight (sexes separately and combined) were analysed using a parametric ANOVA.

For pre-implantation loss, post-implantation loss, early intra-uterine deaths, late intra-uterine deaths and sex ratios, litter based mean percentages were analysed using a parametric ANOVA, following a double arcsine transformation.

Maternal performance (e.g. the proportion of females with live fetuses at termination, abortions, total resorptions) was analysed by a two tailed Fisher's Exact Test, comparing each treated group to the control group.

The incidence of fetal malformations and developmental variations (external, visceral and skeletal) was summarised as the proportion of fetuses affected, the proportion of litters affected and the proportion of fetuses affected within each litter. The proportions of litters affected were analysed by the exact version of the Cochran-Armitage Test. The percentages of fetuses affected within each litter were analysed by the exact version of the Jonckheere Trend Test. In both cases the tests were performed in a step-wise manner; where a test was significant at the 5 % level, the test was repeated after removing the top dose and then subsequent treatment groups, until only the control group was left. Tests were one-sided looking for increase in treated groups versus the control group.

## RESULTS

### Maternal toxicity:

**Mortality and clinical signs:** There were two unscheduled deaths. One female given 100 mg/kg/day showed clinical signs of decreased activity, slow breathing, unsteady and abnormal gait, hunched posture, piloerection, partially closed eyes and was cold to the touch on the first day of dosing (Day 6 of gestation) and was sent for necropsy. There were no macroscopic changes to determine the poor physical condition of this animal and, in isolation, this finding was considered not to be related to treatment. Slow and laboured breathing and piloerection were observed for one female given 10 mg/kg/day on Day 15 of gestation, which

was sent for necropsy; macroscopic findings determined that the clinical signs were due to dosing trauma. Neither of these deaths was related to treatment with SYN545974. There were no clinical signs considered to be related to treatment with the test item.

**Body weight:** There were no adverse effects on body weight gain for treated females, when compared with controls. An initial reduction in body weight gain was evident between Days 6 (the first day of dosing) and Day 10 of gestation, for females given 100 mg/kg/day, when compared with controls. This was resolved by Day 11 of gestation and overall body weight gain at the high dose level was similar to control values. Terminal body weight adjusted for the weight of the gravid uterus was similar in all groups.

**Table 5.6.2-5: Intergroup comparison of body weight gain (g)**

Day of gestation	Dose Level (mg/kg/day)			
	0	10	30	100
<b>0-6</b>	27.6	30.0 (+9)	27.0 (-2)	28.1 (+2)
<b>6-7</b>	6.1	3.5 (-43)	3.4 (-44)	<b>0.6** (-90)</b>
<b>6-8</b>	11.8	10.9 (-8)	9.4 (-20)	<b>8.0* (-32)</b>
<b>6-9</b>	18.0	17.1 (-5)	16.0 (-11)	<b>14.1* (-22)</b>
<b>6-10</b>	24.1	22.1 (-8)	22.8 (-5)	<b>19.7* (-18)</b>
<b>6-11</b>	31.4	30.5 (-3)	30.7 (-2)	29 (-8)
<b>6-12</b>	38	37.2 (-2)	37.1 (-2)	35.7 (-6)
<b>6-13</b>	44.3	44 (-1)	43.8 (-2)	41.1 (-8)
<b>6-14</b>	51	50.3 (-1)	49.9 (-2)	47.6 (-7)
<b>6-15</b>	59.5	59.5 (0)	59.9 (+1)	57 (-4)
<b>6-16</b>	70.5	71.2 (+1)	69 (-2)	67.2 (-5)
<b>6-17</b>	83.3	85.3 (+2)	81.5 (-2)	79.9 (-4)
<b>6-18</b>	98.9	103.2 (+4)	97.4 (-2)	95 (-4)
<b>6-19</b>	113.8	117.7 (+3)	111.7 (-2)	109.3 (-4)
<b>6-20</b>	132.8	135.7 (+2)	132.3 (0)	128.4 (-3)

( ) percentage difference compared with the control group mean

\* Statistically significant difference from control group mean,  $p < 0.05$

\*\* Statistically significant difference from control group mean,  $p < 0.01$

**Food consumption:** There were no adverse effects on food intake for treated females, when compared with controls. An initial reduction in food intake was evident at 100 mg/kg/day between Days 6 and 9 of gestation ( $p < 0.05$ ), however, food intake was similar to controls thereafter.

#### **Sacrifice and pathology:**

**Gross pathology:** There were no macroscopic findings considered to be related to treatment.

**Caesarean section data:** There were no treatment-related differences in pregnancy rates. There were 24, 21, 24 and 23 females in the groups given 0, 10, 30 or 100 mg/kg/day respectively, with live fetuses on Day 20 of gestation. The uterine/implantation data were unaffected by the administration of SYN545974.

There was no effect of treatment on mean fetal, litter or placenta weights. Statistical analysis of the data did not reveal any significant intergroup differences.

Table 6.6.2-6: Caesarean section observations

Observation	Dose level (mg/kg/day)			
	0 (control)	10	30	100
# Animals Assigned (Mated)	24	24	24	24
# Animals Pregnant	24	21	24	24
# Non pregnant	0	3	0	0
# Intercurrent deaths	0	1	0	1
# Died Pregnant	0	0	0	1
# Died Non pregnant	0	1	0	0
# totally resorbed	0	0	0	0
<i>Corpora Lutea</i> /Dam	14.5	14.1	14.3	14.3
Implantations/Dam	13.8	13.8	13.9	13.6
Total # Litters (viable)	24	21	24	23
Live Fetuses/Dam	13.0	12.9	13.2	12.9
Mean number of early deaths	0.8	0.9	0.7	0.7
Mean number of late deaths	0	0	0	0
Mean Litter Weight (g)	50.93	51.32	52.08	51.99
Mean Fetal Weight (g)	3.93	3.98	3.93	4.06
Mean Fetal Weight Males (g)	4.03	4.08	4.03	4.15
Mean Fetal Weight Females (g)	3.84	3.89	3.83	3.93
Sex Ratio (% Males per litter)	47.8	44.0	51.3	54.5
Pre-implantation Loss (%)	4.5	2.2	2.8	5.4
Post-implantation Loss (%)	6.2	5.8	6.0	5.4
No statistically significant differences from control group means				

**Developmental Toxicity:** Major fetal abnormalities were noted in one fetus from each of the control and 10 mg/kg/day groups, two fetuses from two litters in the group given 30 mg/kg/day and in two fetuses from one litter in the group given 100 mg/kg/day. The one severely malformed fetus in the group given 10 mg/kg/day is a reflection of spontaneous malformation; similarly, exencephaly is known to occur in this strain of rat and its occurrence in two fetuses from the same litter in the group given 100 mg/kg/day, does not indicate an adverse effect of treatment (Table 6.6.2-7).

Table 6.6.2-7: Summary of major fetal abnormalities

Dose (mg/kg/day)	Dam	Fetus	Findings
0	16	L5	Diaphragmatic hernia
10	48	L8	Multiply malformed fetus
30	66	L4	Anophthalmia; orbital cavity reduced in size; malformed cervical neural arch; absent cervical neural arches; bent scapula
30	69	L12	Scapulae severely bent; humeri malformed; femurs bowed
100	82	L1	Exencephaly; open eye; cleft palate; malformed inter-parietals, parietals, frontals and nasals; cleft palatine
		R3	Exencephaly; open eye; malformed palate; malformed parietals and frontals; absent inter-parietals; cleft palatine

Historical control data from the conducting laboratory have been provided by the Applicant. They came from either the original study report of this main developmental rat study (K-CA 5.6.2/03 [REDACTED] (2015)) or from an additional report including additional HCD from the conducting laboratory (K-CA 5.6.2/02 [REDACTED], (2016)) which have been submitted later by the applicant. To complete the database, the applicant has also submitted additional historical control data for the [REDACTED]; CD (SD) rat in the report K-CA 5.6.2/07 ([REDACTED] 2015) presented in section 6.6.2-5.

Major and minor fetal abnormalities and variants from external, visceral and skeletal examinations and all corresponding historical control data are presented in Table 6.6.2-8.

Major fetal malformations observed at 100 mg/kg/day were limited to a single litter (Table 6.6.2-7 and -8) and the malformation incidence was within the historical control data.

There were no statistically significant differences in the overall incidences of minor or variant fetal abnormalities in the treated groups, compared with controls. In the groups given 30 or 100 mg/kg/day, there was a significant ( $p < 0.05$ ) increase, compared with the control group, in the number of litters with fetuses showing the minor abnormality, small area of liver protruding into the thorax. HSE asked for further explanation to the applicant on the reason why this anomaly has been considered by the pathologist as a minor defect while diaphragmatic hernia is a malformation according to DevTox. The applicant replied that small area of liver protruding into the thorax is also described as a hepatodiaphragmatic nodule and is considered a congenital lesion in rats. Whilst they appear to be protruding through the diaphragm and extending into the thoracic cavity, they are actually attached to and covered by a thin fibrous portion of the diaphragm<sup>4</sup>. These nodules have normal hepatic architecture. Therefore, small area of liver protruding into the thorax is classified as a minor abnormality, as there is no hole in the diaphragm and therefore this observation has no consequence on post-natal survival and development. This finding is a background lesion that occurs sporadically and is not affected by treatment<sup>5</sup>. Moreover, the finding did not demonstrate a clear dose response and the study incidence at 100 mg/kg/day was within the range seen in control rats at this laboratory (pooled historical control data for this anomaly from reports K-CA 5.6.2/03 [REDACTED] (2015)) and report K-CA 5.6.2/02 [REDACTED], (2016)) including 18 developmental rat studies performed in the conducting laboratory from August 2007 to December 2015).

There were also significant ( $p < 0.05$ ) increases in the number of litters with fetuses showing the minor abnormality, absent costal cartilage and the variant abnormality, left sided umbilical artery at 100 mg/kg/day. However, the number of fetuses showing these minor or variant abnormalities was very small and within the background data ranges and their incidences do not indicate an adverse effect of SYN545974 on fetal development (Table 6.6.2-8).

<sup>4</sup> Thoolen, B., Maronpot, R.R., Harada, T., Nyska, A., Rousseaux, C., Nolte, T., Malarkey, D.E., Kaufmann, W., Küttler, K., Deschl, U., Nakae, D., Gregson, R., Vinlove, M.P., Brix, A.E., Singh, B., Belpoggi, F., Ward, J.M., (2010). Proliferative and nonproliferative lesions of the rat and mouse hepatobiliary system. *Toxicologic Pathology* (38) 5S-81S.




<sup>5</sup> Eustic, S.L., Boorman, G.A., Harada, T., Popp, J.A., (1990). Liver, In *Pathology of the Fischer Rat*. Academic Press, San Diego, 71-94.



**Table 6.6.2-8: Summary of major and minor fetal abnormalities and variants from external/ visceral/ skeletal examination (F: fetal incidence (group mean %); L: litter incidence (group mean %))****Combined examination (external, visceral, skeletal):**

Observations	Dose levels (mg/kg bw/day)			
	0 (control)	10	30	100
<b>Combined examination (external, visceral, skeletal)</b>				
<i>No. of fetuses (F)</i>	311	271	317	296
<i>No. litters (L)</i>	24	21	24	23
Number with major abnormalities	F: 1 (0.3%) L: 1 (4.2%)	F: 1 (0.3%) L: 1 (4.8%)	F: 2 (1.6%) L: 2 (8.3%)	F: 2 (0.7%) L: 1 (4.3%)
Number with minor abnormalities	F: 59 (19.1%) L: 20 (83.3%)	F: 63 (23.6%) 20/21 litters	F: 73 (23.3%) 22/24 litters	F: 45 (15.1%) 19/23 litters
Number with variants	F: 170 (55.1%) L: 24 (100%)	F: 167 (61.7%) L: 21 (100%)	F: 175 (56.0%) L: 24 (100%)	F: 158 (53.2%) L: 23 (100%)

**External examination**

Observations	Type	Dose levels (mg/kg bw/day)				HCD range: incidence (group mean %)		
		0 (control)	10	30	100	 <sup>a</sup>	 <sup>b</sup>	 <sup>c</sup>
External examination								
No. of fetuses (F)		311	271	317	296			
No. litters (L)		24	21	24	23			
Head: eye- uni- or bilateral: open	Major	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (0.7%) <sup>#</sup> L: 1 (4.3%)	F: 0-1 (0-0.4%) L: 0-1 (0-5.3%)	F: 0-1 (0-0.4%) L: 0-1 (0-5.6%)	F: 0-2 (0-0.32%) L: 0-2 (0-4.2%)
Brain: exencephaly	Major	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (0.7%) <sup>#</sup> L: 1 (4.3%)	-	F: 0-1 (0-0.3%) L: 0-1 (0-5%)	F: 0-2 (0-0.36%) L: 0-2 (0-5%)
Oral cavity: palate: cleft or malformed	Major	F: 0 (0%) L: 0 (0%)	F: 1 (0.3%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 2 (0.67%) <sup>#</sup> L: 1 (4.3%)	F: 0-1 (0-0.4%) L: 0-1 (0-5.3%)	F: 0-1 (0-0.3%) L: 0-1 (0-4.2%)	F: 0-4 (0-0.34%) L: 0-4 (0-4.76%)
Runted foetus	Minor	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.3%) L: 1 (4.2%)	F: 0 (0%) L: 0 (0%)	-	-	-

<sup>#</sup> Two fetuses from the same litter present malformations of the oral cavity (cleft palate/ malformed palate, cleft palatine) associated with malformations of the head (inter-parietals, parietals, frontals and nasals), exencephaly and open eye

**Visceral examination**

Observations	Type	Dose levels (mg/kg bw/day)				HCD range from <div></div> <sup>d/e</sup> (incidence (group mean %))
		0 (control)	10	30	100	
Fresh visceral examination						
No. of foetuses (F)		158	137	159	147	
No. litters (L)		24	21	24	23	
Thoracic cavity: Innominate artery absent	Minor	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.6%) L: 1 (4.2%)	F: 0 (0%) L: 0 (0%)	-
Kidney: ↑ pelvic cavitation	Variant	F: 2 (1.2%) L: 1 (4.2%)	F: 4 (2.8%) L: 3 (14.3%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.6%) L: 1 (4.3%)	-
Dilated ureter	Variant	F: 6 (3.5%) L: 4 (16.7%)	F: 7 (5.0%) L: 4 (19%)	F: 2 (1.4%) L: 2 (8.3%)	F: 3 (2.0%) L: 3 (13%)	-
Umbilical artery (left sided)	Variant	F: 1 (0.6%) L: 1 (4.2%)	F: 2 (1.5%) L: 2 (9.5%)	F: 1 (0.6%) L: 1 (4.2%)	F: 6 (3.8%) L: <b>5 (22%*)</b>	F: 0-8 (0-5.4%) <sup>e</sup> L: 1-7 (12-32%)
Visceral examination (Bouins)						
No. of foetuses (F)		158	137	159	147	
No. litters (L)		24	21	24	23	
Brain: cerebral aqueduct enlarged	Minor	F: 0 (0%) L: 0 (0%)	F: 2 (1.4%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.6%) L: 1 (4.3%)	-
Palate: irregular ridging	Minor	F: 6 (4%) L: 5 (20.8%)	F: 5 (3.4%) L: 5 (23.8%)	F: 9 (5.4%) L: 8 (33.3%)	F: 10 (6.8%) L: 9 (39%)	F: 2-12 (1.3-10.5%) <sup>d</sup> L: 2-11 (11.8-57.9%)
Liver: ≥1 lobe: small area protruding into thorax	Minor	F: 0 (0%) L: 0 (0%)	F: 1 (1.0%) L: 1 (4.8%)	F: 4 (2.6%) <b>L: 4 (16.7%)*</b>	F: 3 (2.1%) <b>L: 3 (13%)*</b>	F: 0-3 (0-2.7%) <sup>e</sup> L: 0-3 (0-15.8%)
Kidney: ↑ pelvic cavitation	Variant	F: 14 (9.5%) L: 9/24 litters	F: 20 (15.4%) 12/21 litters	F: 12 (11.3%) 7/24 litters	F: 11 (7.7%) 7/23 litters	-
Dilated ureter	Variant	F: 18 (12.3%) 12/24 litters	F: 30 (23.3%) 12/24 litters	F: 18 (11.5%) 11/24 litters	F: 12 (7.5%) 8/23 litters	-
Umbilical artery (left sided)	Variant	F: 1 (0.8%) L: 1 (4.2%)	F: 5 (3.2%) L: 4 (19%)	F: 7 (4.6%) L: 6 (25%)	F: 5 (7.5%) L: 4 (17.4%)	F: 0-8 (0-5.4%) <sup>e</sup> L: 1-7 (12-32%)

**Skeletal examination**

Observations		Dose levels (mg/kg bw/day)				HCD range (incidence (group mean %))		
		0 (control)	10	30	100	<div></div> <sup>d</sup>	<div></div> <sup>c</sup>	<div></div> <sup>b</sup>
<b>Skeletal abnormalities and variants</b>								
<i>No. of fetuses (F)</i>		158	137	159	147			
<i>No. litters (L)</i>		24	21	24	23			
Skull	Nasal: incomplete ossification	F: 2 (1.4%) L: 2 (8.3%)	F: 11 (7.7%) L: 7 (33.3%)	F: 1 (0.6%) L: 1 (4.2%)	F: 5 (3.3%) L: 4 (17.4%)	F: 0-6 (0-4.0%) L: 0-12 (0-60%)	-	-
	Frontal: incomplete ossification	F: 1 (0.5%) L: 1 (4.2%)	F: 1 (0.5%) L: 1 (4.8%)	F: 1 (0.6%) L: 1 (4.2%)	F: 2 (1.3%) L: 2 (8.7%)	F: 1-12 (0.9-9.5%) L: 1-8 (5.3-42%)	F: - (0-7.41%) L: - (0-19.05%)	F: 0-1 (0-0.7%) L: 0-1 (0-5%)
	Occipital: bipartite ossification	F: 0 (0%) L: 0 (0%)	F: 1 (0.7%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 2 (1.4%) L: 1 (4.3%)	-	-	-
	Zygomatic arch: incomplete ossification	F: 3 (1.9%) L: 2 (8.3%)	F: 8 (5.7%) L: 4 (19%)	F: 10 (5.9%) L: 7 (29.2%)	F: 4 (2.6%) L: 2 (8.7%)	F: 0-23 (0-17.6%) L: 0- 9 (0-52.9%)	-	-
Vertebra:	No. of presacral vertebra 25	F: 0 (0%) L: 0 (0%)	F: 1 (0.8%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.9%) L: 1 (4.3%)	F: 0-2 (0-1.5%) L: 0-1 (0-5.3%)	F: 0-0.8% L: 0-5%	-
Thoracic vertebra:	No. of vertebra: 12	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.6%) L: 1/23 (4.3%)	F: 0-2 (0-1.4%) L: 0-1 (0-5%)	-	-
Lumbar vertebra	No. of vertebra: 5	F: 0 (0%) L: 0 (0%)	F: 1 (0.8%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.9%) L: 1 (4.3%)	F: 0-2 (0-1.5%) L: 0-1 (0-5.3%)	-	-
	No. of vertebra: 7	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.9%) L: 1 (4.3%)	F: 0-1 (0-0.8%) L: 0-1 (0-4.5%)	-	-
Rib	≥1 short rib (1or13)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 3 (3.2%) L: 3 (12.5%)	F: 1 (0.9%) L: 1 (4.3%)	F: 0-6 (0-5.9%) L: 0-3 (0-15.8%)	-	-
	13 <sup>th</sup> rib: vestigial	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.6%) L: 1 (4.2%)	F: 1 (0.6%) L: 1 (4.3%)	F: 0-3 (0-2.3%) L: 0-2 (0-10.5%)	-	-
	≥1 costal cartilage absent	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (1.2%) L: 2 (8.3%)	F: 2 (1.5%) L: 2 (8.7%)*	F: 1-5 (0.7-1.6%) L: 1-2 (4.5 -9.5%)‡	-	-
Sternum	2 <sup>nd</sup> sternebra: incomplete ossification	F: 0 (0%) L: 0 (0%)	F: 2 (1.5%) L: 2 (9.5%)	F: 5 (2.9%) L: 4 (15.7%)	F: 1 (0.5%) L: 1 (4.3%)	F: 0-15 (0-12%) L: 0-7 (0-36.8%)	-	-
	4 <sup>th</sup> sternebra: incomplete ossification	F: 0 (0%) L: 0 (0%)	F: 1 (1.0%) L: 1 (4.8%)	F: 2 (1.2%) L: 1 (4.2%)	F: 1 (0.7%) L: 1 (4.3%)	F: 0-10 (0-8.2%) L: 0-7 (0-36.8%)	-	-
Pelvic girdle	entire: asymmetric insertion	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (2.1%) L: 1 (4.2%)	F: 1 (0.9%) L: 1 (4.3%)	F: 0-1 (0-1.3%) L: 0-1 (0-5.3%)	-	-
	Pubic symphysis: cartilage incomplete	F: 1 (0.6%) L: 1 (4.2%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (1.3%) L: 2 (8.7%)	-	-	-
Forelimb	Metacarpal: ≥1 1-4 <sup>th</sup> incomplete ossification	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 1 (0.6%) L: 1 (4.3%)	F: 0-11 (0-8.9%) L: 0-4 (0-21%)	-	-
<b>Variants</b>								
Skull	Cartilaginous supra-occipital: hole	F: 1 (0.7%) L: 1 (4.2%)	F: 1 (0.6%) L: 1 (4.8%)	F: 0 (0%) L: 0 (0%)	F: 3 (2.1%) L: 2 (8.7%)	-	-	-
	Cartilaginous styloid process: incomplete ossification	F: 4 (2.8%) L: 4 (16.7%)	F: 9 (6.3%) L: 7 (33.3%)	F: 3 (1.8%) L: 3 (12.5%)	F: 10 (7.9%) L: 9 (39%)	-	-	-
Cervical vertebra	≥1 centra ossified (1-3)	F: 3 (1.7%) L: 3 (12.5%)	F: 0 (0%) L: 0 (0%)	F: 4 (2.3%) L: 4 (16.7%)	F: 5 (3.5%) L: 4 (17.4%)	F: 1-17 (0.9-13.2%) L: 1-10/20 (5.3-50%)	-	-
	1 <sup>st</sup> Cartilagenous dorsal arch: not fused	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 0 (0%) L: 0 (0%)	F: 2 (1.4%) L: 1 (4.3%)	-	-	-

	Additional cartilagenous ventral plate on 5 <sup>th</sup> cervical vertebra	F: 2 (1.5%) L: 2 (8.3%)	F: 4 (3.2%) L: 4 (19%)	F: 1 (0.5%) L: 1 (4.2%)	F: 4 (2.6%) L: 4 (17.4%)	-	-	-
Thoracic vertebra	≥1 centra:dumbbell ossification	F: 59 (37.1%) L: 22 (91.7%)	F: 62 (45.1%) L: 18 (85.7%)	F: 58 (36.2%) L: 19 (79.2%)	F: 60 (39.3%) L: 20 (86.9%)	F: 20-71 (12.6-49.0%) L: 11-20 (55-100%)	-	-
Sternum	5 <sup>th</sup> sternebra: incomplete ossification	F: 24 (16.1%) L: 15 (62.5%)	F: 28 (19.9%) L: 16 (76.2%)	F: 27 (16.1%) L: 13 (54.2%)	F: 26 (16.8%) L: 13 (56%)	F: 10-37 (7.5-25.9%) L: 6-19 (31.5-86.4%)	-	-
	Xiphoid cartilage: bifurcated	F: 5 (3.1%) L: 4 (16.7%)	F: 5 (3.7%) L: 3 (14.3%)	F: 8 (5.2%) L: 5 (20.8%)	F: 6 (4%) L: 5 (21.7%)	F: 18/147 (11.7%) L: 11/22 (50%) <sup>‡</sup>	-	-
	Xiphoid cartilage: hole in centre	F: 9 (5.4%) L: 7 (29.2%)	F: 6 (4.4%) L: 5 (23.8%)	F: 13 (7.7%) L: 9 (37.5%)	F: 11 (7.1%) L: 7 (30.4%)	F: 3/147 (2.2%) L: 3/22 (13.6%) <sup>‡</sup>	-	-

\*Statistically significant difference from control group P<0.05 Jonckheere test and/or Cochran-armitage test.

<sup>a</sup> Conducting laboratory HCD in the [REDACTED] Sprague Dawley ([REDACTED]: CD[SD]) strain) : 13 studies performed from Nov 2008 to Nov 2012 including a total of 3206 fetuses and 252 litters (source: [REDACTED] 2015)

<sup>b</sup> Additional HCD in the [REDACTED] Sprague Dawley ([REDACTED]: CD[SD]) strain from [REDACTED] : 36 studies performed from June 2011 to June 2013 including a total of 11543 fetuses and 823 litters (source: [REDACTED] 2015)

<sup>c</sup> Additional HCD in the [REDACTED] Sprague Dawley ([REDACTED]: CD[SD]) strain from [REDACTED] : 118 studies performed from 1996 to 2013 including 35538 fetuses and 2608 litters (source: [REDACTED] 2015)

<sup>d</sup> Additional HCD in the [REDACTED] Sprague Dawley ([REDACTED]: CD[SD]) strain from [REDACTED] laboratory (conducting laboratory): 15 studies performed from Nov 2008 to Dec 2015 including 1542 fetuses and 311 litters (source: [REDACTED] 2016)

<sup>e</sup> Additional HCD in the [REDACTED] Sprague Dawley ([REDACTED]: CD[SD]) strain from [REDACTED] laboratory (conducting laboratory): 18 studies performed from August 2007 to dec 2015 including 2558 fetuses and 357 litters (sources: [REDACTED] 2015 and [REDACTED] 2016)/ <sup>‡</sup>: this skeletal findings was examined in only 3 studies over the 18 performed from Nov 2008 to dec 2015.

Following a request from EFSA (February 2018), additional HCD have been submitted by the applicant. This additional historical control data in the █: CD (SD) rat were from pre-natal developmental toxicity studies after consolidation of other contract research organisations (CRO's) under █ since 2016. According to the information submitted by the applicant, it seems that these additional HCD were from two other developmental studies in rat performed in July and October 2015. Some difficulties were accounted by HSE to assess these data. Indeed, it is not clear if these data were from the conducting laboratory or from other █ and some inconsistencies have been highlighted. Only the incidence for cleft palatine and bent scapula were presented in a summarized table by the applicant (Table 6.6.2-9). However, it should be noted that this additional information does not impact the technical interpretation of the study data and conclusions.

**Table 6.6.2-9: New Historical Control Data (2007-2015) provided by the applicant after the commenting period - Major fetal abnormalities**

Date	No litters	No foetuses	Skeletal	
			Cleft palatine	Bent scapula
Aug 2007	311	25		
Aug 2007	270	21		
Oct 2008	117	19		
Jan 2009	106	17		
Mar 2009	120	19		
Jul 2009	113	20		
Jan 2010	147	22		
Jan 2011	134	19		
May 2011	124	19	1	
Nov 2011	121	20		
Jan 2012	134	20		
Apr 2012	155	20		
Jul 2012	48	7		1
Jul 2012	133	20		
Sep 2012	134	19		
Oct 2012	127	20		
Nov 2012	130	20		1
Jul 2015	122	20		
Oct 2015	152	20		1
Total	377	2835	1 1/377 litters	3 3/377 litters
Range (min-max)			0-1	0-1

Regarding cleft palate finding observed at 100 mg/kg bw/day, HSE would emphasise that these malformations were observed in 2 fetuses from the same litter and these two fetuses presented also other malformations: malformed palate, malformation of the head (inter-parietals, parietals, frontals and nasals), exencephaly and open eye. This increase incidence was within the HCD range provided by the conducting laboratory for litter incidence regarding cleft palate/malformed palate and open eye (Table 6.6.2-8). In addition, no major fetal abnormalities were observed up to 1000 mg/kg bw/d in the preliminary developmental rat study. This finding (cleft palate) was not considered treatment related by HSE.

## CONCLUSION:

In a GLP and guideline PNDT study in SD rats given gavage doses of 0, 10, 30 or 100 mg/kg bw/d pydiflumetofen, statistically significant effects on maternal body weight gain (by 18-90%) and food consumption were seen during gestation days 6-10 at the top dose. None of the developmental findings were considered treatment related. A number of malformations (e.g. exencephaly in 2 foetuses from the same top dose litter) and variations (including ribs with 1 or more absent costal cartilage) observed at 30 and 100 mg/kg bw/d were either not dose-related or within the HCD ranges from animals from the same supplier (██████████) and had not been seen in the dose-ranging study up to the much higher dose of 1000 mg/kg bw/d. Overall, there was no developmental toxicity in the rat. A marginal **NOAEL of 30 mg/kg bw/d** was identified for **maternal toxicity** and a **NOAEL of 100 mg/kg bw/d** was identified for **developmental toxicity**.

HSE notes that the top dose caused insufficient maternal toxicity and questions the adequacy of the study. Although kinetic data (see ADME section) had shown that the systemic dose became non-linear at 100 mg/kg/bw/d, it still increased at higher doses. Therefore, a much higher dose should have been employed to ensure a full investigation of the developmental toxicity potential of pydiflumetofen in the rat.

(██████████, 2015)

## Additional information including HCD on rat PNDT studies

### EFSA Request for additional information (February 2018), Question 30:

Applicant to provide a technical statement and additional appropriate control data (from the conducting laboratory) regarding exencephaly observed in the rat developmental toxicity study.

### Syngenta Response:

The exencephaly observed in the rat developmental toxicity study (██████████, 2015) is considered to be spontaneous in origin and has been observed for this strain in control females at similar incidences. This malformation lacks a clear dose-response relationship, statistical significance when compared with the controls, and is not apparent in the previous preliminary dose-range finding study (██████████, 2011) at significantly higher doses of Pydiflumetofen (100, 200, 500 and 1000 mg/kg bw/d) and therefore demonstrates a distinct lack of dose concordance.

<b>Report:</b>	K-CA 5.6.2/05 ██████████ (2018). Pydiflumetofen - Additional Historical Control Data to Support the Prenatal Developmental Toxicity Study in the Rat. ██████████ ██████████. Unpublished. Syngenta File No. SYN545974_10623.
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**Guidelines:** Not applicable.

**GLP:** This report contains an assessment of data from completed studies and additional scientific information and is not a study per se, a Good Laboratory Practice (GLP) statement as defined by OECD is not appropriate.

## EXECUTIVE SUMMARY

This document refers to additional Historical Control Data (HCD) in the [REDACTED]CD® (SD) rat from pre-natal developmental toxicity studies due to the consolidation of other contract research organisations (CRO's) under [REDACTED] since 2016, and additional external literature HCD which were not included in the original submission.

## RESULTS

### Historical control data comparison with MARTA/[REDACTED] databases and external literature for foetal malformation in the rat:

Historical control data (HCD) from the conducting laboratory are available within the study report for the rat ([REDACTED], 2015). Additional HCD have been included in the study report as references ([REDACTED], 2014; [REDACTED], 2014). These references refer to HCD in the [REDACTED]:CD® (SD) rat from [REDACTED] (study dates June 2011 to June 2013) and Montreal, Canada (study dates 1996 to 2013), which further establishes spontaneous malformations in this strain of rat from the same breeders ([REDACTED], 2015).

However, further HCD in the [REDACTED]:CD® (SD) rat is now available ([REDACTED], 2018; [REDACTED], 2018; [REDACTED], 2018) due to the consolidation of other CRO's under [REDACTED] since 2016, and additional external literature HCD has also been included in this document (Ema, et al., 2014; MARTA, 1996). This wider and extensive HCD demonstrates that the spectrum of malformations apparent in the Pydiflumetofen prenatal developmental toxicity study in the rat at both 30 or 100 mg/kg bw/day are considered to be spontaneous in origin and have been observed for this strain in control females at similar incidences within the historical control data, and therefore, are considered to be incidental to Pydiflumetofen administration.

These foetal malformations lack a clear dose-response relationship, statistical significance when compared with the controls, and were not apparent in the previous preliminary dose-range finding study ([REDACTED], 2011) at significantly higher doses of Pydiflumetofen (100, 200, 500 and 1000 mg/kg bw/d) and therefore demonstrate a distinct lack of dose concordance.

In a typical study using twenty or so pregnant rats per group, there are likely to be only a few fetuses with malformations, and the incidence of each specific malformation even lower. It is not uncommon in the conduct of developmental toxicity studies to observe one or two instances of a malformation in a single group. If these occur in the control then it is obvious that they are attributable to spontaneous background, but if they occur in a treatment group the interpretation is more difficult. Therefore, it is customary to rely on historical control datasets to aid in the interpretation. Contract laboratories and large industry laboratories compile their own historical control data. However, even very active laboratories are limited in the number of studies they can draw from to compile these data, which is clearly a issue with the current HCD at [REDACTED] which only contains only 6108 fetuses and 428 litters in rat pre-natal developmental toxicity studies in the [REDACTED]CD® (SD) rat from 2007 to 2015 as the preferred rat strain for developmental toxicity studies at [REDACTED] is now the [REDACTED]WI (Han) rat for which there is a far large database (>10000 fetuses).

While the HCD within a specific laboratory are useful, they still represent a small sample size for reliably estimating the incidence of relatively rare spontaneous events. Therefore, recognizing this limitation, the Middle Atlantic Reproduction and Teratology Association (MARTA) compiled a large data set from a survey of 21 member organizations for the period of 1989 to 1992. In addition, the Japanese Teratology Society has also compiled a large data set from data obtained from 19 laboratories in Japan, including 10 pharmaceutical and chemical companies and nine contract research organizations for the period 1994 to 2010. Data were summarized separately between 1994 and 2000 and between 2001 and 2010.

These historical control databases consisted of 6102 litters and 88270 fetuses in the [REDACTED]CD® (SD) rat for the period of 1989 to 1992 (MARTA, 1996), and 5747 litters and 79960 fetuses in the [REDACTED]CD® (SD) rat

(Ema et al., 2014) for the period 2001 to 2010 which are sufficiently large enough to provide reasonable estimates for the rates of many individual malformations.

**Table 6.6.2-10: A Comparison of Foetal Gross External Malformations from the Rat Developmental Toxicity Study with SYN545974 (■■■■■, 2015) and HCD Data in ■■■■ CD [SD] Rats**

		Reference	■■■■■	■■■■■	■■■■■	■■■■■	MARTA (MARTA, 1996)	Ema et al., 2014
		Year	2007 to 2015	2007 to 2017	2012-2017	2016-2017	1988-1991	2001-2010
		Strain	■■■■■ CD [SD]	■■■■■ CD [SD]	■■■■■ OFA [SD]	■■■■■ CD [SD]	■■■■■ D [SD]	■■■■■ CD [SD]
Malformation	Study incidence	Dose (mg/kg bw/day)						
Anophthalmia	1/21 litter 1/271 foetuses	30	0/478 litters 0/6108 foetuses	2/1462 litters 2/19277 foetuses	1/563 litters 1/2158 foetuses	0/288 litters 0/3628 foetuses	16/6102 litters 16/88270 foetuses	10†/5702 litters 10/39192 foetuses
Exencephaly	1/24 litters 2/296 foetuses	100	0/478 litters 0/6108 foetuses	1/1462 litters 1/19277 foetuses	1/563 litters 1/7079 foetuses	0/288 litters 0/3628 foetuses	16/6102 litters 26/88270 foetuses	7†/5747 litters 7/79960 foetuses
Open eye	1/24 litters 2/296 foetuses	100	1/478 litters 1/6108 foetuses	1/1462 litters 1/19277 foetuses	0/563 litters 0/7079 foetuses	0/288 litters 0/3628 foetuses	6/6102 litters 9/88270 foetuses	4†/5747 litters 4/79960 foetuses
Cleft palate	1/24 litters 1/296 foetuses	100	2/478 litters 2/6108 foetuses	2/1462 litters 2/19277 foetuses	0/563 litters 0/7079 foetuses	1/288 litters 3/3628 foetuses	9/6102 litters 9/88270 foetuses	9†/5747 litters 9/79960 foetuses
Malformed palate (High arched palate)	1/24 litters 1/296 foetuses	100	1/478 litters 1/6108 foetuses	0/1462 litters 0/19277 foetuses	0/563 litters 0/7079 foetuses	0/288 litters 0/3628 foetuses	2/6102 litters 4/88270 foetuses	0/5747 litters 0/79960 foetuses
Malformed or absent nares	1/24 litters 1/296 foetuses	100	0/478 litters 0/6108 foetuses	1/1462 litters 1/19277 foetuses	0/563 litters 0/7079 foetuses	0/288 litters 0/3628 foetuses	5/6102 litters 5/88270 foetuses	4†/5747 litters 4/79960 foetuses

**CONCLUSION:** In conclusion, this wider and extensive HCD demonstrates that the spectrum of malformations apparent in the Pydiflumetofen prenatal developmental toxicity study in the rat at both 30 or 100 mg/kg bw/day are considered to be spontaneous in origin and have been observed for this strain in control females at similar incidences within the historical control data, and therefore, are considered to incidental to Pydiflumetofen administration.

These foetal malformations lack a clear dose-response relationship, statistical significance when compared with the controls, and were not apparent in the previous preliminary dose-range finding study (■■■■■, 2011) at significantly higher doses of Pydiflumetofen (100, 200, 500 and 1000 mg/kg bw/d) and therefore demonstrate a distinct lack of dose concordance.

## REFERENCES:

■■■■■, E. (2015). SYN545974 - Additional Historical Control Data to Support Developmental Toxicity Studies in Rats. ■■■■■, ■■■■■.

■■■■■. Laboratory Report No. TK0103655. 30 July 2015. Unpublished. Syngenta File No. SYN545974\_10244.

■■■■■ (2015). SYN545974: Oral (Gavage) Prenatal Developmental Toxicity Study in the Rat. ■■■■■. ■■■■■. Laboratory Report No. ■■■■■, 29 May 2015. Unpublished. Syngenta File No. SYN545974\_10190.

Ema, M., Endoh, K. Fukushima, R., Fujii, S., Hara, H., Hirata-Koizumi, M., Hirose, A., Hojo, H., Horimoto, M., Hoshino, N., Hosokawa, Y., Imai, Y., Inada, H., Inawaka, K., Itoh, K., Katsumata, Y., Izumi, H., Kato, H., Maeda, M., Matsumoto, K., Matsuo, S., Matsuoka, T., Matsuura, I., Mineshima, H., Miwa, Y., Nakano, N., Naya, M., Noyori, H., Ohta, T., Oku, H., Ono, A., Shimizu, T., Shimomura, K., Takakura, I., Tanaka, R., Tateishi, T., Tominaga, Y., Uesugi,



T., Urakawa, C., Yabe, K., Yamashita, A., Yamauchi, T., and Yokoi, R. (2014). *Historical Control Data on Developmental Toxicity Studies in Rodents. Congenital Anomalies* 54. pp150-161.

██████████ (2011). SYN545974, SYN546022: Preliminary Oral (Gavage) Prenatal Developmental Toxicity Dose Range Finding Study in the Rat. ██████████ Laboratory Report No. ██████████, 26 October 2011. Unpublished. Syngenta File No. SYN545974\_10003.

██████████. (2018). *Historical Control Data in Rats. ██████████ Embryo-fetal Development Studies, ████ OFA[SD]. 2012-2017.*

MARTA (1996). MARTA, *Historical control data*, in: Hood R.D. (Ed.), *Handbook of Developmental Toxicology*, CRC Press, London, United Kingdom, pp.713-733.

██████████ (2018). *Reproductive and Juvenile Toxicology Historical Control Data in Rats. ██████████ Embryo-fetal Development Studies, CD® IGS (██████:CD[SD]). 2007 to 2017.*

██████████ (2018). *Historical Control Data in Rats. ██████████ Embryo-fetal Development Studies, CD® IGS (██████:CD[SD]). 2016 to 2017.*

(██████████, 2018)

### EU RMS (July 2018):

The applicant provided a comparison table of foetal gross external malformations from the rat developmental toxicity study with pydiflumetofen (██████████ 2015) and HCD data in ██████████:CD(SD rats from other sources (Table 6.6.2-10). Unfortunately, this table includes only the mean litter incidences and the foetus incidences and the min/max range of this HCD were not provided. In addition, some documents cited as reference in the position paper of ██████████ 2018 (K-CA 5.6.2/05) and included in this table, have not been submitted (MARTA 1996, ██████████ 2018, ██████████ 2018, Ema 2014). Therefore, the information provided in these documents cannot be checked since the raw data were not available.

However, to clarify the situation, the EU RMS would like to remind that the Historical Control Data presented in the table 6.6.2-8 were from three different origins and these HCD have been checked by the EU RMS:

- ██████████: corresponds to the HCD from the conducting laboratory. These data were available within the study report for the development toxicity study in the rat (██████████ 2015). These HCD were from 13 studies performed from November 2008 to November 2012 including a total of 3206 fetuses and 252 litters.

After the commenting period (July 2018), HCD from a fourteen study dated from July 2015 has been provided by the applicant. However, this additional information does not impact the technical interpretation of the study data and conclusions.

- ██████████: corresponds to additional HCD which have been submitted by the applicant (January 2017), after a request from the EU RMS. These data were from developmental toxicity studies performed in CR Sprague Dawley (██████: CD(SD)) in ██████████ (sources: ██████████, 2015 and ██████████, 2014). More specifically, these HCD were from 36 studies performed from June 2011 to June 2013 and including a total of 11543 fetuses and 823 litters.
- ██████████: corresponds to additional HCD which have been submitted by the applicant (January 2017), after a request from the EU RMS. These data were from developmental toxicity studies performed in CR Sprague Dawley (██████: CD (SD)) in ██████████ (sources: ██████████, 2015 and ██████████, 2014). More specifically, these HCD were from 118 studies performed from 1996 to 2013 and including a total of 35538 fetuses and 2608 litters.

Regarding exencephaly finding observed at 100 mg/kg bw/day, the EU RMS would reminded that these malformations were observed in 2 fetuses from the same litter and these two fetuses presented also other malformations: cleft palate, malformed palate, malformation of the head (inter-parietals, parietals,

frontals and nasals), exencephaly and open eye. This increase incidence was within the HCD range for litter incidence provided by other [REDACTED] regarding exencephaly (Table 6.6.2-8). In addition, no major fetal abnormalities were observed up to 1000 mg/kg bw/d in the preliminary developmental rat study. This finding (exencephaly) was not considered treatment related by the EU RMS.

**Report:** K-CA 5.6.2/07 [REDACTED] (2015). SYN545974: Additional Historical Control Data to Support Developmental Toxicity Studies in the Rat. [REDACTED]  
[REDACTED] Laboratory Report No. TK0103655, 30 July 2015.  
Unpublished. Syngenta File No. SYN545974\_10244.

**Guidelines:** Not applicable.

**GLP:** This report contains an assessment of data from completed studies and additional scientific information and is not a study per se, a Good Laboratory Practice (GLP) statement as defined by OECD is not appropriate.

## EXECUTIVE SUMMARY

This report compares study data from a developmental toxicity study in the [REDACTED] CD[SD] strain of rat with additional historical control (HCD) data from other laboratories utilising the same strain from [REDACTED].

A small number of fetuses in groups receiving SYN545974 in a developmental toxicity study in the rat had gross external and skeletal malformations as reported in [REDACTED] (2015) (two fetuses from two litters in the group given 30 mg/kg bw/day and two fetuses from one litter in the group given 100 mg/kg bw/day). The malformations are of low incidence and following a comparison of the incidence with HCD, the malformations are considered to be spontaneous and not indicative of an adverse effect of treatment, as the incidence is within the background data ranges in this strain of rat.

## RESULTS

### A Comparison of Malformations in the [REDACTED] CD [SD] Rat with HCD: Fetal Gross External Malformations

A small number of fetuses in groups receiving SYN545974 had gross external malformations as reported in [REDACTED] (2015) (two fetuses from two litters in the group given 30 mg/kg bw/day and two fetuses from one litter in the group given 100 mg/kg bw/day). A comparison of the fetal and litter incidence of gross external malformations with background data in this strain of rat is shown in Table 6.6.2-17.

**Table 6.6.2-17: A comparison of fetal gross external malformations from the rat developmental toxicity study with SYN545974 ([REDACTED], 2015) and HCD data in [REDACTED]: CD [SD] rats**

Malformation	Study incidence	Dose (mg/kg bw/day)	[REDACTED] HCD	[REDACTED]	[REDACTED]
Anophthalmia	1/21 litter 1/271 fetuses	30	0	8/2608 litters 8/35538 fetuses	NR
Exencephaly	1/24 litters 2/ 296 fetuses	100	0	2/2608 litters 2/35538 fetuses	2/823 litters 2/11453 fetuses
Open eye	1/24 litters 2/ 296 fetuses	100	1/252 litters 1/3206 fetuses	2/2608 litters 2/35538 fetuses	1/823 litters 1/11453 fetuses
Cleft palate	1/24 litters 1/296 fetuses	100	2/252 litters 2/3206 fetuses	4/2608 litters 4/35538 fetuses	2/823 litters 2/11453 fetuses
Malformed or absent nares	1/24 litters 1/296 fetuses	100	0	1/2608 litters 1/35538 fetuses	NR

NR – Not reported

### Fetal Skeletal Malformations

A small number of fetuses in groups receiving SYN545974 had skeletal malformations as reported in (2015) (two fetuses from two litters in the group given 30 mg/kg bw/day and two fetuses from one litter in the group given 100 mg/kg bw/day). A comparison of the fetal and litter incidence of skeletal malformations with background data in this strain of rat is shown in Table 6.6.2-18.

**Table 6.6.2-18: A comparison of fetal skeletal malformations from the rat developmental toxicity study with SYN545974 (, 2015) and HCD data in CD [SD] rats**

Malformation	Study incidence	Dose (mg/kg bw/day)	HCD		
Orbital cavity reduced in size	1/21 litters 1/271 fetuses	30	1/68 litters 1/728 fetuses		5/672 litters 5/4814 fetuses
Malformed cervical neural arch (also known as cervical vertebral arch)	1/21 litter 1/271 fetuses	30	0	NR	NR
Absent cervical neural arches (also known as cervical vertebral arch)	1/21 litters 1/271 fetuses	30	2/68 litters 3/728 fetuses (NB 1 or more absent)	2/1801 litters 2/11982 fetuses	NR
Scapulae severely bent	1/21 litters 1/271 fetuses	30	1/233 litters 1/1648 fetuses	1/1801 litters 2/11982 fetuses	1/672 litters 1/4814 fetuses
Humerii malformed	1/21 litters 1/271 fetuses	30	0	NR	NR
Femurs bowed or bent	1/21 litters 1/271 fetuses	30	0	2/2607 litters 3/17933 fetuses	NR
Malformed parietals	1/24 litters 1/296 fetuses	100	0	NR	NR
Malformed frontals	1/24 litters 2/ 296 fetuses	100	0	NR	NR
Cleft palatine	1/24 litters 2/ 296 fetuses	100	1/233 litters 1/1648 fetuses	NR	NR
Malformed or misshapen interparietal	1/24 litters 1/296 fetuses	100	0	1/1801 litters 1/11982 fetuses	NR
Malformed or absent nares	1/24 litters 1/296 fetuses	100	0	1/2608 litters 1/35538 fetuses	NR
Malformed, irregular shaped or absent palate	1/24 litters 1/296 fetuses	100	1/252 litters 1/3206 fetuses	4/2606 litters 4/17787 fetuses	1/672 litters 1/4814 fetuses
Absent interparietal	1/24 litters 1/296 fetuses	100	0	NR	NR

NR – Not Reported

## CONCLUSION:

Exencephaly observed at 100 mg/kg bw/d in 2 fetuses from the same litter in the rat study by (2015) was within the HCD ranges for litter incidence from animals from the same supplier ( ). Notwithstanding this, exencephaly was not seen in the preliminary study up to a much higher dose of 1000 mg/kg bw/d. The finding was not considered treatment-related.

Malformations seen in two foetuses from two litters at 30 and 100 mg/kg bw/d were also within thye HCD ranges from animals from the same supplier ( ) and were not considered treatment-related.

(, 2015)

### B.6.6.2.1. Rabbit studies

#### Range-finding study

<b>Report:</b>	K-CA 5.6.2/06 [REDACTED] (2015a). SYN545974: Preliminary Oral (Gavage) Prenatal Developmental Toxicity Study in the Rabbit (final report amendment 2). [REDACTED] [REDACTED]. Laboratory Report No. [REDACTED], 08 June 2015. Unpublished. Syngenta File No. SYN545974_10192.
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**Guidelines:** Not applicable.

**GLP:** This study was conducted in a Good Laboratory Practice (GLP) compliant facility to a standard based on GLP, however, the study was not conducted according to good laboratory practice standards as defined by OECD. It was not subjected to a quality assurance audit. No claim of GLP compliance was made for this study. The final report fully and accurately reflects the raw data generated during the conduct of the study.

## EXECUTIVE SUMMARY

This preliminary study was designed to investigate the effects of the test item, SYN545974 (purity 99.3% and 98.5%) on the pregnant rabbit and the developing organism when administered by oral gavage daily from Day 6 to Day 27 of gestation, inclusive, in order to select dose levels for a subsequent prenatal developmental toxicity study.

Forty time-mated female New Zealand White rabbits were divided into four groups and dosed orally by gavage, at dose levels of 0, 250, 500 and 1000 mg/kg/day (Groups 1 to 4, respectively) from Days 6 to 27 of gestation with SYN547974 in the vehicle, 1% carboxymethylcellulose. Following a review of the data and to investigate the potential of a treatment related effect on pregnancy at the high dose level, a further 2 groups were added to the study: one group dosed at 1000 mg/kg/day (Group 6) and a group to serve as concurrent controls (Group 5). The dose volume used was 5 mL/kg body weight and was calculated from the most recently recorded body weight.

Clinical observations and body weight were recorded daily throughout the treatment period. Food consumption was recorded daily over Days 4 to 6 of gestation and twice weekly thereafter. All animals were killed as scheduled on Day 28 of gestation and a necropsy was performed, where the major organs were examined. The progress and outcome of pregnancy was assessed and gravid uterus and placenta weights were recorded. The fetuses were removed from the uterus, weighed, the sex determined and then examined for external and visceral abnormalities.

There were no deaths or clinical signs at any dose level that were considered to be related to toxicity of the test item. There was a decrease in body weight gain over Days 6 to 28 of gestation for females given 1000 mg/kg/day (Group 6 only). There was no effect of treatment on food consumption.

There were 10, 9, 9, 5, 9 and 8 females with live fetuses in Groups 1, 2, 3, 4, 5 and 6, respectively, at scheduled termination. There was no effect of treatment at any dose level on the incidence of post-implantation loss, the number of live fetuses per female or on fetal or placental weights.

There were no findings at necropsy at any dose level considered to be related to treatment with the test item and there was no effect of treatment on the overall incidence of major or minor fetal abnormalities and / or variants.

**Administration of SYN545974, once daily, by oral gavage, to pregnant New Zealand White rabbits from Day 6 to 27 of gestation at dose levels up to 1000 mg/kg/day was generally well tolerated, with a decrease in maternal body weight gain at 1000 mg/kg/day. There were no fetal findings at a dose level of 1000 mg/kg/day. The maternal NOAEL was 500 mg/kg/day and the NOAEL for developmental toxicity was 1000 mg/kg/day.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545974
<b>Description:</b>	Technical, white to off white powder
<b>Lot/Batch number:</b>	2637-AA/110 (delivered to testing lab 06 October 2011), SMU2EP12007 (delivered 13 July 2012)
<b>Purity:</b>	99.3% (2637-AA/100); 98.5 % (SMU2EP12007)
<b>CAS#:</b>	CA1228284-64-7
<b>Stability of test compound:</b>	Retest dates 31 July 2013 (2637-AA/110); 30 June 2016 (SMU2EP12007) stored at room temperature in the dark with a desiccant

**Vehicle and/or positive control:** The test item was formulated as a suspension in 1% aqueous carboxymethylcellulose, 500 mPa.s.

<b>Test Animals:</b>	
<b>Species</b>	Rabbit
<b>Strain</b>	New Zealand White
<b>Age/weight at day 0 gestation</b>	Approximately 4 months / 3.00-4.03 kg
<b>Source</b>	
<b>Housing</b>	Females were housed individually in perforated-floor cages over paper lined trays.
<b>Acclimatisation period</b>	At least 2 days
<b>Diet</b>	Pelleted diet, Rabbit Diet (manufactured by ) supplied by , ad libitum
<b>Water</b>	Mains tap water <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 16-20°C Humidity: 51-85% Air changes: Not reported Photoperiod: 12 hours light, 12 hours dark

### Study Design and Methods:

**Experimental dates:** Start: 06 February 2012, End: 30 August 2012

**Mating procedure:** Mating was conducted at the supplier's premises and the day on which mating was observed was designated Day 0 of gestation. Each female was paired with a sexually mature stud male rabbit of the same strain and given an intravenous injection of 25IU chorionic gonadotrophin or luteinising hormone to stimulate ovulation. Females were delivered to by Day 4 of gestation.

**Animal assignment:** On arrival, animals were allocated using a stratified randomisation procedure based on body weights recorded on Day 0 of gestation at the supplier (making sure that females mated with the same male were spread across the groups). Each animal was individually identified by a numbered ear tag. The cages were positioned in the battery using a specific cage-plan.

**Animal numbers and treatment groups**

Group	Number of animals	Dose level (mg/kg/day)	Dose volume (mL/kg body weight)
1	10	0 (control)	5
2	10	250	5
3	10	500	5
4	10	1000	5
5	10	0 (control)	5
6	10	1000	5

Four groups (Groups 1 to 4) were allocated to the study in the first instance. Following a review of the data and to investigate the potential of a treatment related effect on pregnancy at the high dose level, a further 2 groups were added to the study: one group dosed at 1000 mg/kg/day (Group 6) and a group to serve as concurrent controls (Group 5).

**Dose selection rationale:** The dose levels were selected by the Sponsor after examining existing toxicity data and on the basis of a preliminary study performed at [REDACTED].

**Dose preparation and analysis:** The test item was formulated for dosing as a suspension in the vehicle (1 % carboxymethylcellulose). The weighed quantity of the test item was ground using a pestle and mortar. Gradually the vehicle was added to form a smooth paste and then it was transferred to a suitable container and made up to the final volume. This was homogenised to form a uniform suspension. Separate formulations were prepared for each dose level. Formulations were prepared approximately weekly and stored refrigerated prior to use. Test item formulations were stirred for at least 5 minutes prior to use and throughout the dosing period.

Stability of the test item formulations was assessed previously. Formulations of SYN545974, at concentrations between 1 mg/mL and 200 mg/mL, were shown to be stable for up to 10 days when stored refrigerated. Formulations at concentrations of 1 mg/mL and 200 mg/mL were shown to be stable for 3 days when stored at room temperature and for 4 days at a concentration of 20 mg/mL.

On the first preparation occasion for each group, formulations, including the control, were analysed to assess their homogeneity (where applicable) and achieved concentrations. The formulations prepared for use on the last day of dosing for each group were analysed to determine their achieved concentrations only.

**Analysis results:** Test item formulations used to dose animals during Weeks 1 and 3 of the study were considered accurate and homogeneous, where assessed. The measured concentrations of SYN545974 were within 9 % of their nominal values with coefficients of variation no greater than 4.2 % which fulfilled the acceptance criteria ( $\pm 10$  % and  $\leq 5$  % for accuracy and homogeneity, respectively). No SYN545974 was detected in vehicle used to dose Group 1 or 5 animals.

**Dose administration:** The females were dosed once daily, by oral gavage, for 22 days, from Days 6 to 27 of gestation, inclusive, at a dose volume of 5 mL/kg body weight. Individual doses were adjusted according to the most recently recorded body weight. Control animals received the vehicle only, following the same regimen as the other groups.

**Observations:**

**Maternal observations:** Animals were examined twice daily for mortality and morbidity. Following the day of arrival, all animals had a cage-side observation recorded. In addition, all animals were examined daily for clinical signs of toxicity or changes in behaviour and appearance from the start of treatment. During the treatment period, each female was routinely checked pre-dose and immediately after the completion of dosing for that group. On week days, additional observations were made approximately 1

hour after dosing and either approximately 4 hours after dosing and/or at the end of the working day (whichever was sooner). At weekends, additional observations were made approximately 1 hour after dosing or at the end of the working day (whichever was sooner). The time of the 1 hour and 4 hour observations was based each day on completion of dosing for the study.

Body weights were recorded on Day 0 of gestation at the supplier's premises and daily from Days 4 to 28 of gestation, inclusive.

Food consumption was measured over Days 4 to 5, 5 to 6, 6 to 9, 9 to 12, 12 to 15, 15 to 18, 18 to 21, 21 to 24, 24 to 27 and 27 to 28 of gestation.

**Necropsy:** Surviving females on Day 28 of gestation and any animal found dead during the treatment period were subjected to necropsy. Animals were killed by an intravenous injection of sodium pentobarbitone. The thoracic and abdominal cavities were opened by a ventral, mid-line incision and the major organs examined.

The uterus of any apparently non-pregnant female was stained with ammonium sulphide to confirm pregnancy status.

For pregnant females the following observations were made: Pregnancy status; Number of corpora lutea; Number and distribution of implantations in each uterine horn (classified as early intra-uterine deaths, late intra-uterine deaths, dead fetuses or live fetuses). The live fetuses and their placentae were removed and the uterus and ovaries were retained in neutral buffered formaldehyde.

**Fetal observations:** The following observations were made for live fetuses on Day 28 of gestation: fetal weights (live fetuses); fetal sexes; external abnormalities of fetuses. Live fetuses were killed by an intraperitoneal injection of sodium pentobarbitone solution, placed in alcohol for light fixation and subjected to visceral examination and internal sexing.

Abnormalities were assessed as follows; structural congenital abnormalities that impair or potentially impair the survival or constitution of the fetus were classified as major abnormalities. Other external defects were classified as minor abnormalities.

**Indices:** The following indices were calculated from caesarean section records of animals in the study:

Pre-implantation loss (%) = 
$$\frac{(\text{number of corpora lutea} - \text{number of implantation sites})}{\text{number of corpora lutea}} \times 100$$

Post-implantation loss (%) = 
$$\frac{(\text{number of implantation sites} - \text{number of live fetuses})}{\text{number of implantation sites}} \times 100$$

**Data evaluation:** Mean fetal body weights were calculated separately by sex for each litter; group mean body weights were calculated (separately by sex) from the litter means. The percentage of fetuses in each litter exhibiting each classification of abnormality was calculated; group mean percentages were calculated from the litter percentages. The percentage of male fetuses, out of the total number of fetuses, was calculated for each litter.

**Statistical analyses:** The comparison of interest for all parameters was: Group 1 against Groups 2, 3 and 4 Group 5 against Group 6. The data were processed to supply group mean values and standard deviations where appropriate. The following methods were used:

- Analysis of Variance and Dunnett's Test (Body weights and cumulative body weight gains; Body weight gains corrected for gravid uterus weight; Food consumption; Number of corpora lutea; Number of implants; Number of live fetuses; Gravid uterus weight; Total litter weight; Mean fetal weight (sexes were recorded separately and combined).

- Analysis of Variance following double arcsine transformation and Dunnett's Test (Pre-implantation loss, Post-implantation loss, Early intra-uterine deaths, Late intra-uterine deaths, Percentage of male fetuses)
- Fisher's Exact test (fetal abnormalities)

Non-parametric methods were employed if it was considered that the assumptions required for a parametric analysis did not hold. Statistical significance was declared at the  $p < 0.05$  level in all cases, and noted at the  $p < 0.01$  and  $p < 0.001$  levels.

## RESULTS

### Maternal toxicity:

**Mortality and clinical signs:** Female 19 receiving 1000 mg/kg/day (Group 6), was found dead on Day 12 of gestation. Findings at necropsy consisted of dark lungs, pale kidneys, an accentuated lobular pattern of the liver and cream material in the abdominal cavity; the cause of death could not be determined. There were no clinical signs considered to be related to toxicity of the test item at any dose level.

**Body weight:** For Groups 1 to 4 there was no effect of treatment on body weights or body weight gains over the duration of the study; however, increased group mean cumulative body weight gains were apparent over Days 6 to 12 and Days 6 to 13 of gestation ( $p < 0.05$ ) for females given 1000 or 500 mg/kg/day, respectively.

The majority of females in Group 6 given 1000 mg/kg/day had reduced body weight gains / body weight loss between Days 6 and 12 of gestation. Group mean body weight gains remained low throughout the treatment period, compared with the concurrent controls, achieving statistical significance ( $p < 0.05$  to  $p < 0.01$ ). Group mean body weight gain over the treatment period, Day 6 to 28, was significantly lower than for the concurrent controls (-35%;  $p < 0.05$ ). However, after correction for gravid uterus, no statistical significant effect on body weights was observed over the duration of the study (Days 6 to 28) in any groups.

**Table 6.6.2-11: Intergroup comparison of body weight change (g) – selected timepoints**

Day of gestation	Dose Level (mg/kg/day)					
	0	250	500	1000	0	1000
0-6	-0.015	-0.054	-0.072	-0.102	0.060	0.067
6-9	0.022	0.018	0.023	0.064	0.016	-0.007
6-12	0.046	0.049	0.110	0.136*	0.052	-0.006
6-13	0.102	0.094	0.168*	0.174	0.096	0.055
6-15	0.182	0.199	0.216	0.260	0.183	0.139
6-18	0.180	0.194	0.181	0.214	0.183	0.134
6-21	0.190	0.198	0.237	0.242	0.214	0.120*
6-24	0.282	0.283	0.312	0.326	0.290	0.188**
6-28	0.411±0.14	0.343±0.16	0.418±0.08	0.358±0.14	0.410±0.10	0.266±0.11*
6-28 corr	-54.8±78	-134.0±112	-65.9±94	-57.4±169	-51.6±107	-161.8±165

corr = corrected for gravid uterus weight

\* Statistically significant difference from control group mean,  $p < 0.05$

\*\* Statistically significant difference from control group mean,  $p < 0.01$

**Food consumption:** There was no effect of treatment on food consumption.

### Sacrifice and pathology:

**Gross pathology:** There were no findings at necropsy considered to be related to treatment with SYN545974.



**Caesarean section data:** There were 10, 9, 9, 5, 9 and 9 pregnant females at scheduled necropsy in Groups 1, 2, 3, 4, 5 and 6, respectively. In addition, there was 1 female given 1000 mg/kg/day (Group 6) with total resorption of embryos so that there were 8 females with live fetuses in this group.

When the study was initially conducted there was a low pregnancy rate (50%) at the high dose level of 1000 mg/kg/day (group 4). Subsequently, 2 additional groups were added to the study, a further group of 10 females given 1000 mg/kg/day (group 6) and a group of concurrent controls (group 5), to assess whether there was an effect of treatment with the test item on pregnancy. There was no effect of dosing at 1000 mg/kg/day (group 6) on pregnancy rate and therefore the study director considered the initial low pregnancy rate at the high dose level in group 4 to be coincidental.

The mean number of corpora lutea per female was similar across the groups. The group mean number of implantations per female was slightly lower for dams given 1000 mg/kg/day (Groups 4 and 6), compared with concurrent controls, resulting in a higher incidence of preimplantation loss, achieving statistical significance ( $p < 0.01$ ) for Group 6, however, values were within the background data range, these values were considered by the applicant to be due to the small group sizes and not an adverse effect of treatment. However, HSE noted that the size group was reduced in group 4 (only 5 dams) and not in group 6 (9 dams) in which effects (body weight decrease and preimplantation loss) were observed. However, HSE highlighted that the treatment has begun only at day 6 of gestation, the timing where implantation was initiated in New Zealand rabbit. Thus, it can be reasonably stated that the effect on preimplantation observed at 1000 mg/kg/day only in the additional group 6, should not be treatment related. Regarding the body weight decrease, HSE is of opinion that the result obtained at the dose of 1000 mg/kg/day from the additional group 6 cannot be kept out in favour to the one obtained in the initial group 4 (where no effect was observed) which included a smaller number of animals.

There was no effect of treatment on the incidence of post-implantation loss or the number of live fetuses per female.

Mean fetal weights and mean placental weights were similar across the groups. There was no effect of treatment on the percentage of male fetuses.

Table 6.6.2-12: Caesarean section observations for all pregnant females

Observation	Dose level (mg/kg/day)					HCD <sup>a</sup> Mean (range)
	0 (control)	250	500	1000	0	1000
# Animals Assigned (Mated)	10	10	10	10	10	10
# Animals Pregnant	10	9	9	5	9	10
# Non pregnant	0	1	1	5	1	0
% pregnancy incidence	100%	90%	90%	50%	90%	100%
# Intercurrent deaths	0	0	0	0	0	1
# Died Pregnant	0	0	0	0	0	1
# Died Non pregnant	0	0	0	0	0	0
# totally resorbed	0	0	0	0	0	1
<i>Corpora Lutea</i> /Dam	10.6	10.2	10.0	10.6	9.4	11.3
Implantations/Dam	9.1	9.3	9.3	8.0	9.1	8.0
Total # Litters (viable)	10	9	9	5	9	8
Live Fetuses/Dam	7.7	8.6	8.1	6.6	8.4	7.3
Mean number of early deaths	13	7	9	5	6	4
Mean number of late deaths	1	0	2	2	0	1*
Mean Litter Weight (g)	306.0	312.5	314.6	258.1	309.3	273.1
Mean Fetal Weight (g)	39.6	37.0	38.9	39.2	36.6	39.7
Mean Fetal Weight Males (g)	40.1	37.7	38.5	40.0	37.5	39.0
Mean Fetal Weight Females (g)	38.4	36.6	39.4	38.7	35.9	39.1
Sex Ratio (% Males per litter)	55.5	52.4	39.8	53.0	49.8	46.4
Pre-implantation Loss (%)	13.3	7.3	6.6	24.7	3.1	<b>31.5**</b>
Post- implantation Loss (%)	15.8	9.5	13.4	16.5	7.1	9.3

\* Statistically significant difference from control group mean,  $p < 0.05$   
\*\* Statistically significant difference from control group mean,  $p < 0.01$   
<sup>a</sup> Conducting laboratory HCD in the [REDACTED] Sprague Dawley [REDACTED] CD(SD)] strain): 18 studies performed between May 2007 and June 2012 including a total of 96 mated females.

**Fetal examination:** The majority of major abnormalities were seen in the control group (Group 1). Two fetuses in treated groups had abnormalities of the head; one fetus in Group 2 (250 mg/kg/day) with cheilognathopalatoschisis (cleft lip, jaw and palate) and one fetus in Group 4 (1000 mg/kg/day) with proboscis, cyclopia and oral cavity and jaw abnormalities. Although unusual, these abnormalities were considered to be isolated incidences and unrelated to treatment. The other major abnormality (lungs severely reduced) seen in a fetus in Group 2 was also seen in a control fetus, and, therefore, was considered to be unrelated to treatment.

#### Summary of major fetal abnormalities

Dose (mg/kg/day)	Dam	Fetus	Findings
0	41	R1	Thoracogastroschisis; left forelimb amelia; facial cleft on left side; microencephaly, pinna right low set; pinna left anotia. Left eye anophthalmia; left kidney pelvic; ovary bilateral ectopic; heart – persistent truncus arteriosus; intraventricular septum absent; descending aorta enlarged severely.
	44	R5	Heart severely enlarged; transposition of the great vessels; intraventricular septum absent; both lungs severely reduced in size
	46	R1	Left pinna malformed; abdomen – fissure of body wall
	48	L6	Interrupted aortic arch
250	53	R2	Eye bilateral open; nares absent; cheilognathopalatoschisis; pinna bilateral low set.
	55	R1	Both lungs severely reduced in size.
1000	75	R4	Proboscis; cyclopia; nares absent; oral cavity reduced opening; agnathia; malformed palate; microglossia. Interrupted aortic arch.

There was no adverse effect of treatment on the incidences of minor fetal abnormalities or variations.

## CONCLUSION:

In a PNDT range finding study in NZW rabbits, in which groups of 10 pregnant females were given gavage doses of 0, 250, 500 or 1000 mg/kg bw/d pydiflumetofen from day 6 to 27 of gestation, maternal toxicity was observed at the top dose of 1000 mg/kg bw/d as a reduction (by 33%) in body weight gain during day 6-24 of gestation. No developmental toxicity was seen. A top dose level of 500 mg/kg bw/d was proposed for the main study. This dose level was supported by kinetic investigations conducted in pregnant rabbits (see ADME section) in which no increase in systemic exposure was seen between 750 and 1000 mg/kg bw/d.

(██████████, 2015a)

## Main study

<b>Report:</b>	K-CA 5.6.2/07 ██████████ (2015b). SYN545974: Oral (Gavage) Prenatal Developmental Toxicity Study in the Rabbit. ██████████ ██████████ Laboratory Report No. ██████████, 14 May 2015. Unpublished. Syngenta File No. SYN545974_10177.
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**Guidelines:** Prenatal Developmental Study (rabbit) OECD 414 (2001): OPPTS 870.3700 (1998): JMAFF 8147 (2000).

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

In a prenatal developmental toxicity study, three groups of 24 time-mated, approximately 4 month old, female New Zealand White rabbits were dosed by oral gavage from Day 6 to Day 27 of gestation with SYN545974 (purity 98.5%) in the vehicle, 1 % carboxymethylcellulose, at dose levels of 10, 100 and 500 mg/kg/day (Groups 2 to 4, respectively). A fourth group of 24 similar animals (Group 1) was dosed with the vehicle only and served as controls. The dose volume used was 5 mL/kg body weight and was calculated from the most recently recorded body weight. Body weights and clinical observations were recorded daily and food consumption was recorded over Days 5 to 6 of gestation and twice weekly thereafter. Blood samples for toxicokinetic analysis were taken at three time-points on Day 27 of gestation and a terminal sample was obtained on Day 28 of gestation. Animals were killed and subjected to gross necropsy on Day 28 of gestation, internal organs were examined for gross abnormalities. The progress and outcome of pregnancy was assessed and gravid uterus and placenta weights were recorded. The fetuses were removed from the uterus, weighed, the sex determined and then examined for external, visceral and skeletal abnormalities.

There were two unscheduled deaths on the study. One female given 100 mg/kg/day was found dead on Day 12 of gestation due to a dosing trauma. One female given 500 mg/kg/day showed signs of abortion during the study and had early resorptions at necropsy on Day 19 of gestation. There were no test item related clinical signs during the study. There was no effect of treatment on body weight or food intake at any dose level. There were no treatment related maternal macroscopic abnormalities at necropsy. There were 22, 18, 19 and 21 females in the groups given 0, 10, 100 and 500 mg/kg/day, respectively, with live fetuses on Day 28 of gestation. Pregnancy rates were similar in all groups. There were no significant differences in terminal

body weight, gravid uterus weights or body weight adjusted gravid uterus weights for treated animals when compared with controls. The uterine/implantation data were unaffected by treatment with SYN545974. Fetal and placenta weights were similar in all groups. A marginally increased incidence of cartilage variant (one or more costal cartilage interrupted (rib)) was observed in the groups given 100 mg/kg/day or 500 mg/kg/day compared with control. Although there is no dose response, the increase incidence is above the available historical control data from the conducting laboratory provided the applicant. There were no other external, visceral or skeletal fetal abnormalities considered to be test item related. The incidences and intergroup distribution of the major, minor and variant fetal abnormalities were considered not to be related to administration of SYN545974.

**Administration of SYN545974, once daily, by oral gavage, to pregnant New Zealand White rabbits from Day 6 to Day 27 of gestation, inclusive, at dose levels of 10, 100 or 500 mg/kg/day was well tolerated with no clinical signs, no effect on body weight or pregnancy. A marginally increased incidence of cartilage variant (one or more costal cartilage interrupted (rib)) was observed in the groups given 100 mg/kg/day or 500 mg/kg/day compared with control which is above the HCD from the conducting laboratory. On this basis, the No Adverse Observed Effect Level (NOAEL) for maternal toxicity was considered to be 500 mg/kg/day and the NOAEL for embryo-fetal development was considered to be 10 mg/kg/day.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545974
<b>Description:</b>	Technical, off white powder
<b>Lot/Batch number:</b>	SMU2EP12007
<b>Purity:</b>	98.5 %
<b>CAS#:</b>	1228284-64-7
<b>Stability of test compound:</b>	Retest date 30 June 2016 (stored at <30°C)

**Vehicle and/or positive control:** The test item was formulated as a suspension in 1% (w/v) aqueous carboxymethylcellulose.

<b>Test Animals:</b>	
<b>Species</b>	Rabbit
<b>Strain</b>	New Zealand White
<b>Age/weight at dosing</b>	Approximately 4 months / 2.83-4.22 kg
<b>Source</b>	[REDACTED]
<b>Housing</b>	Individually in perforated-floor cages over paper lined trays.
<b>Acclimatisation period</b>	At least 2 days
<b>Diet</b>	Pelleted diet, [REDACTED] Rabbit Diet (manufactured by [REDACTED]) supplied by [REDACTED] 1TP <i>ad libitum</i>
<b>Water</b>	Mains tap water <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 17-21°C Humidity: 33-83% Air changes: Not reported Photoperiod: 12 hours light, 12 hours dark

### Study Design and Methods:

**Experimental dates:** Start: 11 October 2013, End: 27 January 2014

**Mating procedure:** Time-mated females were obtained from the supplier. The day of mating was designated Day 0 of gestation. Each female was mated with a stud male rabbit of the same strain and given an intravenous injection of 25IU Luteinising Hormone to stimulate ovulation.

**Animal assignment:** Each animal was examined upon arrival and appeared outwardly healthy. The animals were acclimatised within the study room for at least two days after arrival. Towards the end of this period the animals were re-examined to confirm their suitability for use. Animals were allocated using a stratified randomisation procedure based on individual body weights recorded on arrival.

#### Animal numbers and treatment groups

Group	Number of animals	Dose level (mg/kg/day)	Dose concentration (mg/mL)
1	24	0 (control)	0
2	24	10	2
3	24	100	20
4	24	500	100

**Dose selection rationale:** The dose levels were selected based on toxicological and kinetic data from previous studies performed at [REDACTED]. Statistical analysis showed that there was no significant difference in systemic exposure at doses of 300, 750 and 1000 mg/kg/day; therefore, 500 mg/kg/day was considered to be an adequate high dose level for use in this study.

**Dose preparation and analysis:** The test item was formulated weekly as a suspension in the vehicle, 1% (w/v) aqueous carboxymethylcellulose, separately for each dose level. A weighed quantity of test item was mixed using a pestle with a small quantity of the vehicle in a mortar to form a paste. Then, via further progressive additions of vehicle and mixing, this was taken up to near final weight. The mortar was rinsed with vehicle and the rinsings were added to the formulation. Once at final weight, the formulation was mixed using a laboratory homogeniser and then dispensed into aliquots for each day of dosing. These were stored refrigerated (approximately 4 °C) until the day of use when they were removed from the refrigerator and stirred for at least 15 minutes before the start of dosing and then throughout the dosing procedure.

Formulations prepared at concentrations of 1, 20 and 200 mg/mL, spanning the concentrations used in this study (2 to 100 mg/mL), had previously been shown to be stable for at least three days at room temperature, at least 10 days when refrigerated and at least 32 days when stored frozen at -18 °C, so confirming that the formulations prepared for this study were stable under their conditions of use.

All test item formulations were sampled. Samples from the first preparation were analysed for SYN545974 to assess their homogeneity and achieved concentration. Homogeneity was confirmed for the first preparation and, therefore, the formulations for use on the last day of dosing were assessed for achieved concentration only. Samples from the vehicle used to dose the control group, were assessed for the absence of test item.

**Analysis results:** Formulations were accurately prepared, with mean formulation concentrations within 6 % of nominal and all individual replicate values within 9 % of nominal on both sampling occasions. Formulations were demonstrated to be homogenous with coefficients of variation between 0.8 % and 1.6 %. No SYN545974 was detected in vehicle used to dose animals in Group 1.

**Dosage administration:** Animals were dosed using a rubber catheter and disposable syringe, once daily from Days 6 to 27 of gestation, inclusive, at a constant dose volume of 5 mL/kg body weight, adjusted according to the most recent body weight.

#### Observations:

**Maternal observations:** Animals were examined twice daily for mortality and morbidity. From the day of arrival, a cageside examination including, but not limited to, changes in behaviour and appearance was made for each animal. In addition, from the start of dosing each animal received a daily detailed clinical examination.

Day 0 of gestation body weight was recorded by the supplier and body weights were recorded for all females on arrival and daily from Day 6 to Day 28 of gestation, inclusive. The amount of food consumed by each animal was recorded over Days 5 to 6 of gestation and twice weekly thereafter.

**Proof of Absorption:** Blood samples (0.1 mL) were taken, via the marginal ear vein, from six animals per group on Day 27 of gestation, at 2, 6 and 12 hours post-dose. Terminal samples (Day 28 of gestation) were taken from the vena cava immediately after each animal was killed. All samples were collected into tubes containing K<sub>2</sub>EDTA anticoagulant. 0.05 mL of whole blood was mixed with exactly 0.05 mL of deionised water and the blood:water samples were stored frozen ( $\leq -70$  °C). The blood:water samples, from animals given the test item, were analysed for SYN545974 by LC-MS/MS.

**Toxicokinetic evaluation:** Toxicokinetic parameters were derived from the individual blood concentration-time profiles by non-compartmental analysis of the individual data using the validated system, Phoenix WinNonLin Professional 6.3. The reported toxicokinetic parameters consisted of the following: C<sub>max</sub> (maximum observed plasma concentration); t<sub>max</sub> (the time of occurrence of C<sub>max</sub>); AUC<sub>0-last</sub> (the area under the plasma concentration versus time curve from time zero to the last time point with measurable concentrations of the test item).

**Necropsy:** One female given 100 mg/kg/day was found dead due to dosing trauma. All other females were killed by an intravenous overdose injection of sodium pentobarbitone solution. One female given 500 mg/kg/day showed signs of abortion and was killed on Day 19 of gestation. All animals found dead or prematurely killed during the study were subjected to the full necropsy procedure. The remaining females were killed at scheduled termination on Day 28 of gestation when dead body weight, gravid uterus and placenta weights were recorded. For all females, the thoracic and abdominal cavities were opened by a ventral mid-line incision and the major organs examined. Organs or tissues showing any macroscopic abnormalities were removed and retained in fixative.

The uterus of any apparently non-pregnant female was stained with ammonium sulphide to confirm pregnancy status.

For pregnant females the following observations were made: Number of corpora lutea; Number and distribution of implantations in each uterine horn (classified as early intrauterine deaths, late intrauterine deaths, dead fetuses or live fetuses). The live fetuses and their placentae were removed and the uterus and ovaries were retained in neutral buffered formaldehyde.

**Fetal observations:** The following observations were made for live fetuses on Day 28 of gestation: fetal weights; fetal sexes; external abnormalities. Live fetuses were killed by an intraperitoneal injection of sodium pentobarbitone solution.

Approximately 50 % of the live fetuses in each litter were decapitated and the heads fixed in Bouin's solution for subsequent serial sectioning to permit examination of the eyes, brain, nasal passages and tongue. The intact fetuses and the bodies of the decapitated fetuses were placed in alcohol for light fixation and later the same day they were skinned and dissected, the viscera were examined and the sex was recorded. The fetuses were then eviscerated and placed back in alcohol. After at least 12 hours fixation, a razor blade cut was made through the head along the frontal parietal suture and the brain was examined.

The carcasses were subsequently cleared in potassium hydroxide, stained with Alizarin red S and Alcian blue to visualise the ossified skeleton and cartilage and examined for variants and abnormalities. Structural congenital abnormalities that impair or potentially impair the survival or constitution of the fetus were classified as major abnormalities. Other defects were classified as minor abnormalities. Commonly observed variations in the position or the location of the origin of the left common carotid artery and the presence of additional minor blood vessels, were classified as variants. After examination, fetal heads were stored in neutral buffered formaldehyde and all skeletal specimens were stored in aqueous glycerol with thymol crystals (to prevent fungal growth).

**Indices:** The following indices were calculated:

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Pre-implantation loss (%) =  $\frac{(\text{number of corpora lutea} - \text{number of implantation sites})}{\text{number of corpora lutea}} \times 100$

Post-implantation loss (%) =  $\frac{(\text{number of implantation sites} - \text{number of live fetuses})}{\text{number of implantation sites}} \times 100$

**Statistical analyses:** All statistical tests were two-sided with minimum significance levels of 5 % and 1 %. Non-parametric statistics were not routinely conducted. The litter, rather than the fetus, was considered as the experimental unit. When used, Dunnett's test was conducted regardless of the outcome of the analysis of variance (ANOVA) or analysis of covariance (ANCOVA).

Data were examined for unusually high or low values which may influence the statistical analysis and interpretation (possible outliers). After examining for any outliers, if the variances were clearly heterogeneous, transformations (e.g. log, double arcsine or square root) were used in an attempt to stabilise the variances. If the transformations failed, the data set was examined and a decision taken on further action.

Body weight, cumulative body weight gain from the start of dosing, food intake, numbers of corpora lutea, implants, live fetuses, gravid uterus weight, total litter weight and mean fetal weight (sexes separately and combined) were analysed using a parametric ANOVA.

For pre-implantation loss, post-implantation loss, early intra-uterine deaths, late intra-uterine deaths and sex ratios, litter based mean percentages were analysed using a parametric ANOVA, following a double arcsine transformation.

Maternal performance (e.g. the proportion of females with live fetuses at termination, abortions, total resorptions) was analysed by a two tailed Fisher's Exact Test, comparing each treated group to the control group.

The incidence of fetal malformations and developmental variations (external, visceral and skeletal) was summarised as the proportion of fetuses affected, the proportion of litters affected and the proportion of fetuses affected within each litter. The proportions of litters affected were analysed by the exact version of the Cochran-Armitage Test. The percentages of fetuses affected within each litter were analysed by the exact version of the Jonckheere Trend Test. In both cases the tests were performed in a step-wise manner; where a test was significant at the 5 % level, the test was repeated after removing the top dose and then subsequent treatment groups, until only the control group was left. Tests were one-sided looking for increase in treated groups versus the control group.

## RESULTS

**Toxicokinetics:** The toxicokinetic parameters of SYN545974 calculated in this developmental rabbit study confirmed the previous result obtained in the toxicokinetic study in the pregnant rabbit (report K-CA 5.1.1/09: [REDACTED], (2014); section 6.6.1). Indeed, the toxicokinetics of SYN545974 were characterised by a sub-proportional increase in peak concentration and total systemic exposure with respect to increases in dose: the AUC increase by only 1.2 and 4.2 fold following a dose increase of 10 (dose of 100 mg/kg/day) and 50 fold (dose of 500 mg/kg/day), respectively (see Table 6.6.2-10). In the [REDACTED] (2014) study, doses of SYN545974 higher than 500 mg/kg/day were tested and no apparent increase in exposure (measured by C<sub>max</sub> and AUC<sub>0-24</sub>) between 750 and 1000 mg/kg/day were observed (see section 6.6.1; report K-CA 6.1.1/09 and figure 6.6.2-1 below). Therefore, 500 mg/kg/day could be considered as an adequate high dose level for this study.

Blood concentrations for individual animals demonstrated a degree of variability at each dose level assessed, with only a limited set of profiles at 10 mg/kg/day.

**Table 6.6.2-13: Mean blood concentrations (ng/mL) and mean toxicokinetic parameters of SYN545974 on day 27 of gestation in female rabbits following oral (gavage) administration of SYN545974**

Dose (mg/kg/day)	10	100	500
Analyte	SYN545974	SYN545974	SYN545974
Timepoint (h) sex	Female	Female	Female
2	32.4	33.1	61.9
6	17.5	51.9	103
12	3.33	21.1	78.4
25	<5.00	<5.00	20.0
C <sub>max</sub> (ng/mL)	33.0	51.9	103
T <sub>max</sub> <sup>1</sup> (h)	2	6	6
AUC <sub>0-tlast</sub> (ng.h/mL)	358	443	1520
Dose-proportionality Ratio <sup>2</sup> (C <sub>max</sub> )		1.6	3.1
Dose-proportionality Ratio <sup>2</sup> (AUC <sub>0-tlast</sub> )		1.2	4.2

<sup>1</sup>: Median for T<sub>max</sub><sup>2</sup>: Dose proportionality versus lowest dose (10:50)

&lt;5.00 ng/mL: below the level of quantification

**Maternal toxicity:**

**Mortality and clinical signs:** There were two early decedents during the study. One rabbit given 100 mg/kg/day was found dead; this death was considered to be a result of dosing trauma. One rabbit given 500 mg/kg/day showed signs of abortion, red stained tray liner was killed on Day 19 of gestation and had early resorptions at necropsy; this was considered to be spontaneous and not related to treatment.

There were no test item-related clinical signs during the study.

**Body weight:** Group mean body weights and body weight gains, including Day 28 of gestation weights adjusted for the gravid uterus weight, were similar to controls at all dose levels.

**Table 6.6.2-14: Intergroup comparison of body weight change (g) – selected timepoints**

Day of gestation	Dose Level (mg/kg/day)			
	0	10	100	500
0-6	0.011	0.041	0.005	0.006
6-9	0.015	0.038	0.021	0.010
6-12	0.050	0.087	0.073	0.061
6-15	0.158	0.191	0.187	0.181
6-18	0.164	0.183	0.179	0.174
6-21	0.204	0.231	0.212	0.202
6-24	0.261	0.298	0.264	0.262
6-28	0.359	0.383	0.366	0.348
6-28 ‡	-0.06±0.17	-0.06±0.14	-0.04±0.14	-0.09±0.11

‡ Terminal body weight adjusted for gravid uterus weight

**Food consumption:** There was no effect of treatment on food intake at any dose level.

**Sacrifice and pathology:**

**Gross pathology:** There were no macroscopic findings considered to be related to treatment.

**Caesarean section data:** There were 22, 18, 20 and 22 pregnant females in the groups given 0, 10, 100 and 500 mg/kg/day, respectively and 22, 18, 19 and 21 females with live fetuses on Day 28 of gestation.

There was no effect on the incidence of pre- or post-implantation loss. The mean number of live fetuses per female was similar across the groups. There was no effect of treatment on mean fetal, litter or placenta weights.



Table 6.6.2-15: Caesarean section observations

Observation	Dose level (mg/kg/day)			
	0 (control)	10	100	500
# Animals Assigned (Mated)	24	24	24	24
# Animals Pregnant	22	18	20	22
# Non pregnant	2	6	4	2
# Intercurrent deaths	0	0	1	1
# Died Pregnant	0	0	1	1
# Died Non pregnant	0	0	0	0
# totally resorbed	0	0	0	1
<i>Corpora Lutea</i> /Dam	9.4	9.6	9.9	9.7
Implantations/Dam	8.0	8.7	7.6	8.0
Total # Litters (viable)	22	18	19	21
Live Fetuses/Dam	7.4	7.9	6.9	7.3
Mean number of early deaths	0.5	0.8	0.6	0.4
Mean number of late deaths	0.2	0	0.1	0.2
Mean Litter Weight (g)	272.75	302.41	259.51	271.42
Mean Fetal Weight (g)	37.72	38.70	38.43	36.93
Mean Fetal Weight Males (g)	37.73	38.94	38.28	37.40
Mean Fetal Weight Females (g)	37.49	37.91	37.87	37.04
Sex Ratio (% Males per litter)	51.3	54.0	54.7	47.7
Pre-implantation Loss (%)	13.0	9.8	23.6	17.0
Post-implantation Loss (%)	8.5	9.5	8.0	7.9
No statistically significant differences from control group means				

**Developmental Toxicity:** Major fetal abnormalities were noted in five fetuses from three litters in the control group, four fetuses from three litters in the group given 10 mg/kg/day, three fetuses from three litters in the group given 100 mg/kg/day and in two fetuses from two litters in the group given 500 mg/kg/day (Table 6.6.2-16). The nature and intergroup distribution of these major fetal abnormalities did not indicate an adverse effect of treatment. In addition, the majority of these major abnormalities have been noted in this strain of rabbit in these laboratories. Diaphragmatic hernia was present in one foetus from one litter (female 80) at 500 mg/kg/day. Although diaphragmatic hernia has not been seen within the background data for this strain of rabbit from this supplier, it has been shown to occur spontaneously in rabbits of this strain from other suppliers and, therefore, it is considered that the instances in this study were incidental to administration of SYN545974 (Table 6.6.2-17a and 6.6.2-17b).

Table 6.6.2-16: Summary of major fetal abnormalities

Dose (mg/kg/day)	Dam	Fetus	Findings
0	8	R3	Flattened right maxillary region of the head; malformed forelimbs; arthrogryposis; malrotated hindlimbs; enlarged right orbital cavity
	17	R1	Severely enlarged aortic arch; transposition of the great vessels, absent intraventricular septum
	22	L3	Severely enlarged aortic arch
		R1	Pulmonary valvular artesia; absent intraventricular septum
		R3	Duplicated sternbrae; sternal and xiphoid cartilage duplicated on 1 <sup>st</sup> and 4 <sup>th</sup> sternbrae
10	34	L1	Interrupted aortic arch; incomplete intraventricular septum
		R1	Interrupted aortic arch; absent intraventricular septum; severely enlarged pulmonary arch, entire heart & superior vena cava; severely fused 1 <sup>st</sup> to 6 <sup>th</sup> sternbrae
	38	R6	Severely enlarged aortic arch
	46	R5	Pulmonary valvular artesia
100	61	L1	Malrotated fore-limbs; severely bent scapula; bowed radii, ulna, tibia and fibula
	65	L4	Malformed and discontinuous lumbar cord; malrotated hindlimbs; filamentous tail; centrally placed kidneys; undescended testes; 10 thoracic vertebrae; 10 pairs of ribs; 10 <sup>th</sup> centra absent; 10 <sup>th</sup> neural arches malformed & fused; absent lumbar sacral & caudal vertebrae
	72	L3	Spina bifida; bifid 7 <sup>th</sup> lumbar to 4 <sup>th</sup> sacral neural arches; malformed 1 <sup>st</sup> to 4 <sup>th</sup> sacral cartilaginous spinous processes; severely fused 6 <sup>th</sup> to 8 <sup>th</sup> caudal centra
500	76	R2	Severely fused 4 <sup>th</sup> to 5 <sup>th</sup> thoracic centra; 4 <sup>th</sup> & 5 <sup>th</sup> right ribs arising from the same neural arch
	80	L1	Diaphragmatic hernia; small lungs; right sided descending aorta.

Table 6.6.2-17a: Diaphragmatic hernia

Observations	Dose levels (mg/kg bw/day)			
	0 (control)	10	100	500
<b>Fresh visceral examination</b>				
No. of fetuses (F)	163	142	132	154
No. litters (L)	22	18	19	21
Liver: diaphragmatic hernia	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	F: 1 (0.6%)
Stomach: diaphragmatic hernia	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	F: 1 (0.6%)

Table 6.6.2-17b: Instances of diaphragmatic hernia (mean %)

Study	1	2	3	3
Date	June 2013	October 2012	October 2012	October 2012
Number of litters	19	20	18	22
Number of fetuses	164	184	158	195
Liver: diaphragmatic hernia	0.7	0.5	-	0.5
Stomach: diaphragmatic hernia	0.7	-	0.5	-

There were no statistically significant differences from control in the overall incidences of minor and variant fetal abnormalities in the treated groups compared with control (Table 6.6.2-18). Significant differences from control were noted as increased incidences in one fetal variant in the groups given 100 mg/kg/day or 500 mg/kg/day (Table 6.6.2-19). The marginally increased incidences of one cartilage variant (one or more costal cartilage interrupted (rib)) in the groups given 100 mg/kg/day or 500 mg/kg/day compared with control, did not demonstrate a dose response but was statistically significant. The interpretation of this skeletal variant is uncertain in the absence of historical control data. The study director concluded that this finding do not represent an adverse effect of treatment. However, HSE is of opinion that a treatment related effect cannot be totally excluded without information on the background data for this variation in this strain of rabbit. No other statistically significant increases in incidences of minor or variant fetal abnormalities were observed.

**Table 6.6.2-18: Summary of overall incidences of major, minor and variant fetal abnormalities**

Observations	Dose levels (mg/kg bw/day)			
	0 (control)	10	100	500
<b>Combined examination (external, visceral, skeletal)</b>				
<i>No. of fetuses (F)</i>	163	142	132	154
<i>No. litters (L)</i>	22	18	19	21
Number with major abnormalities	F: 5 (3%) L: 3 (%)	F: 4 (2.9%) L: 3 (%)	F: 3 (3.4%) L: 3 (%)	F: 2 (1.1%) L: 2 (%)
Number with minor abnormalities	F: 40 (25.8%) L: 17 (%)	F: 26 (18.2%) L: 13	F: 32 (23.6%) 15	F: 26 (16.7%) 13
Number with variants	F: 162 (99.6%) L: 22 (100%)	F: 141(99.1%) L: 18 (100%)	F: 132 (100%) L: 19 (100%)	F: 153 (99.4%) L: 21 (100%)

**Table 6.6.2-19: skeletal variations**

Observations	Dose levels (mg/kg bw/day)			
	0 (control)	10	100	500
<b>Skeletal examination</b>				
<i>No. of fetuses (F)</i>	163	142	132	154
<i>No. litters (L)</i>	22	18	19	21
Rib: one or more: costal cartilage interrupted (variant)	F: 8 (4.4%) L: 6 (27.3%)	F: 8 (5%) L: 6 (33.3%)	F: 14 (14%) L: 12 (63%)*	F: 12 (8%) L: 10 (47.6%)*

**Additional information, including HCD on the rabbit PNDT study, requested by EFSA**

During the commenting period, EFSA requested to the applicant additional information regarding the rabbit developmental study.

**EFSA Request for additional information (February 2018), Question 16:** Applicant to provide HCD for “Ribs - 1 ≥ costal cartilage interrupted” in the rabbit developmental study, and further assessment of the adversity of this finding.

The applicant submitted further assessment of the adversity of the occurrence of costal cartilage in a report (██████████, 2018a), and a summary is provided below.

Following a comparison of the incidence with the HCD ranges from 54 studies, where higher incidences have been observed on numerous occasions, this variation is considered to be spontaneous in origin and is observed in the New Zealand White rabbit irrespective of animal supplier. Subsequently, these incidences of interruption of the costal cartilage of the rib are not indicative of an adverse effect of Pydiflumetofen, as the incidence is within the spontaneous background data ranges in the New Zealand White rabbit from ██████████ between 2007 to 2017.

<b>Report:</b>	K-CA 5.6.2/08 ██████████, (2018a) Pydiflumetofen - Additional Historical Control Data and Assessment to Support the Prenatal Developmental Toxicity Study in the Rabbit, ██████████ ██████████. Syngenta File No. SYN545974_10599.
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**Guidelines:** Not applicable.

**GLP:** This report contains an assessment of data from completed studies and additional scientific information and is not a study per se, a Good Laboratory Practice (GLP) statement as defined by OECD is not appropriate.

**EXECUTIVE SUMMARY**

A prenatal developmental toxicity study in the rabbit was conducted on Pydiflumetofen (SYN545974) at ██████████ in 2013, using the New Zealand White rabbit (██████████, 2015b).

This document refers to Historical Control Data (HCD) in the New Zealand White rabbit from [REDACTED], which establishes the spontaneous occurrence of cartilage variant of interruption within the costal cartilage of the rib in this strain of rabbit between 2007 to 2017.

## RESULTS AND DISCUSSION

### A comparison with additional historical control data and relevant external literature

There were increased incidences in one foetal variant (one or more costal cartilage interrupted (rib)) in the groups receiving 100 mg/kg/day or 500 mg/kg/day Pydiflumetofen compared with Control as reported in [REDACTED], 2015, which attained statistical significance ( $p < 0.05$ ) for the foetal incidence, although there was no dose-response relationship. However, statistical significance was not attained for the litter incidence (the unit of interest for pre-natal developmental toxicity studies (OECD, 2001)).

**Table 5.6.2-20: Variant Skeletal Findings with Statistical Significance**

Finding		Dose level (mg/kg/day)				Foetal Background Data Range (group mean percent)	Litter incidence
		0	10	100	500		
Rib - one or more: costal cartilage interrupted	No Foetuses	8 (4.4)	8 (5.0)	14 (14%) <sup>J</sup>	12 (8%) <sup>J</sup>	4 – 13 (2.6% - 9.4%)	4 – 9
	No Litters	6	6	12	10		

Figures in brackets are group mean percent values.

<sup>J</sup>= $p \leq 0.05$  (Jonckheere Test).

Therefore, following a comparison of the incidence with the HCD ranges from 54 studies as presented above, where incidences have been observed on numerous occasions, this variation is considered to be spontaneous in origin and is observed in the New Zealand White rabbit irrespective of animal supplier. Subsequently, these incidences of interruption of the costal cartilage of the rib are not indicative of an adverse effect of Pydiflumetofen, as the incidence is within the spontaneous background data ranges in the New Zealand White rabbit from [REDACTED] between 2007 to 2017 as shown in Table 6.6.2-20.

Costal cartilage interruption mainly occurs in those ribs that are unattached at the sternum (ribs 8 to 12 or 13) in the rabbit and is used to describe when cartilage is observed on the end of the rib and an unattached section of cartilage is also present. The finding is very similar to “costal cartilage floating”, where there is only an unattached piece of cartilage at the end of the rib. There were no increases in “costal cartilage floating” for any rib components in any Pydiflumetofen treated groups versus the concurrent controls. There are many variations of the costal cartilage (long, interrupted, floating, bifurcated) which can be observed in pre-natal developmental toxicity studies. Following administration of Pydiflumetofen there were no increases in incidence of other cartilage variations of the ribs in Pydiflumetofen treated groups versus controls.

In addition, these findings do not represent malformations, but rather, are variations in skeletal and cartilage development ([REDACTED] *et al.*, 2001; [REDACTED] and [REDACTED], 2007; [REDACTED] *et al.*, 2014), variations are not considered adverse, have no detrimental effect on survival, development, growth, or health postnatally, and are not mechanistic precursors to malformations; therefore, these endpoints should not be used to identify adverse limit doses (i.e. NOAEL's and LOAEL's) ([REDACTED] and [REDACTED], 2009).

A variation is defined as an alternative structure occurring regularly in control populations which may be permanent or transient and have no impact on the survival, growth, development or function of the developing neonate ([REDACTED] *et al.*, 2009). This is further demonstrated in the pre-natal developmental toxicity studies where the overall incidence of variants is consistent across all groups with >99% fetuses and 100% litters in the control group having 1 or more variations recorded.

Furthermore, this is an isolated incidental finding with no associated changes in any other rib parameters such as incomplete or absent ossification. The increased incidence of this cartilage variant does not

demonstrate a dose-response relationship and therefore, this apparent increase cannot be considered to be treatment-related and is incidental to treatment with Pydiflumetofen.

This is further supported by the absence of major foetal abnormalities attributed to administration of Pydiflumetofen, or any effects on the number of live foetuses, foetal weight or sex ratio. There was also no difference from control in the overall foetal and litter incidences of minor abnormalities. In fact, the number of minor foetal abnormalities based on both foetal and litter incidence at 10, 100 and 500 mg/kg/day was lower than concurrent control.

**CONCLUSION:** There were increased incidences in one foetal variant (one or more costal cartilage interrupted (rib)) in the groups receiving 100 mg/kg/day or 500 mg/kg/day Pydiflumetofen (SYN545974) compared with Control as reported in [REDACTED], 2015, which attained statistical significance ( $p < 0.05$ ) for the foetal incidence, but not the litter incidence (the unit of interest for pre-natal developmental toxicity studies (OECD, 2001)) and there was no dose-response relationship.

Therefore, following a comparison of the incidence with the HCD ranges from 54 studies as presented above, where higher incidences have been observed on numerous occasions, this variation is considered to be spontaneous in origin and is observed in the New Zealand White rabbit irrespective of animal supplier. Subsequently, these incidences of interruption of the costal cartilage of the rib are not indicative of an adverse effect of Pydiflumetofen, as the incidence is within the spontaneous background data ranges in the New Zealand White rabbit from [REDACTED] between 2007 to 2017, as shown in Table 6.6.2-20.

## REFERENCES:

Carney E. and Kimmel C. (2007). *Interpretation of skeletal variations for human risk assessment: Delayed ossification and wavy ribs. Birth Defects Res B Dev Reprod Toxicol.* 80:473-496.

Chahoud I. and Paumgarten J. (2009). *Dose-response relationships of rat foetal skeleton variations: Relevance for risk assessment. Environ Res.* 109: 922-929.

Kimmel C., Garry M., and DeSesso J. (2014). *Relationship between bent long bones, bent scapulae, and wavy ribs: Malformations or variations? Birth Defects Res B Develop Reprod Toxicol.* 101:379-392.

OECD (2001). *OECD Guidelines for the Testing of Chemicals, No. 414; "Prenatal Development Toxicity Study" adopted 22nd January 2001.*

Makris S., Solomon H., Clark R., Shiota K., Barbellion S., Buschmann J., Ema M., Fujiwara M., Grote K., Hazelden K., Hew K., Horimoto M., Ooshima Y., Parkinson M., and Wise D. (2009). *Terminology of developmental abnormalities in common laboratory mammals (Version 2). Birth Defects Research, Part B.* 86:227-327.

Solecki R., Bürgin H., Buschmann J., Clark R., Duverger M., Fialkowski O., Guittin P., Hazelden K., Hellwig J., Hoffmann E., Hofmann T., Hübel U., Khalil S., Lingk W., Mantovani A., Moxon M., Müller S., Parkinson M., Paul M., Paumgarten F., Pfeil R., Platzek T., Rauch-Ernst M., Scheecelenbos A., Seed J., Talsness C., Yasuda M., Younes M., and Chahoud I. (2001). *Harmonisation of rat fetal skeletal terminology and classification. Report of the third workshop on terminology in developmental toxicology. Berlin 14-16 September 2000. Reprod. Toxicol.* 15: 713-721.

([REDACTED], 2018a)

## EFSA Request for additional information (February 2018), Question 31:

Applicant provide a technical statement and appropriate additional control data (from the conducting laboratory) regarding diaphragmatic hernia in the rabbit developmental toxicity study.

## Syngenta Response:

Further assessment of the occurrence of diaphragmatic hernia has been conducted in a report ([REDACTED], 2018b), a summary is provided below.

Following a comparison of the incidence with the HCD range from 78 studies where single incidences have been observed on numerous occasions, this abnormality is considered to be spontaneous in origin in the New Zealand White rabbit irrespective of animal supplier. Therefore, this single incidence is not indicative of an adverse effect of treatment, as the incidence is well within the spontaneous background data ranges in the New Zealand White rabbit from [REDACTED] between 2007 to 2017.

<b>Report:</b>	K-CA 5.6.2/09 [REDACTED], (2018b) Pydiflumetofen - Additional Historical Control Data to Support Prenatal Developmental Toxicity Studies in the Rabbit, [REDACTED] [REDACTED] Syngenta File No. SYN545974_10597.
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**Guidelines:** Not applicable.

**GLP:** This report contains an assessment of data from completed studies and additional scientific information and is not a study per se, a Good Laboratory Practice (GLP) statement as defined by OECD is not appropriate.

## EXECUTIVE SUMMARY

A prenatal developmental toxicity study in the rabbit was conducted on Pydiflumetofen (SYN545974) at [REDACTED] in 2013, using the New Zealand White rabbit ([REDACTED], 2015b).

This document refers to Historical Control Data (HCD) in the New Zealand White rabbit from [REDACTED], which establishes the spontaneous major visceral foetal abnormality of diaphragmatic hernia in this strain of rabbit between 2007 to 2017.

## RESULTS AND DISCUSSION

### A comparison with additional historical control data

There was a single incidence of the major visceral foetal abnormality of diaphragmatic hernia in one group receiving Pydiflumetofen (SYN545974) as reported in [REDACTED], 2015b (one foetus from one litter in the group given 500 mg/kg bw/day). In addition, diaphragmatic hernia was not observed in a preliminary pre-natal development study in the rabbit at the same or high any doses (including 500 mg/kg/day and 2 dose groups at 1000 mg/kg/day)([REDACTED], 2015a).

The major visceral foetal abnormality of diaphragmatic hernia was of a low incidence ( $n = 1$ ) and following a comparison of the incidence with the HCD range from 78 studies where single incidences have been observed on numerous occasions, this abnormality is considered to be spontaneous in origin and is observed in the New Zealand White rabbit irrespective of animal supplier.

Therefore, this single incidence is not indicative of an adverse effect of treatment, as the incidence is well within the spontaneous background data ranges in the New Zealand White rabbit from [REDACTED] between 2007 to 2017 as shown in Table 5.6.2-17.

**Table 5.6.2-17: Fresh Visceral Findings Historical Control Data**

Study Start	No. examined		Abdominal cavity: liver-one or more lobe: diaphragmatic hernia		Abdominal cavity: stomach: diaphragmatic hernia	
	No. foetuses	No. litters	Incidence	%	Incidence	%
Aug 2007	193	23				
Sep 2007	204	22				
Mar 2008	140	18				
Aug 2008	139	18				
Oct 2008	143	18				
Feb 2009	150	19				

\* A total of 9720 fetuses from 1167 litters across 78 studies. Of those 78 studies, only 46 studies listed fresh visceral findings

Following a comparison of the incidence with the HCD range from 78 studies where single incidences have been observed on numerous occasions, this abnormality is considered to be spontaneous in origin in the New Zealand White rabbit irrespective of animal supplier. Therefore, this single incidence is not indicative of an adverse effect of treatment, as the incidence is well within the spontaneous background data ranges in the New Zealand White rabbit from [REDACTED] between 2007 to 2017 as shown in Table 5.6.2-17.

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○ **Re-assessment of the increase incidence of the variant “costal cartilage interruption”**

In the prenatal developmental toxicity study in rabbit (■■■■, 2015), it was observed increased incidences in one foetal variant (one or more costal cartilage interrupted (rib)) in the group receiving 100 mg/kg/day or 500 mg/kg/day pydiflumetofen compared with control. Although this increase did not demonstrate a dose response, the statistical significance was attained ( $p < 0.05$ ) for litter incidence (according to the study report) at 100 and 500 mg/kg bw/d.

After the commenting period, the applicant submitted the HCD for this variant from 54 studies performed by the conducting laboratory. In the Tables 6.6.2-21a, the HCD including a limitation to a frame time of +/- five years with respect to the completion date of the study has been also presented by HSE (42 studies performed between 2010 to 2017). Taking into account these data, the increase at the highest dose of 500 mg/kg/d is within the spontaneous background data range for foetus incidence (8% vs 9.4%) and slight above the range for litter incidences (47.6% vs 44.5%). At 100 mg/kg bw/d, the increase in both litter and foetus incidences is above the HCD ranges (see Table 6.6.2-21a).

It should be noted that the incidence of this variant was already high in the control group of the study (4.4% for foetus and 27.3% for litter) when compared with the mean incidence of the HCD (0.9% for foetus and 5.7% for litter). Moreover, regarding these HCD, high incidences for this variant only appears in studies performed after 2015 (Incidence range of 2.6%-9.4% (foetus) obtained from 5 studies) whereas in studies performed before 2015, the majority of them have shown no incidence for this variant (table 6.6.2-21b).

**Table 6.6.2-21a: skeletal variation: rib: one or more: costal cartilage interrupted (variant)**

Observations	Dose levels (mg/kg bw/day)				HCD ■■■■ from the conducting laboratory# (■■■■ 2018a)	
	0 (control)	10	100	500	HCD 2007-2017 Mean % (range)	HCD (2010-2017)* Mean % (range)
<b>Skeletal examination</b>					N= 54 studies (2007-2017)	N = 42 studies (2010-2017)
No. of foetuses (F)	163	142	132	154	7796	6101
No. litters (L)	22	18	19	21	941	732
Rib: one or more: costal cartilage interrupted (variant)	F: 8 (4.4%) L: 6 (27.3%)	F: 8 (5%) L: 6 (33.3%)	F: 14 (14%) L: 12 (63%)*	F: 12 (8%) L: 10 (47.6%)*	F Mean: 54 (0.7%) Range: 0-13 (0-9.4%)	Mean: 54 (0.9%) Range: 0-13 (0-9.4%)
					L Mean: 42 (4.5%) Range: 0-9 (0-44.5%)	Mean: 42 (5.7%) Range: 0-9 (0-44.5%)

\* HSE re-assessed the historical control data for the skeletal variant “rib: one or more: costal cartilage interrupted” taking into account a time frame of +/- five years with respect to the completion date of the study (see for details Table 6.6.2-21).

# Performing laboratory: ■■■■

**Table 6.6.2-21b: Analysis of the distribution of the incidence of rib, costal cartilage interrupted observed in the performing laboratory over time (2007-2017):**

Date	Number of studies examined	Results
After 2015	12 studies	<u>5/12 studies have shown incidences for rib, costal cartilage interrupted:</u> <ul style="list-style-type: none"> <li>- Incidence range of 2.6%-9.4% (foetus) obtained from 5 studies: <ul style="list-style-type: none"> <li>○ Apr. 2015: 2.6% (foetus); 20% (litter)</li> <li>○ Jul. 2016: 7.8% (foetus); 44.5% (litter)</li> <li>○ Sep. 2016: 9.4% (foetus); 43% (litter)</li> <li>○ Oct. 2016: 7.9% (foetus); 43% (litter)</li> <li>○ Jan. 2017: 3.2% (foetus); 27% (litter)</li> </ul> </li> <li>- Zero incidence observed in the other 7 studies</li> </ul>
Before 2015	42 studies	<u>1/42 study have shown incidences for rib, costal cartilage:</u> <ul style="list-style-type: none"> <li>- Incidences of 4.4% (foetus) and 23% (litter) in one study performed in 2013</li> <li>- Zero incidence observed in the other 41 studies (from 2007 to 2015)</li> </ul>



It can be concluded that the increase incidences of the variant costal cartilage interruption are statistically significant at the two highest doses of pydiflumetofen and these increases are above the available HCD from the conducting laboratory.

However, it should be considered that:

- No dose response relationship was observed regarding the increase incidence of the variant costal cartilage interruption
- A clear increase in the spontaneous incidence of this variant is observed in New Zealand White rabbit strain in recent developmental toxicity studies (performed from 2015).
- There was no associated increase in incidence of other cartilage variations of the ribs (long, bifurcated) and in particular there was no increase in “costal cartilage floating”, which is a very similar finding to costal cartilage interruption.
- No associated changes in any other rib parameters such as incomplete or absent ossification have been observed.

Thus, HSE proposes that the relevance of this finding (incidental or treatment related) and its consideration for the setting of the NOAEL of the study be discussed in expert meeting

#### ○ Re-assessment of the increase incidence of diaphragmatic hernia

In the prenatal developmental toxicity study in rabbit (█████, 2015), it was observed a single incidence of the major visceral foetal abnormality of diaphragmatic hernia in one group given 500 mg/kg bw/d.

After the commenting period, the applicant submitted additional HCD for this malformation in New Zealand White rabbit from 46 studies performed in the conducting laboratory (█████) between 2007 and 2017.

This increase incidence is well within the spontaneous background data ranges in the New Zealand White rabbit (Studies from █████ limited (UK) performed between 2007 to 2017 or between 2010 to 2017) as shown in table 6.6.2-22 below. HSE is of opinion that this finding can be considered as incidental and not treatment related.

**Table 6.6.2-22: Diaphragmatic hernia**

Observations	Dose levels (mg/kg bw/day)				HCD █████ from the conducting laboratory <sup>#</sup> (█████ 2018b)	
	0 (control)	10	100	500	HCD 2007-2017 Mean % (range)	HCD 2010-2017** Mean % (range)
<b>Fresh visceral examination</b>					N= 46 studies* (2007-2017)	N = 36 studies (2010-2017)
No. of foetuses (F)	163	142	132	154	5845	3277
No. litters (L)	22	18	19	21	697	385
Liver: diaphragmatic hernia	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	F: 1 (0.6%) L: 1 (4.8%)	F: 0.1% (0-1.6%) L: 0.9% (0-14.3%)	F: 0.18% (0-1.6%) L: 1.6% (0-14.3%)
Stomach: diaphragmatic hernia	F: 0 (0%)	F: 0 (0%)	F: 0 (0%)	F: 1 (0.6%) L: 1 (4.8%)	F: 0.03% (0-1.3%) L: 0.3% (0-11%)	F: 0.06% (0-1.3%) L: 0.5% (0-11%)

\* A total of 9720 foetuses from 1167 litters across 78 studies was analysed by the applicant. Of those 78 studies, only 46 studies listed fresh visceral findings giving a total of 5845 foetuses from 697 litters.

\*\* HSE re-assessed the historical control data for the skeletal variant “rib: one or more: costal cartilage interrupted” taking into account a time frame of +/- five years with respect to the completion date of the study (see for details Table 6.6.2-21).

<sup>#</sup> Performing laboratory: █████

## CONCLUSION:

In a GLP and guideline PNDD study in NWZ rabbits given gavage doses of 0, 10, 100 or 500 mg/kg bw/d pydiflumetofen, no maternal toxicity was observed up to the top dose. Therefore, a **NOAEL of 500 mg/kg bw/d** was identified for **maternal toxicity**. The only developmental findings of concern were the

presence of one single incidence of diaphragmatic hernia (visceral abnormality) at the top dose and the increased litter incidence of a skeletal variant (ribs with 1 or more absent costal cartilage) at 100 and 500 mg/kg bw/d (63% and 47.6% respectively vs 27.3% in controls). The diaphragmatic hernia was well within the laboratory HCD ranges from 46 studies performed between 2010 and 2017. Therefore, it was not considered treatment-related. The skeletal variant was statistically significant and although not dose-related, was outside the laboratory HCD range (25% - 42.8%) from 54 studies conducted between 2007 and 2017. Relation to treatment could therefore not be excluded and a **NOAEL of 10 mg/kg bw/d** was identified for **developmental toxicity**.

However, this skeletal variant has no impact on normal growth or function; and was not associated with changes in other rib parameters or any retardation in skeletal development. Overall, HSE agrees with RAC that this minor skeletal variation in the rabbit was insufficient to trigger classification for developmental toxicity. For further information on classification, please see the [GB MCL Technical Report](#).

HSE notes that the top dose caused no maternal toxicity and questions the adequacy of the study in fully exploring the developmental toxicity potential of pydiflumetofen in the rabbit.

(██████, 2015b)

### B.6.6.3. Summary of reproductive toxicity

Pydiflumetofen has been evaluated for the potential to cause effects on fertility and reproductive performance in a GLP and guideline multi-generation reproductive toxicity study in the Wistar (Han) rat. The developmental toxicity of pydiflumetofen was also investigated in the (SD) rat and (NZW) rabbit in GLP and guideline compliant studies with preliminary range-finding studies for both.

In the two-generation study, rats were given pydiflumetofen at dietary levels of 0, 150, 450 and 1500 ppm (females, equivalent to 0, 11.9, 36.1 and 116.2 mg/kg bw/d) or 0, 150, 750 and 4500 ppm (males, equivalent to 0, 11.9, 59.1 and 276.6 mg/kg bw/d). The dose levels were selected based on non-proportionality of the kinetics with respect to dose due to dose limited absorption of pydiflumetofen (see ADME section).

#### Reproductive toxicity

There were no effects on fertility and mating performance or gestation length for either generation at any dietary concentration. Sperm parameters were unaffected. All pregnant females gave birth to live litters with a similar number of pups born, and there was no effect of treatment on the postnatal survival of P or F1 generation litters to Day 21 of age.

The mean length of the oestrous cycle was statistically significantly increased at the top dose (4.05 d vs 3.93 d in controls) in the P generation only. The increase was driven by 2 females with longer cycle length (5 and 4.5 d) and was well within the laboratory historical control data (HCD) mean range (4.0 – 4.3 d) from four studies conducted between 2009 and 2014. Therefore, the effect was considered unrelated to treatment.

A delay in sexual maturation was noted in both sexes at the top dose in the F1 generation. Mean age at preputial separation (PS) was statistically significantly increased by approx. 3 days (45.9 d vs 43.0 in controls). However, when excluding from the analysis a clear outlier, with an age at PS of 57 days, the top dose mean was 45.4 d. This increase was at the upper bound of the laboratory HCD mean range (43.0 – 45.3 d; mean = 44.2 d) from 6 studies conducted between 2008 and 2014. However, when excluding from the HCD 3 studies from 2008 and 2009 (because > 5 years from study's year), the increase (45.4 d) was clearly above the more time-restricted and more relevant HCD mean range (43.0 – 43.5 d). In addition, it is unclear whether the outlier was a spontaneous aberration or was caused by the test substance. It is

therefore uncertain whether the very high PS value in one top-dose animal should have been excluded from the analysis.

Mean age at vaginal opening (VO) was statistically significantly increased by approx. 3 days (33.0 d vs 30.3 d in controls). This increase was well within the laboratory HCD mean range (29.3 – 34.1 d; mean = 31.5 d) from 6 studies conducted between 2008 and 2014. However, when excluding from the HCD 3 studies from 2008 and 2009 (because > 5 years from study's year), the increase (33.0 d) was clearly above the more time-restricted and more relevant HCD mean range (29.3 – 31.3 d).

Therefore the delay in PS and VO at the top dose was considered to be treatment related. Pup body weights were statistically significantly reduced at the top dose compared with controls in both sexes of the F1 generation from day 7 to day 21 of lactation (by 10-12%). However, the reduction was within the laboratory HCD range from 6 studies conducted between 2008 and 2014, and more importantly was not replicated in the F2 generation. Therefore, the decrease in pup body weight was unconvincing and hence the delay in sexual maturation could not be considered the secondary unspecific consequence of the reduced pup body weight development.

In agreement with RAC, HSE concludes that although these pups went on to mate and reproduce successfully and despite the absence of endocrine effects and the lack of effects on other developmental landmarks, ano-genital distance and other reproductive parameters and organs, the delay in puberty onset at the top dose was seen in both sexes, was clear (statistically significant and outside time-relevant HCD) and specific (i.e. independent of reductions in pup body weight development). Based on this analysis, HSE agrees that classification of pydiflumetofen for adverse effects on fertility and sexual function in category 2 (H361f) is warranted. For further information on classification, please see the [GB MCL Technical Report](#). A **NOAEL for reproductive toxicity** was therefore set at 450/750 ppm in females/males (equivalent to **36.1 mg/kg bw/d in females and 59.1 mg/kg bw/d in males**) based on a delay in sexual maturation at the top dose of 1500/4500 ppm in females/males (equivalent to 116.2 mg/kg bw/d in females and 276.6 mg/kg bw/d in males).

#### Parental toxicity

In males, there were decreases in body weight gains (10%) in both generations, reductions in food consumption (8%) in the F1 generation and statistically significant increases in liver and thyroid weights with associated hypertrophy in both generations at the top dose. In top dose females, there was a statistically significant increase in liver weight with associated hypertrophy in both generations. Therefore the **NOAEL for parental toxicity** was set at 450/750 ppm in females/males (equivalent to **36.1 mg/kg bw/d in females and 59.1 mg/kg bw/d in males**).

#### Offspring toxicity

There were no effects on offspring up to the top dose of 1500/4500 ppm in males/females. Therefore the **NOAEL for offspring toxicity** was set at 1500/4500 ppm (equivalent to **116.2 mg/kg bw/d in females and 276.6 mg/kg bw/d in males**).

#### Adequacy of top dose

RAC concluded that only minimal parental toxicity was evident at the top dose, especially in females. Therefore RAC agreed that the top dose was inadequate and that the study had not fully investigated the reproductive toxicity potential of pydiflumetofen. HSE considers that the top dose was adequate in males, with decreases in body weight gains (10%) in both generations, reductions in food consumption (8%) in the F1 generation and statistically significant increases in liver and thyroid weights with associated hypertrophy in both generations. The top dose should have been higher in females; however, the Agency notes that the OECD TG (No. 416, 2001) states '*the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering*'. Attainment of the MTD is not specified in the OECD TG. In top dose females, there was a statistically significant increase in liver weight with associated hypertrophy in both generations, which is the most sensitive effect of the toxicity profile of the substance. Hence, target organ toxicity in the liver was induced at the top dose

in both generations in females. Therefore, HSE concludes that the requirements of the OECD TG for selection of the top dose in females was also met, as ‘toxicity’ was induced.

#### Developmental toxicity

##### Rat

In the main PNDT study in SD rats given gavage doses of 0, 10, 30 or 100 mg/kg bw/d pydiflumetofen, statistically significant effects on maternal body weight gain (by 18-90%) and food consumption were seen during gestation days 6-10 at the top dose. None of the developmental findings were considered treatment related. A number of malformations (e.g. exencephaly in 2 foetuses from the same top dose litter) and variations (including ribs with 1 or more absent costal cartilage) observed at 30 and 100 mg/kg bw/d were either not dose-related or within the HCD ranges from animals from the same supplier (██████████) and had not been seen in the dose-ranging study up to the much higher dose of 1000 mg/kg bw/d. Overall, there was no developmental toxicity in the rat. A marginal **NOAEL of 30 mg/kg bw/d** was identified for **maternal toxicity** and a **NOAEL of 100 mg/kg bw/d** was identified for **developmental toxicity**.

HSE notes that the top dose caused insufficient maternal toxicity and questions the adequacy of the study. Although kinetic data (see ADME section) had shown that the systemic dose became non-linear at 100 mg/kg/bw/d, it still increased at higher doses. Therefore, a much higher dose should have been employed to ensure a full investigation of the developmental toxicity potential of pydiflumetofen in the rat.

##### Rabbit

In the main PNDT study in NWZ rabbits given gavage doses of 0, 10, 100 or 500 mg/kg bw/d pydiflumetofen, no maternal toxicity was observed up to the top dose. Therefore, a **NOAEL of 500 mg/kg bw/d** was identified for **maternal toxicity**. The only developmental findings of concern were the presence of one single incidence of diaphragmatic hernia (visceral abnormality) at the top dose and the increased litter incidence of a skeletal variant (ribs with 1 or more absent costal cartilage) at 100 and 500 mg/kg bw/d (63% and 47.6% respectively vs 27.3% in controls). The diaphragmatic hernia was well within the laboratory HCD ranges from 46 studies performed between 2010 and 2017. Therefore, it was not considered treatment-related. The skeletal variant was statistically significant and although not dose-related, was outside the laboratory HCD range (25% - 42.8%) from 54 studies conducted between 2007 and 2017. Relation to treatment could therefore not be excluded and a **NOAEL of 10 mg/kg bw/d** was identified for **developmental toxicity**.

However, this skeletal variant has no impact on normal growth or function; and was not associated with changes in other rib parameters or any retardation in skeletal development. Overall, HSE agrees with RAC that this minor skeletal variation in the rabbit was insufficient to trigger classification for developmental toxicity. For further information on classification, please see the [GB MCL Technical Report](#).

HSE notes that the top dose caused no maternal toxicity and questions the adequacy of the study in fully exploring the developmental toxicity potential of pydiflumetofen in the rabbit.

The most sensitive **maternal NOAEL is 30 mg/kg bw/d in the rat** and the most sensitive **developmental NOAEL is 10 mg/kg bw/d in the rabbit**.

The table below summaries the results of the reproductive toxicity studies.

Study & Acceptability	Mode of Dosing	Test material & Dose Levels	NO(A)EL (mg/kg bw/day)	LOAEL (mg/kg/day)	Effects at the LOAEL
Two generation reproductive toxicity study in the rat [REDACTED], (2015)  <i>Modern, valid, guideline study</i>	Dietary	Pydiflumetofen 98.5% Males: 0, 150, 750 & 4500 ppm Females: 0, 150, 450 & 1500 ppm	<i>Parental:</i> <u>Males</u> 750 ppm (46 mg/kg bw/d) <u>Females</u> 450 ppm (31.6 mg/kg bw/d)	<i>Parental:</i> <u>Males</u> 4500 ppm (276.6 mg/kg bw/d) <u>Females</u> 1500 ppm (116 mg/kg/d)	<i>Parental:</i> ↓(10%) bwg in males in P0 and F1; ↓(8%) food con in males in F1; ↑liver wt and associated hypertrophy in males and females in P0 and F1; ↑thyroid wt and associated hypertrophy in males in P0 and F1;
			<i>Reproduction:</i> <u>Males</u> 750 ppm (46 mg/kg bw/d) <u>Females</u> 450 ppm (31.6 mg/kg bw/d)	<i>Reproduction:</i> <u>Males</u> 4500 ppm (276.6 mg/kg bw/d) <u>Females</u> 1500 ppm (116 mg/kg/d)	<i>Reproduction</i> Delays in Vo and PS in F1 pups
			<i>Offspring:</i> <u>Males</u> 4500 ppm (276.6 mg/kg bw/d) <u>Females</u> 1500 ppm (116 mg/kg bw/d)	<i>Offspring:</i> <u>Males</u> >4500 ppm (>276.6 mg/kg bw/d) <u>Females</u> >1500 ppm (>116 mg/kg bw/d)	<i>Offspring</i> No treatment-related effects
Range-finding Developmental toxicity in the rat. [REDACTED], (2011)  <i>Supportive study</i>	Gavage	Pydiflumetofen 99.5% 0, 100, 200, 500 & 1000 mg/kg bw/d	Not applicable – range-finding study	Not applicable – range-finding study	<i>Maternal:</i> Transient effect on bwg was seen at 500 and slight body weight loss at 1000 mg/kg bw/day during gestation days 6-9 only. <i>Developmental:</i> None.
Main Developmental toxicity in the rat [REDACTED], (2015)  <i>Modern, guideline study but top dose inadequate</i>	Gavage	Pydiflumetofen 98.5% 0, 10, 30 & 100 mg/kg bw/d	<i>Maternal:</i> 30 mg/kg bw/d  <i>Developmental:</i> 100 mg/kg bw/d	<i>Maternal:</i> 100 mg/kg bw/d  <i>Developmental:</i> >100 mg/kg bw/d	<i>Maternal:</i> Marginal effects on bodyweight and food consumption during gestation days 6-9.  <i>Developmental:</i> None.
Range-finding Developmental toxicity in the rabbit [REDACTED], (2015a)  <i>Supportive study</i>	Gavage	Pydiflumetofen 99.3% & 98.5% Phase 1: 0, 250, 500 & 1000 mg/kg bw/d Phase 2: 0 & 1000 mg/kg bw/d	Not applicable – range-finding study	Not applicable – range-finding study	<i>Maternal:</i> ↓ bwg at 1000 mg/kg bw/d  <i>Developmental:</i> None.
Developmental toxicity in the rabbit [REDACTED], (2015b)  <i>Modern, guideline study</i>	Gavage	Pydiflumetofen 98.5% 0, 10, 100 & 500 mg/kg bw/d	<i>Maternal:</i> 500 mg/kg bw/d  <i>Developmental:</i> 10 mg/kg bw/d	<i>Maternal:</i> >500 mg/kg bw/d  <i>Developmental:</i> 100 mg/kg bw/d	<i>Maternal:</i> None.  <i>Developmental:</i> Increased incidence of one skeletal variant (rib costal cartilage interrupted) at 100 and 500 mg/kg bw/d without clear dose

Study & Acceptability	Mode of Dosing	Test material & Dose Levels	NO(A)EL (mg/kg bw/day)	LOAEL (mg/kg/day)	Effects at the LOAEL
<i>but top dose inadequate</i>					response but incidence above the HCD

### B.6.7. NEUROTOXICITY

The acute neurotoxicity of pydiflumetofen has been investigated in the rat in an acute neurotoxicity (single oral gavage) study. Since findings in this study were only apparent in females, a further modified acute neurotoxicity study (single oral gavage) was subsequently carried out in females only.

#### B.6.7.1. Neurotoxicity studies in rodents

<b>Report:</b>	K-CA 5.7.1/01 [REDACTED] (2015). SYN545974 – Acute Oral (Gavage) Neurotoxicity Study in the Wistar Rat. Report Amendment 1. [REDACTED] Laboratory Report No. [REDACTED]. Issue date: 15 September 2015. Unpublished. Syngenta File No. SYN545974_10198.
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**Guidelines:** Acute Oral Neurotoxicity (rat) OECD 424 (1997); OPPTS 870.6200 (1998)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

**HSE comment:** the study was considered acceptable

### EXECUTIVE SUMMARY

The purpose of this study was to assess the potential neurotoxic effects of SYN545974 when administered to rats as a single oral (gavage) dose. Han-Wistar rats in groups 1 - 4 (10 rats/sex/group) were administered a single oral dose of SYN545974 at dose levels of 0 (control), 100 (females only), 300 (males only), 1000, or 2000 mg/kg body weight, respectively. The animals were observed for 15 days, with general cage-side observations performed once daily. A functional observational battery (FOB) was performed once during acclimatization (pre-treatment), on day 1 (approximately 6 hours after treatment) and on days 8 and 15. The FOB included detailed clinical examinations as well as quantitative assessment of landing foot splay, body temperature and grip strength. Locomotor activity (LMA) was assessed over a time period of 30 minutes after each FOB evaluation. Food consumption was recorded once during acclimatization and on days 1 - 2, 2 - 3, 8 - 9, and 14 - 15. Body weights were recorded once during acclimatization, daily on days 1 - 8 and on day 15. Fifteen days after treatment (day 16), the animals were sacrificed and 5 rats/sex/group were perfusion-fixed in situ, the brain weights were measured and selected nervous system tissues were collected. Histopathological examination was performed on the nervous tissues collected from the perfusion-fixed control and high dose rats.

For males, treatment-related findings were limited to slight decreases in body weight gain over the first day following dosing at 1000 and 2000 mg/kg. For females, treatment-related findings were noted primarily on the day of dosing at 1000 and 2000 mg/kg. In these dose groups, various clinical signs, including recumbency, hunched posture, piloerection, reduced activity, abnormal gait, skin cold on touch, and mydriasis were observed within 3 - 6 hours post-dose. There was no dose-response relationship for clinical signs between 1000 and 2000 mg/kg and one 1000 mg/kg female showing marked convulsions, recumbency, heavy breathing, and skin cold on touch was euthanized at approximately 3.5 hours post-dose. At 6 hours post-dose decreased body temperature and significantly decreased LMA were recorded for the 1000 and 2000 mg/kg females. A few clinical signs (piloerection, hunched posture) were also observed in a limited number of 100 mg/kg females at 2 and 6 hours post-dose. Mean body temperature and LMA were slightly decreased at 6 hours post-dose when compared to the control group, these decreases were not statistically significant. Clinical signs occurred only during a short time after the test item administration and changes in body temperature and LMA were slight and transient.

All in-life findings for males and females attributed to treatment were transient and limited to the first day following treatment. All animals recovered by the end of the two-week observation period. There were no test item-related effects on brain weights, no test item-related macroscopic findings, and no test item-related neurohistopathologic findings.

Based on the results of this study and the above-mentioned considerations, 2000 mg/kg is considered to be the no-observed-adverse-effect level (NOAEL) for males and 100 mg/kg is considered to be the NOAEL for females.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545974 (SYN545974 technical)
<b>Description:</b>	Off white powder
<b>Lot/Batch number:</b>	SMU2EP12007
<b>Purity:</b>	98.5%
<b>CAS#:</b>	1228284-64-7
<b>Stability of test compound:</b>	Stable under storage conditions. Retest date: End of June 2016

**Vehicle and control item:** 1% (w/v) carboxymethylcellulose (CMC) in purified water

<b>Test Animals:</b>	
<b>Species:</b>	Rat
<b>Strain:</b>	RccHan <sup>TM</sup> :WIST (SPF)
<b>Age at dosing:</b>	7 weeks
<b>Source:</b>	
<b>Housing:</b>	Standard Laboratory Conditions. In groups of five in Makrolon type-4 cages with wire mesh tops and standard softwood bedding (J. Rettenmaier & Söhne GmbH & Co. KG, 73494 Rosenberg, Germany, imported by Provimi Kliba AG, 4303 Kaiseraugst, Switzerland) including paper enrichment (Enviro-dri from Lillico, Biotechnology, Surrey, United Kingdom). The animals were housed individually in Makrolon type-3 cages (in randomized order) during FOB evaluation.
<b>Acclimatisation period:</b>	6 - 7 days
<b>Diet:</b>	Pelleted standard 2914C rodent maintenance diet, batch no. 53/13 ( ) <i>ad libitum</i> .
<b>Water:</b>	Community tap water from ( ) <i>ad libitum</i> .
<b>Environmental conditions:</b>	Temperature: 20.5-22.1 °C Humidity: 34 - 60 % Air changes: 10 - 15 / h Photoperiod: 12 hours light and 12 hours dark

### Study Design and Methods:

**In-life dates:** Start: 18 Mar 2014      End: 08/09 Apr 2014 (males/females)

**Animal assignment and treatment:** The animals received a single dose of the test item by oral

## Group identification and animal numbers

Group:	Group 1	Group 2	Group 3	Group 4
Description:	Control*	Low	Mid	High
Dose (mg/kg bw):	0*	300 – Males 100 – Females	1000	2000
Dose Volume (mL/kg bw)	10	10	10	10
Concentration** (mg/mL):	0*	30 – Males 10 – Females	100	200
Males	1 - 10	11 - 20	21 - 30	31 - 40
Females	41 - 50	51 - 60	61 - 70	71 - 80

\* Control animals received the dosing solution (vehicle) without the test item.

\*\* Dose concentrations are in terms of test item as supplied. No correction factor for purity was used for preparation of dose formulations.

Viability/mortality was checked twice daily. General cage-side clinical observations were recorded once daily. A functional observational battery (FOB) was performed once during acclimatization, on day 1 (at approximately 6 hours after dosing), and on days 8 and 15. The FOB included detailed clinical observations of animals in the cage, during handling and in a standard arena (open field), tests for reflexes and other reactions to stimuli, test for hearing ability, body temperature, landing foot splay and grip strength. Locomotor activity using an automatic open field device was recorded over 30 minutes following conduct of the FOB during acclimatization and on days 1, 8 and 15. Food consumption was recorded over a 24-hour period once during acclimatization and on days 1 - 2, 2 - 3, 8 - 9 and 14 - 15. Body weights were recorded in all animals once during the acclimatization period, daily during the first week of the observation period (days 1 - 8), and then at two weeks post-dose (day 15). 5 animals per group and sex were sacrificed on day 16 by intraperitoneal injection of pentobarbitone and perfusion fixation *in situ*. The brains from these animals were weighed after perfusion fixation and nervous tissues were collected for histopathology. The remaining animals were killed and discarded. Histopathology was performed on tissues collected from control and high-dose animals.

**Statistics:** All statistical tests were performed using appropriate computing devices or programs. The following statistical approaches were used in this study: All analyses were two-tailed for significance levels of 5% and 1% and all means were presented with standard deviations. Square root transformations were used for analysis of motor activity data in an attempt to stabilise the variances. Body weights, cumulative body weight gain, quantitative FOB measurements (grip strength, landing foot splay, body temperature), motor activity data at each measurement interval and overall activity, and absolute organ weights were analyzed initially by a one-way analysis of variance (ANOVA). Organ weights were also analyzed by analysis of covariance (ANCOVA) on final body weight. Summary values of organ to body weight ratios were presented but these were not analyzed statistically. Summary values of food consumption data were presented but no statistical analysis was performed due to the low number of cages per group (n = 2). For all parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test was used to compare the control and test item-treated groups. No statistical analysis of macropathology incidence data was conducted since there were no macroscopic findings. Micropathology incidence data were tabulated for each treatment group, but they were not analyzed statistically. Qualitative functional observational battery parameters were presented as summary data, but were not analyzed statistically.

## RESULTS

**Analysis of dose formulations:** The mean concentrations of SYN545974 in the group 2 - 4 dose formulations were determined to be within 90.4% to 99.5% of target. The formulations were homogeneous; coefficients of variation (CV) for the mean concentration determined from the individual concentrations obtained from the top, middle and bottom of each dose formulation were between 0.2% and 3.9%. Stability of dose formulations at 16 and 200 mg/mL for at least 7 days following both storage at refrigerator temperature ( $5 \pm 3^\circ\text{C}$ ) and at room temperature ( $20 \pm 5^\circ\text{C}$ ) was determined under GLP prior to initiation of dosing. The results obtained from storage stability samples did not deviate more than 1.6% from time-



zero reference (content or mean of homogeneity samples). No test item was detected in the vehicle control formulation.

**Mortality:** One 1000 mg/kg female (no. 65) was killed *in extremis* approximately 3.25 hours after treatment. The moribund condition of this animal was considered test item-related since no findings indicative of a gavage error were observed at necropsy and clinical signs for stress/toxicity were also observed in other females several hours after treatment with SYN545974.

**General cage-side clinical observations:** No clinical signs were observed for males and females at the daily scheduled cage-side examinations. The observations made for some females on day 1 between treatment and FOB examination which are considered to be related to test item administration are presented in Table 6.7.1-1.

**Table 6.7.1-1: Cage-side observations noted for females on day 1 at unscheduled intervals**

Animal number	Dose level (mg/kg)	Post-dose time	Clinical signs
53	100	2 hrs	Ruffled fur (grade 2), hunched posture
61	1000	4 hrs, 45 min	Lateral recumbency, both eyes closed, labored breathing, pale, ruffled fur (grade 1)
		5 hrs, 45 min	Repetitive chewing in cage
65	1000	3 hrs, 15 min	Marked convulsions, lateral recumbency, heavy breathing, skin cold on touch (animal killed for ethical reasons)
74	2000	3 hrs	Repetitive chewing in cage
75	2000	4 hrs, 30 min	Ruffled fur (grade 2), hunched posture, unsteady gait

No clinical signs were observed in the surviving animals during the cage-side observations at the end of the working day on day 1 and during days 2 - 15 of the observation period.

#### **Functional observational battery (FOB):**

**Detailed clinical observations:** For the 300, 1000 and 2000 mg/kg males there were no clinical signs seen during detailed clinical observations that were attributed to administration of the test item at any of the assessment intervals.

For the females, a number of findings were recorded during the detailed examinations at 6 hours post-dose (day 1). These findings are summarized below Table 6.7.1-2.

**Table 6.7.1-2: Cage-side observations noted for females on day 1 at unscheduled intervals**

FOB finding (max. severity grade)	Females			
	0 mg/kg	100 mg/kg	1000 mg/kg	2000 mg/kg
Recumbency (1)	0	0	1/9 (1.0)	0
Hunched posture (1)	0	1/10 (1.0)	0	2/10 (1.0)
Piloerection (2)	2/10 (1.0)	3/10 (1.0)	4/9 (1.3)	4/10 (1.3)
Vocalization (2)	3/10 (1.0)	0	1/9 (1.0)	2/10 (1.0)
Reduced muscle tone (1)	0	0	1/9 (1.0)	0
Reduced activity (2)	0	1/10 (1.0)	2/9 (1.5)	1/10 (1.0)
Decreased rearing (1)	2/10 (1.0)	1/10 (1.0)	2/9 (1.0)	1/10 (1.0)
Abnormal gait (2)	0	0	1/9 (1.0)	1/10 (1.0)
Skin cold on touch (1)	0	0	1/9 (1.0)	0
Pupillary reflex impaired (1)	0	0	1/9 (1.0)	0

	Females			
FOB finding (max. severity grade)	0 mg/kg	100 mg/kg	1000 mg/kg	2000 mg/kg
Mydriasis (1)	0	0	1/9 (1.0)	0

The following findings noted at the detailed clinical observations for 1000 and/or 2000 mg/kg females on day 1 were attributed to the treatment with the test item: recumbency, hunched posture, piloerection, reduced muscle tone, reduced activity, abnormal gait, skin cold-on-touch, impaired pupil reflex, and mydriasis.

The observation of vocalization at touching (grade 1) for one 1000 mg/kg female and two 2000 mg/kg females was not attributed to treatment with the test item as this observation was also noted for three control females. The observation of decreased rearing noted for two 1000 mg/kg females and one 2000 mg/kg female could not be conclusively attributed to treatment with the test item as this observation was also noted for two control females. However, it should be noted that during LMA testing on day 1, statistically significant decreases in the mean total number of rearings were noted for the 1000 and 2000 mg/kg females, relative to the control group.

One out of the ten 100 mg/kg females showed hunched posture, slight piloerection, reduced activity, and decreased rearing at the detailed FOB examinations 6 hours post-dose on day 1. Slight piloerection noted for two other 100 mg/kg females at 6 hours post-dose on day 1 was considered to be incidental because a similar observation was made for two control females at this time point. None of these above-mentioned FOB findings observed for the females at approximately 6 hours post-dose on day 1 were observed at the later examinations (days 8 or 15).

**Body temperature:** For the 300, 1000, and 2000 mg/kg males there were no statistically significant differences in mean body temperatures at any of the assessment intervals (days 1, 8 and 15).

For the females, a dose-dependent, minimal to moderate decrease in mean body temperature was observed on day 1 at approximately 6 hours post-dose when compared with the control group, attaining statistical significance at 2000 mg/kg (Table 6.7.1-3). This decrease in mean body temperature was attributed to treatment with the test item.

**Table 6.7.1-3: Mean body temperatures for females at 6 hours post-dose on day 1**

	Females			
	0 mg/kg	100 mg/kg	1000 mg/kg	2000 mg/kg
Mean body temperature (°C) ± standard deviation	38.2 ± 0.7	37.9 ± 0.5	37.2 ± 1.3	37.0 ± 1.3*
% Change from control		-0.8	-2.6	-3.1

\* Statistically significant at  $p < 0.05$

Individual females treated with 1000 or 2000 mg/kg had a markedly decreased body temperature on day 1, for which several findings were noted at the detailed examinations. Although not statistically different from the controls, the lower mean body temperature noted for the 1000 mg/kg females is attributed to treatment. The slight decrease in mean body temperature for the 100 mg/kg females noted at 6 hours post-dose was not statistically significantly different from the control group and was within the range of the historical control data.

On study days 8 and 15, mean body temperatures for the 100, 1000, and 2000 mg/kg females were similar to both control and pre-dose values.

**Grip strength:** There were no test item-related effects on fore- and/or hind-limb grip strength.

**Landing foot splay:** Landing foot splay was not affected by treatment with the test item at any of the assessment intervals (days 1, 8 and 15). Although statistically significantly increased values were recorded for the 100 mg/kg females (relative to controls) on days 1 and 8, the values were not dose-related and were lower than pre-dose values. Therefore, this increase was considered not to be related to administration of the test item.

**Locomotor activity (LMA):** For males there were no test item-related effects on LMA at any dose level. For the 1000 and 2000 mg/kg females, statistically significant, dose-related decreases in mean total distances were evident at approximately 6 hours post-dose (Table 6.7.1-4). Although the results for the 3-minute intervals were not themselves dose-related, these decreases were apparent from the start of the measuring period, with noticeable decreases in distances moved. Similarly, statistically significant, dose-related decreases in the mean number of rearings were noted for the 1000 and 2000 mg/kg females at 6 hours post-dose. While not statistically significant, the decrease in the amount of time spent in the center of the chamber by the 2000 mg/kg females was less (-74%) than control.

Although a slight decrease in LMA (all parameters) was also noted for the 100 mg/kg females at 6 hours post-dose on day 1, the differences from the control group did not attain statistical significance.

**Table 6.7.1-4: Mean LMA parameters obtained over a 30 minute session at 6 hours post dose on day 1**

	Females			
Dose level (mg/kg)	0	100	1000	2000
Total distance (cm)	2581.3	1767.1	1345.5**	1060.2**
% Change from Control	---	-32	-48	-59
Total center time (sec)	129	82	101	34
% Change from Control	---	-36	-22	-74
Total no. of rearings	47	25	16**	9**
% Change from Control	---	-47	-66	-81

\*\* Statistically significant at  $p < 0.01$

For both sexes, on days 8 and 15 of the study, all parameters for locomotor activity of test item-treated animals were similar to those of control animals and also within a range considered to be normal based on historical control data for rats of this strain and age.

**Body weight:** Statistically significant losses in mean body weights were noted for the 1000 and 2000 mg/kg males (relative to control) over the first day following dosing (days 1 - 2), with 8/10 and 7/10 males, respectively, losing weight over this period.

By day 3 of the study, all animals in the affected groups had gained weight, resulting in a group mean body weight gain similar to that of the controls, at both dose levels. There was no further effect on body weight/body weight gain during the course of the study.

No test item-related effects on body weight gain and body weight were observed in females.

**Food consumption:** The amount of food consumed by animals treated with the test item was similar to that of control animals during the whole observation period. During days 1 - 2, food consumption was reduced in all groups (including control group), which was likely related to the delayed placing of the animals into their home cages on day 1 (after FOB and LMA testing at 6 hours post-dose).

**Organ weights:** No test item-related effects on absolute, relative and adjusted brain weights were evident.

**Macroscopic findings:** No gross lesions were observed at necropsy.

**Microscopic findings:** There were no treatment-related histopathological findings.

**Discussion:** In this study, Han-Wistar rats of both sexes were administered a single oral dose of SYN545974 (prepared in a 1% carboxymethylcellulose aqueous vehicle) at 0, 100 (females only), 300 (males only), 1000 or 2000 mg/kg body weight and observed for 15 days.

In males, no test item-related effects on mortality, clinical signs, locomotor activity (LMA), body temperature, grip strength, landing foot splay, food consumption, or brain weight were observed, and there were no neurohistopathologic findings. Slight body weight loss occurred between days 1 and 2 in males treated with 1000 or 2000 mg/kg. This was considered to be not adverse since the effect was transient, and all males gained body weight again from day 2. No effect on body weight gain was observed in males treated with 300 mg/kg.

Females were generally more affected by treatment with the test item than males, predominantly at 1000 and 2000 mg/kg. On day 1 at approximately 3 - 6 hours post-dose, clinical signs indicative of stress/toxicity, such as recumbency, piloerection, hunched posture and/or skin cold at touch, were noted for some 1000 and 2000 mg/kg females. There was no dose-response relationship for these observations, and one 1000 mg/kg female was euthanized due to severe clinical signs at approximately 3.25 hours post-dose.

At approximately 6 hours post-dose some 1000 and 2000 mg/kg females had markedly decreased body temperature, and LMA was significantly decreased in these dose groups when compared to the control group.

A few clinical signs (including piloerection, hunched posture) were also observed in 2/10 females treated with the low dose of 100 mg/kg on day 1 (for animal no. 53 signs were present at cageside observations; for animal no. 58 signs were present during FOB assessment). Furthermore, mean body temperature and LMA were decreased at 6 hours post-dose on day 1 for the 100 mg/kg females when compared to the control group. These effects were considered not to be adverse since clinical signs occurred only during a short time after the test item-administration (observed only at 2 and 6 hours post-dose, respectively), and changes in body temperature and LMA were slight and transient (observed only on day 1).

No test item-related effects on food consumption, body weight, grip strength or landing foot splay were observed at any assessment interval in the females, and there were no test item-related effects on brain weight or neuropathological findings after the 15-day observation period. The test item-related clinical signs and effects on body temperature and LMA in females were only observed on the day of treatment, i.e. reversible.

## CONCLUSION:

In an acute neurotoxicity study conducted according to GLP and OECD TG 424 (1997), Han Wistar rats (10/sex/group) were administered a single oral gavage dose of 0, 100 (females), 300 (males), 1000 or 2000 mg/kg bw of pydiflumetofen. Over the subsequent 15 days, animals were observed via once daily cage-side general observations and a functional observational battery (FOB), performed pre-treatment and on days 1, 8 and 15 thereafter. Histopathological examination of nervous tissues was also included.

One female at 1000 mg/kg bw was sacrificed *in extremis* approximately 3.25 hours following treatment; this female presented with clinical signs comprising marked convulsions, lateral recumbency, heavy breathing and cold skin. Treatment related clinical signs of toxicity were also noted in surviving females in the period between dosing and FOB (i.e., within six hours following dosing). Clinical signs were indicative of general toxicity and comprised ruffled fur and hunched posture at 100 mg/kg bw, lateral recumbency, closed eyes, laboured breathing, pale ruffled fur, and repetitive cage chewing at 1000 mg/kg bw and ruffled fur, hunched posture, unsteady gait, and repetitive cage chewing, at 2000 mg/kg bw. There were no deaths or clinical signs of toxicity in males and no clinical signs were observed in surviving females at any other time-point.

With regard to the functional observational battery (FOB), there were no treatment related signs observed in males during the detailed clinical observations at any dose or time-point. A number of findings were recorded for females, however, only during the detailed examination conducted at 6 hours post-dose. These findings were indicative of general toxicity and are summarised in the table below:

<b>FOB finding (max. severity grade)</b>	<b>0 mg/kg bw</b>	<b>100 mg/kg bw</b>	<b>1000 mg/kg bw</b>	<b>2000 mg/kg bw</b>
Recumbency (1)	0	0	1/9	0
Hunched posture (1)	0	1/10	0	2/10
Piloerection (2)	2/10	3/10	4/9	4/10
Vocalisation (2)	3/10	0	1/9	2/10
Reduced muscle tone (1)	0	0	1/9	0
Reduced activity (2)	0	1/10	2/9	1/10
Decreased rearing (1)	2/10	1/10	2/9	1/10
Abnormal gait (2)	0	0	1/9	1/10
Skin cold to touch (1)	0	0	1/9	0
Pupillary reflex impaired	0	0	1/9	0
Mydriasis	0	0	1/9	0

In females at 1000 and/or 2000 mg/kg bw, treatment related findings comprising recumbency, hunched posture, piloerection, reduced muscle tone, reduced activity, abnormal gait, skin cold-on-touch, impaired pupil reflex and mydriasis were noted on day one (6-hours post-dose). Other observations (vocalisation and decreased rearing) were also noted at similar incidences in the control group and therefore were not related to treatment with pydiflumetofen. Similarly, findings at the low dose (hunched posture, piloerection, reduced activity, and decreased rearing) were also observed at similar incidences in the control group and therefore were not treatment related. No unusual clinical findings were seen at the day 8 or day 15 FOB examinations.

A dose-dependent decrease in body temperature was noted in females at 6-hours post-dose when compared with the control group (-0.8%, -2.6% and -3.1% at 100, 1000 and 2000 mg/kg bw), with the change being statistically significant in the high-dose group. The change at 100 mg/kg bw was slight, not statistically different from control and was within the range of historical control data; therefore, only the findings from 1000 mg/kg bw are related to treatment. Body temperature was not affected in either males or females at the later measurements.

There was no effect on grip strength or landing foot splay. In mid- and high-dose females, locomotor activity (LMA) was affected at 6-hours post-dose, as evidenced by a dose-related, statistically significant decrease in mean total distance (-32%, -48% and -59% compared with control at 100, 1000 and 2000 mg/kg bw respectively) and mean number of rearings (-47%, -66% and -81% compared with control at 100, 1000 and 2000 mg/kg bw respectively). A decrease in the time spent in the chamber centre was also seen in high-dose females, although the change was not statistically significant (-74% compared with control). There were no unusual findings in males or in females at either the day 8 or day 15 examinations.

There was no effect on body weight or food consumption; initial body weight losses in males following dosing had recovered by day 3 resulting in group mean body weights that were similar to controls. Similarly, initial reductions in food consumption did not persist beyond day 3 and overall food consumption was not affected.

There was no effect on organ weights and no unusual macroscopic or microscopic findings; in particular, no lesions were noted on the tissues relating to the central or peripheral nervous system.

Overall, administration of pydiflumetofen to rats via a single oral gavage dose, resulted in clinical signs, decreased body temperature, and decreased locomotor activity in females only at 1000 and 2000 mg/kg bw/d, being apparent on day 1 only (6-hours post dose). The clinical signs observed were transient and were indicative of general systemic toxicity and not a specific effect on the nervous system. Signs that could indicate potential neurotoxicity (reduced body temperature and locomotor activity) were also transient as they were only noted at the first measurement (6-hours post-dose) and had fully recovered by

the next measurements on day 8). Furthermore, there were no unusual histopathological findings on any tissues relating to the central or peripheral nervous system.

Overall, a **NOAEL for potential acute neurotoxicity of 100 mg/kg bw was determined in females** from this study. In males the NOAEL for acute neurotoxicity was determined to be 2000 mg/kg bw (the highest dose tested).

(██████████, 2015)

<b>Report:</b>	K-CA 5.7.1/02 ██████████ (2015a). SYN545974 - An Abbreviated Acute Oral (Gavage) Neurotoxicity Study in the Female Wistar Rat. Report Amendment 1. ██████████ ██████████ Laboratory Report No. ██████████. Issue date: 15 September 2015. Unpublished. Syngenta File No. SYN545974_10197.
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**Guidelines:** Modified Acute Oral Neurotoxicity (rat) - non-guideline

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

The purpose of this study was to further assess the potential neurotoxic effects of SYN545974 when administered to female rats as a single oral (gavage) dose at 0, 100, 300 or 1000 mg/kg. The study was largely based on the results of a previously conducted acute neurotoxicity study of SYN545974 (██████████, 2015a) in which the no-observable-adverse-effect (NOAEL) for males was 2000 mg/kg (highest dose tested) whereas the NOAEL for females was 100 mg/kg (lowest dose tested). This study was designed to better define the NOAEL in female rats by including a dose level between the previously determined NOAEL (100 mg/kg) and LOAEL (lowest-observable adverse effect level; 1000 mg/kg) and confirm the treatment-related observational findings noted for the female rats, which were seen exclusively post-dose on day 1. Female Han-Wistar rats in groups 1 - 4 (10 females/group) were administered a single oral dose of SYN545974 (prepared in 1% carboxymethylcellulose aqueous vehicle) at dose levels of 0 (control), 100, 300, or 1000 mg/kg body weight, and observed for 8 days. General cage-side observations were performed once daily, with additional observations performed several times on day 1 prior to the detailed examinations. A functional observational battery (FOB) was performed once during acclimatization (pre-treatment), on day 1 (approximately 6 hours after treatment), and on day 8. The FOB included detailed clinical examinations as well as quantitative assessment of landing foot splay, body temperature and grip strength. Locomotor activity (LMA) was assessed over a time period of 30 minutes after each FOB evaluation (LMA evaluations included distance, center time and number of rearings). On day 1, a blood sample was collected from every animal after LMA measurement for possible analysis. Food consumption was recorded once during acclimatization and on days 1 - 2, 2 - 3, and 7 - 8. Body weights were recorded once during acclimatization and on days 1 - 8. On day 9, the animals were sacrificed and a gross examination was performed. The lungs, trachea, samples of liver, and any macroscopic abnormalities were collected and preserved for possible future examination.

Treatment-related findings were noted on the day of dosing at 100 and 1000 mg/kg. In these dose groups, various clinical signs, including recumbency, piloerection, reduced activity, skin cold on touch, and impaired extensor thrust reflex were observed within 2 - 6 hours post-dose. There was no dose-response relationship for clinical signs. At 6 hours post-dose markedly decreased body temperature and decreased LMA were recorded for individual animals at 100 and 1000 mg/kg. A slight trend towards decreased mean body temperature was observed for all test item-treated groups (dose levels  $\geq 100$  mg/kg), and a trend towards decreased mean LMA was observed for groups treated at dose levels  $\geq 300$  mg/kg.

No test item-related effects on food consumption and body weight were observed at any dose level.

All in-life findings attributed to treatment were transient and limited to the first day following treatment and no clinical observations were observed as part of the cage side observations at the end of Day 1.

There were no macroscopic abnormalities observed at necropsy on day 9.

Based on the results of this study, the lowest observed effect level (LOAEL) was 100 mg/kg in this acute neurotoxicity study based on post-dose findings on study day 1. However, all test item-related effects were transient and reversed quickly.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545974 (SYN545974 technical)
<b>Description:</b>	Off white powder
<b>Lot/Batch number:</b>	SMU2EP12007
<b>Purity:</b>	98.5%
<b>CAS#:</b>	1228284-64-7
<b>Stability of test compound:</b>	Stable under storage conditions. Retest date: End of June 2016

**Vehicle and control item:** 1% (w/v) carboxymethylcellulose (CMC) in purified water

<b>Test Animals:</b>	
<b>Species:</b>	Rat
<b>Strain:</b>	RccHan <sup>TM</sup> :WIST (SPF)
<b>Age at dosing:</b>	7 weeks
<b>Source:</b>	
<b>Housing:</b>	Standard Laboratory Conditions. In groups of five in Makrolon type-4 cages with wire mesh tops and standard softwood bedding (J. Rettenmaier & Söhne GmbH & Co. KG, 73494 Rosenberg, Germany, imported by Provimi Kliba AG, 4303 Kaiseraugst, Switzerland) including paper enrichment (Enviro-dri from Lillico, Biotechnology, Surrey, United Kingdom). The animals were housed individually in Makrolon type-3 cages (in randomized order) during FOB evaluation.
<b>Acclimatisation period:</b>	6 days
<b>Diet:</b>	Pelleted standard 2914C rodent maintenance diet, batch no. 46/14 ( ) <i>ad libitum</i> .
<b>Water:</b>	Community tap water from ( ) <i>ad libitum</i> .
<b>Environmental conditions:</b>	Temperature: 19 - 22 °C Humidity: 44 - 59 % Air changes: 10 - 15 / h Photoperiod: 12 hours light and 12 hours dark

### Study Design and Methods:

**In-life dates:** Start: 27 Nov 2014      End: 12 Dec 2014

**Animal assignment and treatment:** The animals received a single dose of the test item by oral gavage administration according to the scheme in the table below and were observed for 8 days.

**Group identification and animal numbers**

Group:	Group 1	Group 2	Group 3	Group 4
Description:	Control*	Low	Mid	High
Dose (mg/kg bw):	0*	100	300	1000
Dose Volume (mL/kg bw)	10	10	10	10
Concentration** (mg/mL):	0*	10	30	100
Females	1 - 10	11 - 20	21 - 30	31 - 40

\* Control animals will receive the dosing solution (vehicle) without the test item.

\*\* Dose concentrations are in terms of test item as supplied. No correction factor for purity was used for preparation of dose formulations.

Viability/mortality was checked twice daily. General cage-side clinical observations were recorded once daily. On the day of treatment (day 1), cage-side observations were made at the end of the working day. Cage-side observations were additionally made on day 1 several times before FOB testing. A functional observational battery (FOB) was performed once during acclimatization, on day 1 (at approximately 6 hours after dosing), and on day 8. The FOB included detailed clinical observations of animals in the cage, during handling and in a standard arena (open field), tests for reflexes and other reactions to stimuli, test for hearing ability, body temperature, landing foot splay and grip strength. Locomotor activity (LMA) using an automatic open field device was recorded over 30 minutes following conduct of the FOB during acclimatization and on day 1. A blood sample for possible future examination was collected from each animal following the conduct of the LMA assessment on day 1. Food consumption was recorded over a 24-hour period once during acclimatization and on days 1 – 2 (except during the interval from dosing through LMA testing when the rats were individually housed), 2 - 3, and 7 – 8. Body weights were recorded in all animals once during the acclimatization period and daily during the observation period (days 1 - 8). On day 8 all animals were euthanized by intraperitoneal injection of pentobarbitone. A gross examination was performed on all animals. Lungs, trachea, and samples of liver were collected for possible future examination.

**Statistics:** All statistical tests were performed using appropriate computing devices or programs. The following statistical approaches were used in this study: All analyses were two-tailed for significance levels of 5% and 1% and all means were presented with standard deviations. Square root transformations were used for analysis of LMA data in an attempt to stabilise the variances. Body weights, cumulative body weight gain, quantitative FOB measurements (grip strength, landing foot splay, body temperature), motor activity data at each measurement interval and overall activity were analyzed initially by a one-way analysis of variance (ANOVA). Summary values of food consumption data were presented but no statistical analysis was performed due to the low number of cages per group (n = 2). For all parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test was used to compare the control and test item-treated groups. Qualitative functional observational battery parameters were presented as summary data, but were not analyzed statistically.

**RESULTS**

**Analysis of dose formulations:** The mean concentrations of SYN545974 in the group 2 - 4 dose formulations were determined to be within 99.3 to 107.6% of target. The formulations were homogeneous; coefficients of variation (CV) for the mean concentration determined from the individual concentrations obtained from the top, middle and bottom of each dose formulation were between 0.3% and 1.1%. Stability of dose formulations at 16 and 200 mg/mL for at least 7 days following both storage at refrigerator temperature ( $5 \pm 3$  °C) and at room temperature ( $20 \pm 5$  °C) was determined under GLP prior to initiation of dosing. The results obtained from storage stability samples did not deviate more than 1.6% from time-zero reference (content or mean of homogeneity samples). No test item was detected in the vehicle control formulation.

**Mortality:** No mortality occurred.



**General cage-side clinical observations:** No clinical signs were observed at the daily scheduled cage-side examinations. Observations made for some animals on day 1 between treatment and FOB examination are presented below in Table 6.7.1-5.

**Table 6.7.1-5: Cage-side observations noted on day 1 at unscheduled intervals**

Animal number	Dose level (mg/kg)	Post-dose time	Clinical signs
15	100	2 hrs, 15 min	Ruffled fur, eyes half closed, ventral recumbency
17	100	4 hrs, 45 min	Ruffled fur, eyes half closed, ventral recumbency
34	1000	3 hrs	Ruffled fur, eyes half closed, ventral recumbency

These observations in the females were attributed to the test item-administration due to the post-dose time point (within several hours after treatment), and the fact that several test item-related findings were noted for these animals at the functional observational battery (FOB) and locomotor activity (LMA) assessments at approximately 6 hours post-dose.

No clinical signs were observed in the animals during the cage-side observations at the end of the working day on day 1 or during days 2 - 8 of the post-dose observation period.

#### **Functional observational battery (FOB):**

**Detailed clinical observations:** A number of findings were recorded during the detailed examinations at 6 hours post-dose (day 1). These findings are summarized below in Table 6.7.1-6.

**Table 6.7.1-6: Incidences of detailed clinical observations for females at 6 hours post-dose on day 1 (total number of animals affected and mean severity grade)**

FOB finding (max. severity grade)	Females			
	0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
Piloerection (2)	0	1/10 (2.0)	0	1/10 (2.0)
Vocalization (2)	1/10 (1.0)	1/10 (1.0)	1/10 (1.0)	2/10 (1.0)
Reduced activity (2)	1/10 (1.0)	4/10 (1.0)	1/10 (1.0)	3/10 (1.0)
Decreased rearing (1)	4/10 (1.0)	7/10 (1.0)	4/10 (1.0)	5/10 (1.0)
Tremor (2)	0	0	0	1/10 (1.0)
Skin cold on touch (1)	0	2/10 (1.0)	0	1/10 (1.0)
Pupillary reflex impaired (1)	0	0	1/10 (1.0)	0
Miosis (1)	0	0	1/10 (1.0)	0
Extensor thrust reflex impaired (1)	0	2/10 (1.0)	0	1/10 (1.0)

The following findings noted at the detailed clinical observations for 100 and/or 1000 mg/kg females on day 1 were attributed to the treatment with the test item: piloerection, tremor, skin cold-on-touch, and extensor thrust reflex impaired. These findings were recorded for animals which had also shown several clinical signs during cage-side observations between test item administration and FOB testing (nos. 15 and 17 at 100 mg/kg and no. 34 at 1000 mg/kg).

For animal nos. 15 (100 mg/kg) and 34 (1000 mg/kg), the gait could not be assessed during the detailed examinations in the open field as these animals only showed a few movements.

The observation of vocalization on touching (grade 1) for four animals at  $\geq 100$  mg/kg was not attributed to treatment with the test item as this observation was also noted for one control animal.

The observation of reduced activity and/or decreased rearing noted for several animals at  $\geq 100$  mg/kg could not be conclusively attributed to treatment with the test item as this observation was also noted in the control group. However, it should be noted that during LMA testing on day 1, markedly decreased LMA (total

distance and number of rears) was recorded for animals with test item-related clinical signs at FOB testing, i.e. numbers 15, 17 and 34.

Miosis and impaired pupillary reflex noted for one 300 mg/kg female at 6 hours post-dose were considered to be incidental since this finding occurred in isolation and without dose-response relationship.

None of the test item-related FOB findings observed at approximately 6 hours post-dose on day 1 were observed at the examination on day 8.

**Body temperature:** A minimal decrease in mean body temperature was observed on day 1 for animals in all treated groups at approximately 6 hours post-dose when compared with the control group (Table 6.7.1-7). However, only animal numbers 15, 17 (100 mg/kg) and 34 (1000 mg/kg) had body temperatures outside of the range seen in the concurrent control group; these animals also had several findings at the detailed clinical observations. When these animals were excluded as outliers for the statistical analysis, the differences between groups 3 and 4 (300 and 1000 mg/kg) and the control group maintained statistical significance, which is consistent with the findings from the previous acute neurotoxicity study for females treated with SYN545974 at 2000 mg/kg. Therefore the decrease in mean body temperatures for the 300 and 1000 mg/kg females on day 1 was considered test item-related.

**Table 6.7.1-7: Mean body temperatures at 6 hours post-dose on day 1**

	Females			
	0 mg/kg	100 mg/kg	300 mg/kg	1000 mg/kg
Mean body temperature (°C) ± standard deviation	38.0 ± 0.3	37.0 ± 1.6	37.6 ± 0.2	37.2 ± 1.2
% Change from control		-2.6	-1.1	-2.1
<i>Exclusion of animal nos. 15, 17 and 34 as outliers:</i>				
Mean body temperature (°C) ± standard deviation	38.0 ± 0.3	37.7 ± 0.3	37.6 ± 0.2*	37.6 ± 0.3*
% Change from control		-0.8	-1.1	-1.1

\* Statistically significant at  $p < 0.05$

On day 8, mean body temperatures for the 100, 300, and 1000 mg/kg animals were similar to both control and pre-dose values.

**Grip strength:** There were no test item-related effects on fore- and/or hind-limb grip strength at any of the assessment intervals (days 1 and 8).

The mean hindlimb grip strength was slightly decreased on day 8 in the group treated at 1000 mg/kg (group 4) when compared to the control group. This change was considered as incidental, because the results for group 4 animals were similar to the pretest data.

**Landing foot splay:** Landing foot splay was not affected by treatment with the test item at any of the assessment intervals (days 1 and 8).

**Locomotor activity (LMA):** For the 300 and 1000 mg/kg females, slightly decreased mean total distance and mean number of rears were evident at approximately 6 hours post-dose (Table 6.7.1-8). The differences relative to the control group did not attain statistical significance but they were consistent with the LMA results in the previous acute neurotoxicity study with SYN545974. Furthermore, markedly decreased LMA was measured for animal nos. 15 and 17 at 100 mg/kg and no. 34 at 1000 mg/kg, which is consistent with the other findings recorded for these animals at the FOB examination. Therefore, it was considered that the

treatment with the test item at dose levels  $\geq 100$  mg/kg resulted in the decreased LMA noted at 6 hours post-dose on day 1.

**Table 6.7.1-8: Mean LMA parameters obtained over a 30 min session at 6 hrs post-dose on day 1**

	Females			
Dose level (mg/kg)	0	100	300	1000
Total distance (cm)	2533.3	2444.2	1874.1	1821.8
% Change from Control	---	-4	-26	-28
Total center time (sec)	103	169	70	117
% Change from Control	---	+64	-32	+14
Total no. of rears	51	51	32	30
% Change from Control	---	0	-37	-41

On day 8, all parameters for locomotor activity of test item-treated animals were similar to those of control animals.

**Body weight:** No test item-related effects on body weight gain and body weight were observed.

Slight body weight loss over days 1 - 2 was noted for animals that had shown test item-related effects on day 1 (nos. 15, 17 and 34). However, this could not be conclusively attributed to treatment with the test item as some body weight loss was also noted for some control females during this period. At the end of the observation period, mean values for body weight gain and body weight were similar in test item-treated groups and control group.

**Food consumption:** The amount of food consumed by animals treated with the test item was similar to that of control animals during the whole observation period. During days 1 - 2, food consumption was slightly reduced in all groups (including control group), which is likely related to the delayed placing of the animals into their home cages following dosing and the FOB/LMA assessments on day 1.

**Macroscopic findings:** No gross lesions were observed at necropsy.

**Discussion:** In this study, female Han-Wistar rats were administered a single oral dose of SYN545974 (prepared in a 1% carboxymethylcellulose aqueous vehicle) at 0, 100, 300, or 1000 mg/kg body weight and observed for 8 days.

On day 1 at approximately 2 - 6 hours post-dose, clinical signs indicative of stress/toxicity, such as recumbency, piloerection, skin cold at touch, and impaired extensor thrust reflex, were noted for two 100 mg/kg animals and one 1000 mg/kg animal. The 1000 mg/kg animal additionally showed slight tremor at approximately 6 hours post-dose. Furthermore, all three animals had markedly decreased body temperature and decreased LMA on day 1. A slight trend towards decreased mean body temperature was observed for all test item-treated groups ( $\geq 100$  mg/kg), and a trend towards decreased mean LMA was observed for groups treated at  $\geq 300$  mg/kg.

No test item-related effects on food consumption, body weight, grip strength or landing foot splay were observed at any assessment interval. Test item-related clinical signs and effects on body temperature and LMA were only observed on the day of treatment (i.e. the effects were reversible).

The observed test item-related effects are similar to those observed for females in the previously conducted acute neurotoxicity study of SYN545974 (██████████, 2015a).

## CONCLUSION:

In the previous acute neurotoxicity study in rats (■■■■■ 2015) a NOAEL for potential neurotoxicity of 100 mg/kg bw was determined in females (the neurotoxicity NOAEL for males was 2000 mg/kg bw, the highest dose tested). Therefore, to further investigate the potential neurotoxic effects in females (seen only on day 1) and to better define the NOAEL, a further acute neurotoxicity study was conducted in female rats only.

The modified acute neurotoxicity study was conducted according to GLP but not according to any particular test guideline. Female Han Wistar rats (10/group) were administered a single oral gavage dose of pydiflumetofen at dose levels of 0, 100, 300 or 1000 mg/kg bw followed by an observation period of eight days (once daily cage-side observations were carried out as well as FOB which was conducted pre-treatment and then on day 1 (post-dose) and day 8.

There were no deaths; clinical signs of toxicity noted in some animals at 100 and 1000 mg/kg bw during the general cage-side observations comprised ruffled fur, eyes half-closed and ventral recumbency, and were seen on day one only (within 6-hours post-dose). A number of clinical signs indicative of general toxicity were also noted during the detailed clinical observations conducted as part of the FOB on day 1 (6-hours post-dose). These are summarised in the table below.

<b>FOB finding (max. severity grade)</b>	<b>0 mg/kg bw</b>	<b>100 mg/kg bw</b>	<b>300 mg/kg bw</b>	<b>1000 mg/kg bw</b>
Piloerection (2)	0	1/10	0	1/10
Vocalisation (2)	1/10	1/10	1/10	2/10
Reduced activity (2)	1/10	4/10	1/10	3/10
Decreased rearing (1)	4/10	7/10	4/10	5/10
Tremor (2)	0	0	0	1/10
Skin cold to touch (1)	0	2/10	0	1/10
Pupillary reflex impaired	0	0	1/10	0
Miosis (1)	0	0	1/10	0
Extensor thrust reflex impaired	0	2/10	0	1/10

Piloerection, tremor, skin cold-on-touch and impaired extensor thrust reflex at 100 and/or 1000 mg/kg bw were related to treatment with pydiflumetofen; the same animals had also presented with severe clinical signs during the cage-side observations. Other findings were not treatment related as they were either also found in the control group (vocalisation) or were noted in isolation without a clear dose response (miosis and impaired pupillary reflex). Decreased activity and decreased rearing were also noted in the control group but were nevertheless attributed to treatment as they were consistent with findings in the locomotor activity assessment.

In all treated groups, body temperature was slightly decreased compared with controls on day 1 (6-hours post-dose), being statistically significantly different from controls at 300 and 1000 mg/kg bw/d. Body temperature was comparable with controls in all treated groups at the next assessment on day 8.

There were no treatment related effects on grip strength or landing foot splay. Locomotor activity (LMA) assessments revealed a slight decrease in the mean total distance and the mean number of rears at 300 and 1000 mg/kg bw (not statistically significant). Additionally, two animals at 100 mg/kg bw showed the same reduction in LMA activity (similar to the findings recorded at the FOB assessment). Therefore, the effect on locomotor activity was treatment related from the low dose of 100 mg/kg bw. All LMA parameters had returned to normal by the next scheduled assessment on day 8.

There was no effect on body weight development or food consumption and no unusual findings at necropsy, notably, there were no unusual findings in tissues related to the central or peripheral nervous system.

Overall, when pydiflumetofen was administered to female rats via a single oral gavage dose of 0, 100, 300 or 1000 mg/kg bw, effects consistent with the previous acute neurotoxicity study were seen in all treated groups. Clinical signs indicative of general toxicity were observed at the cage side observations and the FOB assessments but were transient as they only occurred at the time of peak effect (i.e., within 6-hours of

dosing). Signs potentially related to neurotoxicity (decreased body temperature and decreased locomotor activity) were also transient and reversible as they were only noted at the 6-hour post-dose measurement and had fully recovered by the next measurement on day 8. Furthermore, a clear dose response was not always evident (some effects were seen at 100 and 1000 mg/kg bw but not at 300 mg/kg bw) and no histopathological findings indicative of an effect on the nervous system were evident.

**A LOAEL for neurotoxicity of 100 mg/kg bw** was determined from this acute neurotoxicity study, albeit based on transient and reversible effects.

(██████████, 2015a)

#### B.6.7.2. Overall summary of neurotoxicity

In an acute oral (gavage) neurotoxicity study in male and female rats, there was no effect on males up to and including the highest dose tested; in females, however, clinical signs of toxicity were noted from 1000 mg/kg bw at both the cage-side observation and the FOB assessment, with one female at 1000 mg/kg bw being sacrificed in extremis. Clinical signs seen within 2-6 hours of dosing (ruffled fur, hunched posture, lateral recumbency, closed eyes, laboured breathing, pale/ruffled fur, repetitive cage chewing and unsteady gate) were transient and indicative of general toxicity, and not a specific neurotoxic effect.

Accompanying the clinical signs from 1000 mg/kg bw were transient signs potentially indicative of neurotoxicity, comprising decreased body temperature and an effect on locomotor activity (decreased mean total distance and mean number of rearings). However, the findings were transient and reversible. No unusual findings were seen at necropsy, particularly on those tissues relating to the central or peripheral nervous system.

Overall, a **NOAEL for acute neurotoxicity of 100 mg/kg bw** was ascertained in females in this study.

In a second oral (gavage) acute neurotoxicity study, conducted in females only, similar transient effects were seen. Clinical signs of toxicity were noted from 100 mg/kg bw during the cage-side observations (within 6-hours post-dose) and from 100 mg/kg bw at the first FOB assessment at 6-hours post-dose only. All effects had reversed by the next examination. Consistent with the findings of the first study, body temperature was statistically significantly reduced on day 1 (6-hours post dose) and an effect on locomotor activity (reduced mean total distance and mean number of rears) was noted from 100 mg/kg bw. No clear dose response was seen between the 100 and 300 mg/kg bw dose groups and the effects were transient and reversible (in all cases they were only observed at the day one measurements).

Overall, the low dose of **100 mg/kg bw** was considered the **LOAEL for acute neurotoxicity** in this confirmatory study. Taken together, a **LOAEL of 100 mg/kg bw** has been identified for pydiflumetofen for potential acute neurotoxicity.

Overall, in two acute neurotoxicity studies, transient effects (day 1 only) were seen in females, comprising clinical signs of toxicity, reduced body temperature and reduced locomotor activity. In a 90-day toxicity study in the rat (██████████ and ██████████, 2015), no similar effects were seen during detailed clinical examinations, functional observational battery (FOB) parameters or locomotor activity (LMA) assessments up to and including the highest dose tested of 16000 ppm (1322/1174 mg/kg bw/d in males and females).

Owing to the transient nature of the effects, no classification for STOT SE is warranted for pydiflumetofen. This was confirmed in a recent [GB MCL Technical Report](#) for pydiflumetofen which concluded (in agreement with RAC) that no classification for STOT SE was necessary for pydiflumetofen, owing to the transient and reversible nature of the observed effects.

The table below summarises the main findings in the acute neurotoxicity studies.

Study & Acceptability	Test material & Dose levels	NOAEL	LOAEL	Effects at LOAEL
Acute neurotoxicity study (██████, 2015)  <i>Acceptable modern study</i>	Pydiflumetofen  Males: 0, 300, 1000 & 2000 mg/kg bw  Females: 0, 100, 1000 & 2000 mg/kg bw	<i>Neurotoxicity &amp; general toxicity</i> 100 mg/kg bw in females	<i>Neurotoxicity &amp; general toxicity</i> 1000 mg/kg bw in females	1 F sacrificed in extremis  Clinical signs on day 1 in F:  Ruffled fur, laboured breathing, recumbency, piloerection, reduced muscle tone, reduced activity, abnormal gait, skin cold-to-touch, impaired pupil reflex, and mydriasis  ↓ Body temperature  ↓ Locomotor activity (mean total distance and mean number of rearings)
Acute neurotoxicity study in females only (██████, 2015a)  <i>Acceptable modern study</i>	Pydiflumetofen  Females: 0, 100, 300 & 1000 mg/kg bw	<i>Neurotoxicity &amp; general toxicity</i> <100 mg/kg bw	<i>Neurotoxicity &amp; general toxicity</i> 100 mg/kg bw	Clinical signs on day 1:  Ruffled fur, ventral recumbency, piloerection, skin cold-to-touch & impaired extensor thrust reflex, decreased activity, and decreased rearing  ↓ Body temperature  ↓ Locomotor activity (mean total distance and mean number of rearings)

### B.6.7.3. Delayed polyneuropathy studies

None provided and none required.

### B.6.8. OTHER TOXICOLOGICAL STUDIES

### B.6.8.1. Toxicity studies on metabolites and relevant impurities

#### Studies on metabolites

A number of toxicity studies have been conducted on metabolites of SYN545974 which were detected in livestock (CSAA798670 and SYN508272) and summaries of the data are presented below. These metabolites (CSAA798670 and SYN508272) are common to a number of SDHI molecules. In addition, 2, 4, 6-trichlorophenol (2, 4, 6-TCP)<sup>6</sup> and its related metabolites (hydroxyl TCP sulphate and 2, 4, 6-TCP sulphate) were identified in animal commodities and are included in the definition of residue only for animal commodities. However, in the rat and mouse, 2, 4, 6-TCP was the major circulating metabolite of SYN545974 in plasma (██████████ and ██████████, 2015 and ██████████ *et al.*, 2015 see Section 6.1.1/05 and 6.1.1/08 respectively). Therefore, the mammalian toxicity database on SYN545974 also assesses the toxicity of 2, 4, 6-TCP and risk assessment endpoints for SYN545974 are considered appropriate also for 2, 4, 6-TCP. In accordance with Article 8(5) of Regulation (EC) No 1107/2009 published literature data on relevant metabolites are required, therefore summaries of relevant literature data identified in MCA Section 9 – Toxicology are included in this section.

During the commenting period, EFSA requested additional information in regards to several pydiflumetofen metabolites.

**EFSA Request for additional information (February 2018), Question 35:** Applicant to provide further assessment of the toxicological profile of the metabolite SYN548263.

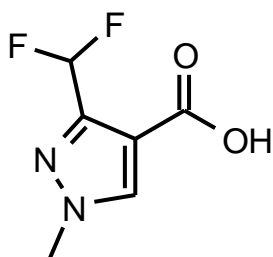
Additional genotoxicity studies on SYN548263 and accompanying summaries have been provided by the applicant and assessed by HSE later in this section.

**EFSA Request for additional information (February 2018), Question 41:**

Applicant to provide a detailed assessment of the QSAR analysis as well read across analysis (including genotoxicity and other toxicological endpoints) in order to characterize the toxicological profile of the metabolite SYN547897.

A detailed assessment of the QSAR analysis as well read across analysis for genotoxicity of metabolite SYN547897 has been provided by the applicant and assessed later in this section by HSE.

#### **B.6.8.1.1. Metabolite CSAA798670 glucuronide/sulphate**



3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxylic acid (CSAA798670 aglycon)

Metabolite CSAA798670 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, CSAA798670. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

<sup>6</sup> From here on in 2,4,6-TCP refers to “2,4,6-TCP and its related metabolites particularly hydroxyl TCP sulphate and 2,4,6-TCP sulphate”, due to the rapid conjugation of 2,4,6-TCP *in vivo*.

Toxicity (acute oral toxicity study, 28-d study, 90-d study, rabbit PNDT study) and standard in vitro genotoxicity studies have been conducted with the metabolite CSAA798670, also known as DF-pyrazole acid. Synonyms for this metabolite are CA4312 and NOA449410. The same metabolite has also been tested in studies undertaken by [REDACTED] under the test substance code M700F001 and hence the summaries for studies conducted by [REDACTED] (for which Syngenta has co-ownership) refer to the test substance as M700F001.

#### Acute oral toxicity study

<b>Report:</b>	K-CA 5.8.1/01 [REDACTED] (2008). DF-Pyrazole Acid (CA4312): Screening Acute Oral Toxicity Study in the Rat. [REDACTED] [REDACTED]. Laboratory Report No. [REDACTED] 2364/0169 issue date 18 Jan 2008. Amendment 1 2011. Unpublished. Syngenta File No.CA4312/0003.
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**Guidelines:** This study was a screening study and was not conducted according to a specific regulatory guideline.

**GLP:** The study was conducted according to the current version of the UK Principles of GLP (The United Kingdom GLP Regulations) and the OECD Principles of Good Laboratory Practice 1997 (ENV/MC/CHEM(98) 17) except that the report was not subject to Quality Assurance Audit.

**HSE comment:** the study was considered acceptable

### EXECUTIVE SUMMARY

In an acute oral toxicity study, four fasted Sprague-Dawley CD strain rats (two males and two females) were treated with DF-pyrazole acid (CA4312) at a dose level of 2000 mg/kg bw.

Clinical signs and body weight development were monitored during the study.

All animals were subjected to gross necropsy.

There were no deaths and no signs of systemic toxicity.

All animals gained weight and no effect was observed on bodyweight in surviving animals. There were no abnormalities seen at study termination

**The acute oral median lethal dose (LD<sub>50</sub>) of DF-pyrazole acid (CA4312) in the Sprague-Dawley CD strain rat was estimated as being >2000 mg/kg bw.**

### MATERIALS AND METHODS

#### Materials:

Test Material:	CA4312 DF-pyrazole acid
Description:	Not reported
Lot/Batch number:	Not reported
Purity:	Not reported
CAS#:	Not reported
Stability of test compound:	Not reported

**Vehicle and/or positive control:** Dimethyl sulphoxide



<b>Test Animals:</b>	
<b>Species:</b>	Rat (male and female)
<b>Strain:</b>	Sprague-Dawley CD
<b>Age/weight at dosing:</b>	Age not specified. Weight at dosing Males: 299-312 g; Females: 204-252 g
<b>Source:</b>	Not reported
<b>Housing:</b>	Not reported
<b>Acclimatisation period:</b>	Not reported
<b>Diet:</b>	Not reported
<b>Water:</b>	Not reported
<b>Environmental conditions:</b>	Not reported

### Study Design and Methods:

**In-life dates:** Start: 17 December 2007 End: 25 December 2007

**Animal assignment and treatment:** In an acute oral toxicity study, 2 male and 2 female Sprague-Dawley CD strain rats were fasted before receiving a single oral dose of 2000 mg/kg bw of DF-Pyrazole (CA4312) by gavage. The test substance was formulated as a solution in dimethyl sulphoxide.

The animals were examined during the course of the study for clinical signs and bodyweight development. All animals were subjected to gross necropsy.

### RESULTS

**Mortality:** There were no deaths.

**Clinical observations:** There were no clinical effects noted.

**Bodyweight:** All animals gained weight and no effect was observed on bodyweight.

**Necropsy:** There were no abnormalities seen in animals at study termination.

### CONCLUSION:

The potential acute oral toxicity of metabolite CSAA798670 was investigated in a screening GLP compliant study similar to OECD 425 guideline. Two male and two female SD rats were administered 2000 mg/kg bw of the test item by oral gavage.

There were no mortalities, no clinical signs of toxicity, non effects on body weights and no abnormalities at necropsy. As a result, HSE agrees with the EU evaluation, that **the acute oral LD<sub>50</sub> of CSAA798670 was >2000 mg/kg bw.**

(██████████, 2008)

### Ames test

<b>Report:</b>	K-CA 5.8.1/02 ██████████ (2007). <i>Salmonella typhimurium</i> and <i>Escherichia coli</i> Reverse Mutation Assay with DF-pyrazole Acid (CA4312). RCC, Cytotest Cell Research GmbH (RCC-CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1077403; 24 May 2007. Unpublished. Syngenta File No. SYN520453/0096.
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**Guidelines:** OECD 471 (1997); OPPTS 870.5100 (1998); 2000/32/EEC B.13/B.14 (2000)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

**EFSA Request for additional information (February 2018), Question 36:** Applicant to provide the summary tables of the results regarding the Ames test, the chromosome aberration test, and the cell mutation assay in mammalian cells with CSAA798670.

Additional summary tables have been included to the OECD summaries of the Ames test, the chromosome aberration test, and the cell mutation assay in mammalian cells with CSAA798670.

## EXECUTIVE SUMMARY

In a reverse gene mutation assay in bacteria, the potential of DF-pyrazole acid (CA4312) to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strains WP2 uvrA (pKM 101), and WP2 (pKM 101), was investigated.

The assay was performed in two independent experiments both with and without liver microsomal activation. Due to a questionable minor increase in strain WP2 uvrA (pKM 101) without metabolic activation in experiment I, this part was repeated (reported as Exp. IA). Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations:

Pre-Experiment/Experiment I / IA: 3, 10, 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

The plates incubated with the test item showed normal background growth with and without metabolic activation in all strains in all experiments.

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with DF-pyrazole acid (CA4312) at any dose level, neither in the presence nor absence of metabolic activation (S9 mix).

Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

**DF-pyrazole acid (CA4312) did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used and, therefore, is considered to be non-mutagenic in this *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	DF-pyrazole acid (CA4312)
<b>Description:</b>	Technical; white solid (small needles)
<b>Lot/Batch number:</b>	AMS 1234/1
<b>Purity:</b>	99.6 % a.i
<b>CAS#:</b>	176969-34-9
<b>Stability of test compound:</b>	Reanalysis date December 2008

<b>Control Materials:</b>	
<b>Negative:</b>	Concurrent untreated and solvent controls were performed
<b>Solvent control (final concentration):</b>	10 µL/plate
<b>Positive control:</b>	Non-activation: Sodium azide 10.0 µg/plate TA100, TA1535 4-nitro-o-phenylene-diamine, 4-NOPD 50.0 µg/plate in TA 1537; 10.0 µg/plate in TA98 Methyl methane sulfonate, MMS 3.0 µL/plate WP2 (pKM101) WP2 <i>uvrA</i> (pKM101)
	Activation: 2-Aminoanthracene, 2-AA 2.5 µg/plate TA 1535, TA 1537, TA100, TA98 10 µg/plate WP2 (pKM101), WP2 <i>uvrA</i> (pKM101)

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM MgCl<sub>2</sub>

33 mM KCl

5 mM Glucose-6-phosphate

5 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment the S9 mix was stored in an ice bath.

**Test organisms:***S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

*E. coli* strains

X	WP2 (pKM101)	X	WP2 <i>uvrA</i> (pKM101)						
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Properly maintained?

☒

Yes

☐

No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?☒

Yes

☐

No

**Test compound concentrations used:** In the pre-experiment the concentration range of the test item was 3 - 5000 µg/plate. The pre-experiment is reported as experiment I. Since no toxic effects were observed, 5000 µg/plate were chosen as maximal concentration.

The concentration range included two logarithmic decades. The following concentrations were tested in experiment II:

33; 100; 333; 1000; 2500; and 5000 µg/plate

For each strain and dose level including the controls, three plates were used.

**Study Design and Methods:**

**In-life dates:** Start: 7 March 2007      End: 20 March 2007

**TEST PERFORMANCE****Type of Bacterial assay:**

- X standard plate test (both experiments –S9, initial experiment +S9)
- X pre-incubation (60 minutes) (second experiment +S9)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Protocol:**

Bacterial cultures were prepared from frozen stocks by incubating in shaking water bath for 4 hours at 37°C.

The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL                      Test solution at each dose level, solvent (negative control) or reference mutagen (positive control);
- 500 µL                      S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL                      Bacteria suspension (cf. test system, pre-culture of the strains)

- 2 mL Overlay agar containing 10.5 mg/L-histidine or 2.5 mg tryptophan as appropriate.

In the pre-incubation assay 100 µL test solution, 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspension were mixed in a test tube and shaken at 37°C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45°C) was added to each tube. The mixture was poured on selective agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37°C in the dark.

For each strain and dose level, including the controls three plates were used.

Following incubation all plates were counted using the Petri Viewer Mk2 with the software program Ames Study Manager. The counter was connected to an IBM AT compatible PC with printer to print out the individual values and the means from the plates for each concentration together with standard deviations and enhancement factors as compared to the spontaneous reversion rates.

**Statistical analysis:** None – see Evaluation Criteria below.

**Evaluation criteria:** A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, WP2 uvrA (pKM 101), and WP2 (pKM 101)) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed.

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

## RESULTS

**Mutagenicity assay:** The plates incubated with the test item showed normal background growth with and without metabolic activation in all strains in all experiments.

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with DF-pyrazole acid (CA4312) at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). In experiment I strain WP2 uvrA (pKM 101) showed a minor increase in revertant colony numbers. The number of colonies exceeded the threshold of twice the number of the corresponding solvent control at 10, 100, 1000 - 5000 pg/plate. However, no dose dependency was observed at these concentrations, therefore a confirmatory experiment was performed under identically conditions. No increase in revertant colony numbers was observed in this experiment.

In experiment I without metabolic activation, the data in the negative control of strain WP2 uvrA (pKM 101) were slightly above the laboratory's historical control range. In strain WP2 (pKM 101) the data in the negative control (exp. I with and without S9 Mix, exp. II without S9 mix) and in the solvent control (exp. I and II with S9 mix) were slightly above the historical control range. Since this deviation was small, the effect was considered to be based upon biologically irrelevant fluctuations in the number of colonies.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

**Table 6.8.1-2: Results Obtained in the Absence and Presence of Metabolic Activation:**

**Experiment 1**

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)						
			TA 1535	TA 1537	TA 98	TA 100	WP2 uvrA pkm 101	WP2pKm 101	WP2 uvrA pkm 101*
Without Activation	DMSO		16±7	16±3	34±3	130±9	146±15	106±8	152±12
	Untreated		20±4	9±3	37±1	122±12	271±13	118±8	236±28
	DF - Pyrazole	3 µg	14±6	10±3	27±1	129±9	140±12	133±17	154±34
	Acid	10µg	19±5	13±3	31±4	130±22	298±34	131±5	150±4
	(CA4312)	33 µg	21±7	10±6	30±7	141±14	275±16	140±22	147±21
		100 µg	13±4	10±2	30±6	124±5	290±45	130±10	142±20
		333 µg	14±1	9±4	23±4	129±11	243±19	120±11	170±23
		1000 µg	17±5	15±1	26±4	124±18	293±29	108±3	173±24
		2500 µg	13±8	17±9	32±4	144±7	326±13	131±17	154±9
		5000 µg	20±9	14±6	25±7	141±5	298±18	124±17	142±26
	NaN3	10µg	2097±84		2459±89				
	4-NOPD	10µg			480±22				
	4-NOPD	50 µg	115±12						
	MMS	3.0µL					5238±225	3698±341	4581±139
With Activation	DMSO		19±7	14±7	41±11	154±10	238±27	135±2	
	Untreated		17±8	13±7	36±11	143±15	228±12	156±9	
	DF - Pyrazole	3 µg	16±5	14±1	36±1	146±12	236±25	110±7	
	Acid	10µg	12±3	18±9	34±4	131±9	214±31	100±5	
	(CA4312)	33 µg	19±5	18±3	40±13	140±11	231±30	113±24	
		100 µg	18±5	21±1	36±6	148±17	227±8	112±5	
		333 µg	17±3	11±3	36±9	132±5	204±24	113±7	
		1000 µg	16±5	9±1	36±6	133±14	222±30	112±6	
		2500 µg	20±4	19±7	39±11	149±12	216±16	114±10	
		5000 µg	17±7	17±8	29±1	180±40	218±5	106±11	
	2-AA	2.5 µg	257±16	152±19	1214±168	1511±49			
	2-AA	10.0 µg					2354±96	406±45	

**Key to Positive Controls**

NaN3	Sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sultanate

\*Repeated WP2 uvrA pkm 101 experiment.

**Table 6.8.1-3: Results Obtained in the Absence and Presence of Metabolic Activation:**

**Experiment 2**

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 uvrA pkm 101	WP2pKm 101

Without Activation	DMSO		18 ± 3	8 ± 6	33 ± 10	114 ± 12	129 ± 2	77 ± 5
	Untreated		14 ± 5	16 ± 7	39 ± 1	133 ± 23	226 ± 28	114 ± 20
	DF-Pyrazole	33 µg	16 ± 1	12 ± 3	38 ± 8	122 ± 12	159 ± 17	100 ± 14
	Acid	100 µg	17 ± 3	10 ± 4	35 ± 4	122 ± 9	108 ± 7	80 ± 4
	(CA4312)	333 µg	19 ± 3	11 ± 4	30 ± 11	128 ± 9	102 ± 7	84 ± 17
		1000 µg	15 ± 1	11 ± 2	32 ± 3	111 ± 11	167 ± 25	93 ± 8
		2500 µg	21 ± 3	10 ± 4	38 ± 6	122 ± 13	138 ± 26	86 ± 8
		5000 µg	15 ± 1	9 ± 1	35 ± 2	126 ± 7	123 ± 5	79 ± 12
	NaN3	10 µg	2064 ± 166			2377 ± 49		
	4-NOPD	10 µg			677 ± 61			
With Activation	4-NOPD	50 µg		171 ± 24				
	MMS	3.0 µL				593 ± 38	1813 ± 121	
	DMSO		17 ± 4	14 ± 7	39 ± 2	140 ± 6	133 ± 15	121 ± 11
	Untreated		26 ± 6	16 ± 1	50 ± 6	157 ± 7	152 ± 25	103 ± 4
	DF - Pyrazole	33 µg	19 ± 4	14 ± 6	43 ± 6	129 ± 4	156 ± 29	117 ± 12
	Acid	100 µg	13 ± 6	16 ± 4	35 ± 13	118 ± 13	140 ± 14	132 ± 28
	(CA4312)	333 µg	19 ± 10	13 ± 4	43 ± 7	141 ± 5	129 ± 13	137 ± 4
		1000 µg	17 ± 6	12 ± 5	41 ± 10	127 ± 23	152 ± 28	116 ± 22
		2500 µg	16 ± 1	20 ± 6	41 ± 4	132 ± 18	143 ± 33	112 ± 13
		5000 µg	16 ± 7	21 ± 7	44 ± 3	139 ± 13	114 ± 12	82 ± 20
	2-AA	2.5 µg	162 ± 13	106 ± 19	657 ± 60	1130 ± 50		
	2-AA	10.0 µg					1740 ± 60	447 ± 24

## Key to Positive Controls

NaN3	Sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sultanate

## CONCLUSION:

In a GLP and OECD compliant Ames test, the potential of CSAA798670 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strains WP2 uvrA (pKM 101), and WP2 (pKM 101), was investigated.

The assay was performed in two independent experiments both with and without liver microsomal activation. Due to a questionable minor increase in strain WP2 uvrA (pKm 101) without metabolic activation in experiment I, this part was repeated (reported as Exp. IA). Each concentration, including the controls, was tested in triplicate. The test item was tested at the following concentrations:

Pre-Experiment/Experiment I / IA: 3, 10, 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

No toxic effects, evident as a reduction in the number of revertants, occurred in the test groups with and without metabolic activation. No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with CSAA798670 at any concentration, neither in the presence nor absence of metabolic activation. Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

As a result, HSE agrees with the EU evaluation, that **CSAA798670 is not mutagenic in bacteria when tested up to the limit concentration.**

(██████████, 2007)

In vitro chromosome aberration test

<b>Report:</b>	K-CA 5.8.1/03 ██████████ (2009). CSAA798670: Chromosome Aberration Test in Human Lymphocytes <i>In Vitro</i> . Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1266902, issue date: 26 November 2009. Unpublished. Syngenta File No. NOA449410_10001.
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**Guidelines:** OECD 473 (1997); OPPTS 870.5375 (1998); EC 440/2008 B10 (2008); Kampoan No. 287; Eisei No. 127; Heisei 09/10/31; JMAFF No. 12

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

**EXECUTIVE SUMMARY**

This *in vitro* assay was performed to assess the potential of CSAA798670 to induce structural chromosomal aberrations in the absence and presence of an exogenous metabolic activation system (liver S9 mix from phenobarbital/β-naphthoflavone treated male rats).

In each experimental group two parallel cultures were analysed. Per culture 100 metaphase plates were scored for structural chromosomal aberrations. The highest applied concentration in this study (1810.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the current OECD Guideline 473.

Dose selection of the cytogenetic experiments was performed considering the toxicity data and in accordance with OECD Guideline 473. In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In both independent experiments, no biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ( $p < 0.05$ ) in cells with structural chromosome aberrations.

**In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce structural chromosomal aberrations in human lymphocytes *in vitro*. Therefore, CSAA798670 is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation when tested up to the highest required concentration.**



## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	CSAA798670
<b>Description:</b>	Light brown, solid (powder)
<b>Lot/Batch number:</b>	LT-DFPA09001
<b>Purity:</b>	97.4 %
<b>CAS#:</b>	176969-34-9
<b>Stability of test compound:</b>	Stability in DMSO for several hours at ambient temperature was verified by sponsor via NMR

<b>Control Materials:</b>	
<b>Negative:</b>	-
<b>Solvent control (final concentration):</b>	DMSO (0.5 %)
<b>Positive control:</b>	Absence of S9 mix: Ethylmethane sulfonate, 825 µg/mL (Experiment I), 660 µg/mL (Experiment II)
	Presence of S9 mix: Cyclophosphamide 7.5 µg/mL

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM	MgCl <sub>2</sub>
33 mM	KCl
5 mM	glucose-6-phosphate
4 mM	NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

### Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

**Test compound concentrations used:**

Absence of S9 mix	Experiment 1 Experiment 2	591.0, 1034.3, 1810.0 µg/mL 591.0, 1034.3, 1810.0 µg/mL
Presence of S9 mix	Experiment 1 Experiment 2	591.0, 1034.3, 1810.0 µg/mL 591.0, 1034.3, 1810.0 µg/mL

**Study Design and Methods:**

**In-life dates:** Start: 23 July 2009 End: 24 August 2009

**TEST PERFORMANCE**

**Preliminary Cytotoxicity Assay:** Not performed.

**Cytogenetic Assay:****Cell exposure time**

Cell exposure time:		Test Material	Solvent Control	Positive Control
- S9 mix	Experiment 1	4h	4h	4h
+ S9 mix		4h	4h	4h
-S9 mix	Experiment 2	22h	22h	22h
+ S9 mix		4h	4h	4h

**Spindle inhibition**

Spindle inhibition:	
Inhibitor used/ concentration:	Colcemid 0.2 µg/mL
Administration time:	3 hours (before cell harvest)

**Cell harvest time after termination of treatment**

Cell harvest time after termination of treatment:	Test Material	Solvent Control	Positive Control
- S9 mix (4 hour treatment)	18h	18h	18h
+ S9 mix (4 hour treatment)	18h	18h	18h
- S9 mix (22 hour treatment)	0h	0h	0h

**Details of slide preparation:** About 70 h after seeding for each test group 2 blood cultures (10 mL each) were set up in parallel in 25 cm<sup>2</sup> cell culture flasks. The culture medium was replaced with serum-free medium, containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL medium were used. Concurrent solvent and positive controls were performed. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant with the dissolved test item was discarded and the cells were re-suspended in "saline G". The washing procedure was repeated once as described.

The "saline G" solution was composed as follows (per litre):

NaCl 8000 mg  
 KCl 400 mg  
 glucose•H<sub>2</sub>O 1100 mg  
 Na<sub>2</sub>HPO<sub>4</sub>•2H<sub>2</sub>O 192 mg  
 KH<sub>2</sub>PO<sub>4</sub> 150 mg  
 pH was adjusted to 7.2

After washing the cells were re-suspended in complete culture medium and cultured until preparation. The culture medium at continuous treatment was not changed until preparation of the cells.

Three hours before harvesting, colcemid was added to the cultures (final concentration 0.2 µg/mL). The cultures were harvested by centrifugation 22 h after beginning of treatment. The supernatant was discarded and the cells were re-suspended in approximately 5 mL hypotonic solution (0.0375 M KCl). The cell suspension was then allowed to stand at 37° C for 20 to 25 minutes. After removal of the hypotonic solution by centrifugation the cells were fixed with a mixture of methanol and glacial acetic acid (3 parts plus 1 part). At least two slides per experimental group were prepared by dropping the cell suspension onto a clean microscope slide. The cells for evaluation of cytogenetic damage were stained with Giemsa.

#### Metaphase analysis

No. of cells examined per dose: 200				
Scored for structural?	X	Yes		No
Scored for numerical?		Yes (polyploidy noted if observed)	X	No
Coded prior to analysis?	X	Yes		No

X indicates those that apply

**Evaluation criteria:** The percentages of aberrant metaphases were calculated for each treatment scored, both including and excluding cells with only gap-type aberrations.

A test item is classified as non-mutagenic if:

- the number of induced structural chromosome aberrations in all evaluated dose groups is in the range of laboratory historical control data.
- no significant increase of the number of structural chromosome aberrations is observed.

A test item is classified as mutagenic if:

- the number of induced structural chromosome aberrations is not in the range of laboratory historical control data.

and

- either a concentration-related or a significant increase of the number of structural chromosome aberrations is observed.

Statistical significance was confirmed by means of the Fisher's exact test. However, both biological and statistical significance should be considered together. If the above mentioned criteria for the test item are not clearly met, the classification with regard to the historical data and the biological relevance is discussed and/or a confirmatory experiment is performed.

**Statistical analysis:** Data were evaluated for statistical significance using the Fisher Exact Probability Test (one-sided).

## RESULTS

**Preliminary cytotoxicity assay:** Not performed.

**Cytogenetic assay:** In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying structural chromosome aberrations was observed. The aberration rates of the cells after treatment with the test item (0.5 – 2.5 % aberrant cells, excluding gaps) were close to the solvent control values (1.0 – 3.0 % aberrant cells, excluding gaps) and were within the range of the laboratory's historical solvent control data.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures.

In both experiments, either EMS (660 or 825 µg/mL) or CPA (7.5 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

**Table 6.8.1-4: Summary of results of the chromosomal aberration study with CSAA798670**

Exp.	Preparation interval	Test item concentration in µg/mL	Mitotic indices in % of control	incl. gaps*	Aberrant cells in % excl. gaps	carrying exchanges
<b>Exposure period 4 hrs without S9 mix</b>						
I	22 hrs	Solvent control <sup>1</sup>	100	1	1	0
		Positive control <sup>2</sup>	71.3	16	15.0 <sup>S</sup>	4.5
		591	105.8	1	1	0
		1034.3	91.6	2.5	2.5	0
		1810	82.2	2	2	0
<b>Exposure period 22 hrs without S9 mix</b>						
II	22 hrs	Solvent control <sup>1</sup>	100	3	3	0
		Positive control <sup>3</sup>	50.2	22	22.0 <sup>S</sup>	5.5
		591	112	1.5	1.5	0
		1034.3	92	1.5	1	0
		1810	80.9	2	1.5	0
<b>Exposure period 4 hrs with S9 mix</b>						
I	22 hrs	Solvent control <sup>1</sup>	100	2	2	0
		Positive control <sup>4</sup>	47.2	11.5	11.0 <sup>S</sup>	1.5
		591	85.3	1.5	1	0
		1034.3	93.1	2	1.5	0
		1810	93.4	2.5	2	0
II	22 hrs	Solvent control <sup>1</sup>	100	1.5	1.5	0
		Positive control <sup>4</sup>	58.1	20.5	20.0 <sup>S</sup>	2.5
		591	104.2	1	0.5	0
		1034.3	108.5	1	1	0
		1810	101.9	0.5	0.5	0

\* Including cells carrying exchanges

S Aberration frequency statistically significant higher than corresponding control values

1 DMSO 0.5 % (v/v)

2 EMS 825.0 µg/mL

3 EMS 660.0 µg/mL

4 CPA 7.5 µg/mL

## CONCLUSION:

In a GLP and OECD compliant in vitro chromosome aberration test, the potential of CSAA798670 to induce structural chromosomal aberrations in human lymphocytes in the absence and presence of an exogenous metabolic activation system was assessed.

In each experimental group two parallel cultures were analysed. Per culture 100 metaphase plates were scored for structural chromosomal aberrations. The highest applied concentration was 1810.0 µg/mL (approx. 10 mM), the limit concentration for the test. Exposure was for 4 hrs with and without S9 in Experiment I and for 4 hours with S9 and for 22 hrs without S9 in Experiment II.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In both independent experiments, no biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. No evidence of an increase in polyploid metaphases was noticed after treatment with the test item as compared to the control cultures. Appropriate mutagens were used as positive controls. They induced statistically significant increases ( $p < 0.05$ ) in cells with structural chromosome aberrations.

As a result, HSE agrees with the EU evaluation, that **CSAA798670 is considered to be non-clastogenic in this chromosome aberration test in the absence and presence of metabolic activation when tested up to the highest required concentration.**

(██████████, 2009)

#### In vitro mouse lymphoma test

<b>Report:</b>	K-CA 5.8.1/04 ██████████ (2009). CSAA798670: Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells. Harlan Cytotest Cell Research GmbH, (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1266901, 30 November 2009. Unpublished. Syngenta File No. NOA449410_10000.
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**Guidelines:** OECD 476 (1997); OPPTS 870.5300 (1998); 2008/440/EC B.17 (2008) Kampoan No. 287; Eisei No. 127; Heisei 09/10/31; JMAFF No. 12

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

#### **EXECUTIVE SUMMARY**

In a mammalian cell gene mutation assay, the potential of CSAA798670 (purity 97.4%) to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y was investigated. The assay was performed in two independent experiments, using two parallel cultures each. The main experiments were performed with and without liver microsomal activation and a treatment period of 4 hours. The cells cultured *in vitro* were exposed at the following concentrations: 113.1, 226.3, 425.5, 905.0 and 1810.0 µg/mL

The highest applied concentration (1810 µg/mL) was chosen with regard to the molecular weight of the test item corresponding to a molar concentration of about 10 mM.

No reproducible cytotoxic effects occurred in both main experiments up to the maximum concentration in the absence and presence of metabolic activation.

No substantial and reproducible dose dependent increase in mutant colony numbers was observed up to the maximum concentration with and without metabolic activation.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

**In conclusion it can be stated that during the mutagenicity test described and under the experimental conditions reported CSAA798670 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation. Therefore, CSAA798670 is considered to be non mutagenic in this mouse lymphoma assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	CSAA798670
<b>Description:</b>	Light brown powder
<b>Lot/Batch number:</b>	LT-DFPA09001
<b>Purity:</b>	97.4% a.i (by HPLC)
<b>CAS#:</b>	176969-34-9
<b>Stability of test compound:</b>	Reanalysis end March 2011 (Storage condition of test material <30°C)

<b>Control Materials:</b>	
<b>Solvent control (final concentration):</b>	DMSO
<b>Positive control:</b>	Without metabolic activation: Methyl Methane Sulfonate (MMS), 19.5 µg/mL = 0.18 mM
	With metabolic activation: Cyclophosphamide (CPA); 3.05 µg/mL = 10.7 µM and 4.5 µg/mL = 16.1 µM

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared from 8-12 weeks old male Wistar HsdCpb rats, induced by applications of 80 mg/kg bw phenobarbital i.p. and β-naphthoflavone p.o. each on three consecutive days. The livers were prepared 24 hours after the last treatment. The S9 fractions were produced by dilution of the liver homogenate with a KCl solution (1:4 v/v) followed by centrifugation at 9000 g. Aliquots of the supernatant were frozen and stored. The protein concentration of the S9 preparation was 35.6 mg/mL (pre-experiment and experiment I) and 32.3 mg/mL in experiment II.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM MgCl<sub>2</sub>

33 mM KCl

5 mM Glucose-6-phosphate

4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment, the S9 mix was stored in an ice bath. The concentration in the final test medium was 5% (v/v).

### Test cells: mammalian cells in culture

X	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media:</b> RPMI 1640			
Properly maintained?		X	Yes
Periodically checked for Mycoplasma contamination?		X	Yes
Periodically checked for karyotype stability?		X	Yes
Periodically "cleansed" against high spontaneous background?		X	Yes

X indicates those that apply

### Experimental Design

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na+/K+ ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	Trifluorothymidine (TFT)				

X indicates those that apply

### Test compound concentrations used:

#### Experiment I:

without S9 mix: 113.1, 226.3, 425.5, 905.0 and 1810.0 µg/mL  
 with S9 mix: 113.1, 226.3, 425.5, 905.0 and 1810.0 µg/mL

#### Experiment II:

without S9 mix: 113.1, 226.3, 425.5, 905.0 and 1810.0 µg/mL  
 with S9 mix: 113.1, 226.3, 425.5, 905.0 and 1810.0 µg/mL

### Study Design and Methods:

**Experimental dates:** Start: 22 July End: 31 August 2009

**Pre-test on toxicity:**  $1 \times 10^7$  cells were exposed to each of 8 concentrations (14.1 to 1810 µg/mL) of CSAA798670 for 4 hours with and without metabolic activation. Following treatment the cells were washed twice by centrifugation and re-suspended in "saline G". Subsequently the cells were resuspended in 30 mL complete culture medium for a 2-day growth period. The cell density was determined immediately after treatment and at each day of the growth period and adjusted to  $3 \times 10^5$  cells/mL, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated at the end of the growth period according to the method of *Clive and Spector (1975)*<sup>7</sup>. The doses of the main experiments were selected according to the results obtained in the pre-experiment.

### Mutation experiment:

**Cell treatment:** In the mutation experiment  $1 \times 10^7$  cells/flask (80 cm<sup>2</sup> flasks) suspended in 10 mL RPMI medium with 3% horse serum were exposed to various concentrations of the test item either in the presence or absence of metabolic activation. After 4 h the test item was removed by centrifugation (425 × g, 10 min) and the cells were washed twice with "saline G". Subsequently the cells were resuspended in 30 mL complete culture medium and incubated for an expression and growth period of 48 h.

The cell density was determined each day and adjusted to  $3 \times 10^5$  cells/mL, if necessary. The relative suspension growth (RSG) of the treated cell cultures was calculated by the day 1 fold-increase in cell number multiplied by the day 2 fold-increase in cell number according to the method of *Clive and Spector*

<sup>7</sup> Clive D, Spector JFS Laboratory procedure for assessing specific locus mutation at the TK locus in cultured L5178Y mouse lymphoma cells Mutat. Res. 31, 17-29, 1975

(1975). One sample of the cells was taken at the end of treatment, diluted and seeded into microtiter plates, to determine the viability of the cells after treatment (cloning efficiency 1).

After the expression period the cultures were selected. Cells from each experimental group were seeded into 2 microtiter plates so that each well contained approximately  $4 \times 10^3$  cells in selective medium with TFT. The viability (cloning efficiency 2) was determined by seeding about 2 cells per well into microtiter plates (same medium without TFT). The plates were incubated at  $37 \pm 1.5^\circ\text{C}$  in 4.5%  $\text{CO}_2$ /95.5% water saturated air for 10-15 days. Then the plates were evaluated.

Colonies were counted manually. In accordance with their size the colonies were classified into two groups. The colony size distribution was determined in the controls and at all concentrations of the test item. Criteria to determine colony size were the absolute size of the colony (more than 1/3 of a well for large colonies) and the optical density of the colonies (the optical density of the small colonies is generally higher than the large colonies).

**Evaluation criteria:** A test item is classified as mutagenic if the induced mutation frequency reproducibly exceeds a threshold of 126 colonies per  $10^6$  cells above the corresponding solvent control or negative control, respectively.

A relevant increase of the mutation frequency should be dose-dependent.

A mutagenic response is considered to be reproducible if it occurs in both parallel cultures.

However, in the evaluation of the test results the historical variability of the mutation rates in negative and vehicle controls and the mutation rates of all negative and vehicle controls of this study were taken into consideration.

Results of test groups are generally rejected if the relative total growth and the cloning efficiency 1 is less than 10% of the vehicle control unless the exception criteria specified by the IWGT recommendations are fulfilled.

Whenever a test item is considered mutagenic according to the above mentioned criteria, the ratio of small versus large colonies is used to differentiate point mutations from clastogenic effects. If the increase of the mutation frequency is accompanied by a reproducible and dose dependent shift in the ratio of small versus large colonies clastogenic effects are indicated.

**Statistical methods:** A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT®11 statistics software. The number of mutant colonies obtained for the groups treated with the test item was compared to the solvent control groups. A trend was judged as significant whenever the p-value (probability value) was below 0.05. However, both, biological relevance and statistical significance were considered together.

## RESULTS

**Preliminary toxicity assay:** Toxic effects leading to RSG values below 50% were not observed up to the maximum concentration in the absence and presence of metabolic activation. The test medium was checked for precipitation or phase separation at the end of the treatment period (4 hours) before the test item was removed. No precipitation occurred up to the highest concentration with and without metabolic activation.

Both pH value and osmolarity were determined in the pre-experiment at the maximum concentration of the test item and in the solvent control without metabolic activation. No relevant increase in the osmolarity or pH value was observed (solvent control: 311 mOsm, pH 7.30 versus 317 mOsm and pH 7.35 at 1810  $\mu\text{g/mL}$ ).

Since no relevant cytotoxic effects were noted in the pre-experiment the maximum concentration of 1810  $\mu\text{g/mL}$  equal to approximately 10 mM was also used in both main experiments. The lower doses of both main experiments were spaced by a factor of two.



**Mutation assay:** The test medium was checked for precipitation visible to the naked eye at the end of the 4 hours treatment just before the test item was removed. No precipitation meeting the criteria mentioned above was noted in the main experiments at analysed concentrations.

No relevant toxic effects indicated by a relative cloning efficiency 1 (survival) or a relative total growth (RTG) of less than 50% in both cultures occurred in experiment I and II up to the maximum concentration with and without metabolic activation. An isolated reduction of the relative cloning efficiency 1 to 49.3% was noted in the second culture of the first experiment at 1810 µg/mL without metabolic activation. However, the corresponding RTG level was 80.6% and no cytotoxic effect was observed in the parallel culture under identical conditions. A similar effect occurred in the first culture of the second experiment at 1810 µg/mL. The RTG was reduced to 37.4% but the corresponding cloning efficiency 1 was 150.2%. Again, no reduction was noted in both parameters of cytotoxicity in the parallel culture under identical experimental conditions.

No substantial and reproducible dose dependent increase of the mutation frequency was observed in experiment I and II up to the maximum concentration with and without metabolic activation. The threshold of 126 above the corresponding solvent control was not reached or exceeded at any test point with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT® statistics software. A single significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in culture I of the second experiment without metabolic activation. Since the mutation frequency did not exceed the threshold in any of the concentrations tested, as indicated above, the statistical result was considered as biologically irrelevant.

In this study the range of the solvent controls was from 114 up to 198 mutant colonies per 10<sup>6</sup> cells; the range of the groups treated with the test item was from 98 up to 267 mutant colonies per 10<sup>6</sup> cells. The solvent controls of the first culture of experiment I without metabolic activation, the second culture of the first experiment with metabolic activation, and the first culture of the second experiment with metabolic activation exceeded the upper limit of the acceptance criteria somewhat (170 mutant colonies/10<sup>6</sup> cells). The data are acceptable however, since the mutation frequency of each parallel culture remained within the range of 50–170 mutant colonies/10<sup>6</sup> cells.

MMS (19.5 µg/mL) and CPA (3.0 µg/mL and 4.5 µg/mL in both main experiments) were used as positive controls and showed a distinct increase in induced total mutant colonies at acceptable levels of toxicity with at least one of the concentrations of the controls.

**Table 6.8.1-5: Summary of results of the L5178Y study in the absence and presence of metabolic activation**

	conc.	S9	relative cloning efficiency 1	relative total growth	mutant colonies 10 <sup>6</sup> cells	threshold	relative cloning efficiency 1	relative total growth	muta coloni 10 <sup>6</sup> cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
<b>Experiment I / 4 h treatment</b>			culture I				culture II			
Solv. control with		-	100.0	100.0	179	305	100.0	100.0	114	240
Pos. control with MMS	19.5	-	82.1	10.8	1318	305	72.8	34.0	265	240
Test item	56.6	-	108.6	culture was not continued <sup>#</sup>			100.0	culture was not continued <sup>#</sup>		
Test item	113.1	-	86.4	63.0	197	305	85.1	101.6	132	240
Test item	226.3	-	82.1	72.6	195	305	66.2	87.6	143	240
Test item	425.5	-	102.0	87.0	234	305	90.1	123.5	98	240
Test item	905.0	-	100.0	90.2	173	305	76.5	101.5	102	240
Test item	1810.0	-	121.9	75.8	253	305	49.3	80.6	108	240
Solv. control with		+	100.0	100.0	15	285	100.0	100.0	171	297
Pos. control with CPA	3.0	+	100.0	49.4	23	285	44.8	46.6	157	297

	conc.	S9	relative cloning efficiency 1	relative total growth	mutant colonies 10 <sup>6</sup> cells	threshold	relative cloning efficiency 1	relative total growth	muta coloni 10 <sup>6</sup> cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Pos. control with CPA	4.5	+	65.9	45.5	35	285	44.8	41.2	376	297
Test item	56.6	+	86.9	culture was not			66.3	culture was not		
Test item	113.1	+	101.6	97.4	161	285	82.0	104.8	154	297
Test item	226.3	+	157.4	111.2	161	285	77.5	91.9	138	297
Test item	425.5	+	80.6	101.6	182	285	78.9	88.9	201	297
Test item	905.0	+	98.4	94.4	211	285	72.1	105.7	146	297
Test item	1810.0	+	110.6	102.3	196	285	77.5	110.1	136	297
<b>Experiment II / 4 h treatment</b>			culture I				culture II			
Solv. control with		-	100.0	100.0	166	292	100.0	100.0	155	281
Pos. control with MMS	19.5	-	102.9	54.3	431	292	98.6	49.5	338	281
Test item	56.6	-	119.5	culture was not continued <sup>#</sup>			107.7	culture was not continued <sup>#</sup>		
Test item	113.1	-	112.5	50.8	167	292	83.0	88.2	233	281
Test item	226.3	-	155.6	63.9	138	292	90.4	115.2	172	281
Test item	425.5	-	125.1	58.1	186	292	104.5	89.6	197	281
Test item	905.0	-	129.2	58.1	250	292	100.0	93.7	187	281
Test item	1810.0	-	150.2	37.4	267	292	89.1	105.9	185	281
Solv. control with DMSO		+	100.0	100.0	198	324	100.0	100.0	148	274
Pos. control with CPA	3.0	+	36.4	53.9	238	324	77.3	49.1	199	274
Pos. control with CPA	4.5	+	55.5	24.7	505	324	68.0	43.6	394	274
Test item	56.6	+	90.4	culture was not continued <sup>#</sup>			101.6	culture was not continued <sup>#</sup>		
Test item	113.1	+	80.7	78.1	171	324	130.5	95.5	156	274
Test item	226.3	+	114.4	69.8	200	324	96.9	64.3	218	274
Test item	425.5	+	107.7	79.6	162	324	82.0	67.5	195	274
Test item	905.0	+	80.7	87.8	171	324	112.4	102.2	151	274
Test item	1810.0	+	78.4	80.9	217	324	148.5	80.1	147	274

# Culture was not continued since a minimum of only four analysable concentrations are required

## CONCLUSION:

In a GLP and OECD compliant mammalian cell gene mutation assay, the potential of CSAA798670 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y was investigated. The assay was performed in two independent experiments, using two parallel cultures each. The main experiments were performed with and without liver microsomal activation and a treatment period of 4 hours. The cells were exposed at the following concentrations: 113.1, 226.3, 425.5, 905.0 and 1810.0 µg/mL.

The highest applied concentration (1810 µg/mL, approx. 10 mM) was the limit concentration for this test. No reproducible cytotoxic effects occurred in both main experiments up to the maximum concentration in the absence and presence of metabolic activation. No substantial and reproducible dose dependent increase in mutant colony numbers was observed up to the maximum concentration with and without metabolic activation. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

As a result, HSE agrees with the EU evaluation, that **CSAA798670 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation when tested up to the highest required concentration.**

(██████, 2009)

## 28-day oral toxicity study in rats

<b>Report:</b>	K-CA 5.8.1/05 [REDACTED] (2010). CSAA798670: 28-Day Oral (Dietary) Toxicity Study in the Wistar Rat. [REDACTED]. Laboratory Report No. [REDACTED]. Issue date 11 January 2010. Syngenta File No. NOA449410_10003.
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**Guidelines:** OECD 407 (1995); U.S. EPA OPPTS 870.3050 (2000); 96/54/EC B.7 (1996); JMAFF 12-Nousan-No. 8147 (2000)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable. The analytical method for determination of pydiflumetofen in rodent diet has been assessed and validated (see DAR Vol. 3 B. 5).

## EXECUTIVE SUMMARY

In this oral dietary toxicity study, groups of five male and five female RccHan:Wistar (SPF) rats were fed CSAA798670 admixed to their diet at concentrations of 0 (control), 2000, 6000, and 12000 ppm for at least 28 days.

General cageside observations were made in all animals prior to treatment start and daily throughout the study. Detailed clinical observations comprising open field evaluation of clinical signs were performed once prior to treatment start and once weekly (during weeks 1 to 3) thereafter. Functional observation batteries (FOBs) including the quantitative assessment of grip strength in the fore- and hindlimbs were performed in all animals in randomized order in week 4. Locomotor activities were assessed after the FOB evaluation during week 4. Food consumption values were recorded once prior to treatment start and daily throughout the study. Body weights were recorded twice prior to treatment start, weekly thereafter and before necropsy. Ophthalmoscopic examinations were performed in all animals once prior to treatment start and in all animals in week 4. At the end of the treatment period, all surviving animals were necropsied and selected organs and tissues were processed and examined microscopically.

All animals survived. There were no treatment-related effects on clinical observations, ophthalmoscopic findings and FOB findings including grip strength and locomotor activity investigations, food consumption, food utilization, body weights, body weight gains, clinical pathology findings, macroscopic findings and/or organ weight changes. Hepatocellular hypertrophy was noted in livers of males and females at 6000 and 12000 ppm, and this was accompanied by a lower incidence of glycogen deposition at 12000 ppm. Considering the absence of any effect on liver weight or clinical biochemistry parameters related to liver function, minimal hypertrophy is an adaptive response that does not represent an adverse effect.

CSAA798670 provided to Wistar rats as a dietary admix at 0, 2000, 6000, and 12000 ppm, respectively, did not produce any specific evidence of toxicity. The average achieved dose levels at 2000, 6000, and 12000 ppm were approximately 167, 511, and 1007 mg/kg bw/day for males, and 175, 572, and 1043 mg/kg bw/day for females, respectively.

No effects on liver histopathology or any other parameters measured in this study were observed at 2000 ppm.

Based on the results of this study, the no observed adverse effect level (NOAEL) associated with dietary CSAA798670 exposure in rats is 12000 ppm, corresponding to 1007 mg/kg bw/day in males and 1043 mg/kg bw/day in females.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	CSAA798670 (Synonym: CA4312D)
<b>Description:</b>	Light brown powder
<b>Lot/Batch number:</b>	LT-DFPA09001
<b>Purity:</b>	97.4%
<b>CAS#:</b>	176969-34-9
<b>Stability of test compound:</b>	Expiry date: 31 Mar 2011

**Vehicle:** The test substance was administered via powdered standard [REDACTED] 3433 rodent maintenance diet, batch no.42/09 ([REDACTED]).

<b>Test Animals:</b>	
<b>Species:</b>	Rat
<b>Strain:</b>	RccHan: WIST (SPF)
<b>Age/weight at dosing:</b>	7 weeks/ Males: 195.5 - 227.5 g, Females: 143.7 - 175.9 g
<b>Source:</b>	[REDACTED]
<b>Housing:</b>	In groups of five in Makrolon type-4 cages with wire mesh tops and standard softwood bedding
<b>Acclimatisation period:</b>	7 days
<b>Diet:</b>	standard [REDACTED] 3433 rodent maintenance diet <i>ad libitum</i>
<b>Water:</b>	<i>ad libitum</i>
<b>Environmental conditions:</b>	Temperature: 22 ± 3 °C Humidity: 30 - 70% Air changes: 10 - 15 air changes per hour Photoperiod: 12-hour fluorescent light/12-hour dark cycle with music during the light period.

### Study Design and Methods:

**In-life dates:** Start: 26 August 2009, End: 30 September 2009

**Animal assignment:** The animals were assigned to each group following a computer-generated random algorithm. The group identification and animal nos. assigned are stated in the table below.

### Study design

Test group	Dietary concentration (ppm)	Dose to animal (mg/kg bw/day) M/F	# Male	# Female
Control	0	--	01-05	21-25
Low	2000	167/175	06-10	26-30
Mid	6000	511/572	11-15	31-35
High	12000	1007/1043	16-20	36-40

### Diet preparation and analysis:

Diet admixtures were prepared with the test item as supplied without correction for chemical purity. Fresh batches of the diet admixtures for this study were prepared weekly and aliquoted into seven portions for daily administration. Experimental diets were prepared by adding required weighed quantity of test item to approximately 200 g blank diet and transferred into a 1000 mL glass bottle which was tightly closed with a cap. Then, the bottle was subjected to a linear shaker for 3 minutes. The mixture was transferred to the

toxicology department where it was mixed with required amount of blank diet using a kitchen aid mixer (Kenwood Chief) for 5 minutes.

Control diet for the animals of group 1 was taken directly from stock and not treated any further.

Analysis of dose concentration and homogeneity was carried out on all dose groups. The samples were delivered to Dr. [REDACTED] ([REDACTED]) and stored there at  $-20 \pm 5$  °C until analysis, except that the samples prepared on 21 Sep 2009 were not stored frozen but directly analyzed after delivery. The samples were analyzed using a method determined at [REDACTED].

The identity of CSAA798670 was confirmed by the retention time of CSAA798670. In blank samples no peak appeared at the retention time of CSAA798670 and, therefore, it was confirmed that only control diet was supplied to group 1 (control) animals. The results indicate that all diet batches prepared were suitable in terms of both achieved concentration and homogeneity.

**Statistics:** The statistical analyses were performed with SAS version 9.1.3. All procedures were done with proc glm (for diagnostic homogeneity tests of the variance according to Bartlett) and with proc mixed (since by this approach the Dunnett's tests can be directly assessed). The following statistical approaches were used in this study: All analyses were two-tailed for significance levels of 5% and 1%.

- All means were two-tailed for significance levels of 5% and 1%.
- All means were presented with standard deviations.
- If the variances were clearly heterogeneous, appropriate transformations (e.g. log, square root, double arcsine) were used in an attempt to stabilize the variances. In the final report, any transformations that were utilized were indicated in the specific results tables and/or the statistical methods section.
- **For quantitative data:** Body weights, cumulative body weight gain, food utilization, quantitative FOB measurements (grip strength), motor activity data at each measurement interval and overall activity, and absolute organ weights were analyzed initially by a one-way analysis of variance (ANOVA).
- Organ weights were also analyzed by analysis of covariance (ANCOVA) on final body weight). This statistical analysis provided an adjusted organ weight value, which has been displayed in the results table in the final report along with flags for statistical significance. Adjusted organ weights were calculated.
- Summary values of organ to body weight ratios were presented but not analyzed statistically.
- For all of the parameters evaluated initially by ANOVA or ANCOVA, the Dunnett's test was used to compare the control and treated groups, based on the error mean square in the ANOVA or ANCOVA. The Dunnett's test was performed for all continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant, and statistical flags are presented in the tables of results in the final report.
- **For qualitative data ( e.g. possible values of 0, 1, 2 or present/absent):** Qualitative functional observational battery parameters or any other parameters not specifically mentioned above that yield qualitative data have been presented as summary data, but were not analyzed statistically.

Individual values were rounded before printing. All derived values that appear in the report tables represent the rounded results of calculations that are based on the exact (non-rounded) raw data values. Statistical analyses also were carried out on the exact raw data values.

**Observations:** Observations for viability / mortality were recorded twice daily. The animals were observed for clinical signs once daily during acclimatization as well as once daily during the treatment period. The animals were observed in their home cages, outside their home cages in a standard arena and in the hand. These observations were performed in random sequence once before commencement of administration and once weekly (weeks 1 to 3) thereafter. During week 4, relevant FOB parameters from a modified Irwin screen test including grip strength in the fore- and hindlimbs and locomotor activity measurement were evaluated in all animals in random order.

**Bodyweight:** The bodyweight of each animal was recorded twice weekly during acclimatization and weekly during treatment periods and before necropsy.

**Food consumption and test substance intake:** Food consumption for each animal was determined and listed daily in the report. During treatment week 3 the grid insert in the food hoppers was removed to give the animals an easier access to the diet admixtures. This measure was not applied during treatment week 4. Food utilization was calculated based on daily food consumption and body weight gain per cage. Food utilization is defined as the body weight gain over a certain time period divided by the food consumption during that period. Units are grams of body weight gain per 100 grams of food.

**Ophthalmoscopic examination:** Eyes of all animals were examined during the acclimatization period and during the final week of the test item administration. The ophthalmoscopic examinations of both eyes of the animals were performed by an experienced veterinary ophthalmologist after the application of a mydriatic solution (Ciba Vision AG, 3172 Niederwangen/Switzerland) using an indirect ophthalmoscope (Miroflex 2 Ophthalmoscope, Eisenhut Vet. AG, 4123 Allschwil / Switzerland).

**Haematology and clinical chemistry:** Blood samples were drawn from the retro-orbital plexus from all animals under light isoflurane anesthesia after 4 weeks administration of test diet. The animals were fasted in metabolism cages for approximately 18 hours before blood sampling but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms.

**Haematology:** The following parameters were examined:

haemoglobin	differential leukocyte count:
haematocrit	neutrophils
erythrocyte count	eosinophils
mean corpuscular volume	basophils
mean corpuscular haemoglobin	lymphocytes
mean corpuscular haemoglobin concentration	monocytes
reticulocyte count	large unstained cells
erythrocyte morphology	platelet count
haemoglobin distribution width	total leukocyte count
prothrombin time (=thromboplastin time)	activated partial thromboplastin time

**Clinical chemistry:** The following parameters were examined:

urea	alkaline phosphatase activity
creatinine	aspartate aminotransferase activity
glucose	alanine aminotransferase activity
albumin	calcium
total protein	phosphorus (as phosphate)
total cholesterol	sodium
triglycerides	potassium
total bilirubin	chloride
globulin	albumin/globulin ratio

**Urinalysis:** The following parameters were examined:

urine volume (18 hours)	glucose
colour	ketones
appearance	protein
specific gravity (relative density)	bilirubin

pH value  
microscopy of sediment  
nitrite

urobilinogen  
blood: erythrocytes and leukocytes

### Investigations *post mortem*:

**Macroscopic examination:** All animals were examined *post mortem*. This involved an external observation and an internal examination of all organs and structures.

**Organ weights:** From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

adrenal glands  
brain  
epididymides  
heart  
kidneys  
liver

ovaries  
spleen  
testes  
thymus  
uterus (with cervix)

Paired organs were weighed together.

**Tissue submission:** The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

gross lesions including masses  
adrenal gland  
brain (medulla/pons, cerebral and cerebellar cortex)  
Bone (sternum, femur including joint)  
bone marrow (sternum, femur)  
cecum  
coagulating glands  
colon  
duodenum  
epididymis (fixed in Bouin's solution)  
eyes (retina, optic nerve) (fixed in Davidson's solution)  
Harderian gland  
heart  
ileum with Peyer's patches  
jejunum with Peyer's patches  
kidneys  
larynx  
liver  
lungs (filled with formalin at necropsy)  
lymph node - mandibular  
lymph node - mesenteric  
nerve - sciatic

ovaries  
oviducts  
pancreas  
parathyroid gland  
pharynx  
pituitary gland  
prostate gland  
rectum  
salivary gland  
seminal vesicle  
spinal cord (cervical, midthoracic, lumbar)  
spleen  
skeletal muscle  
stomach  
testes (fixed in Bouin's solution)  
thymus  
thyroid gland  
trachea  
urinary bladder (filled with formalin at necropsy)  
uterus (with cervix)  
vagina  
testes (fixed in Bouin's solution)

**Microscopic examination:** All processed tissues were examined by light microscopy.

## RESULTS

**Diet analysis:** *Concentration analysis results:* The achieved concentration of CSAA798670 in diet batches was found to range from 89.0% to 109.8% of the nominal concentration. Two batches were only marginally outside the acceptable range of  $\pm 10\%$  of the nominal concentration (-10.2% and -11% for the 6000 and 12000 ppm diet batches prepared for the fourth week of dosing, respectively) and, therefore, did not adversely affect the outcome of the study.

*Homogeneity results:* CSAA798670 was found to be homogeneously distributed in the diet preparations as single sample results did not deviate more than 10% from the corresponding mean, with the exception of the 2000 ppm diet batch prepared for the fourth week of dosing in which one out of three samples deviated marginally from the corresponding mean by more than 10% (+14.1%). This minor deviation did not adversely affect the outcome of the study.

**Stability results:** Stable at room temperature ( $20 \pm 5$  °C) for 24 hours under artificial light conditions and stable for 15 days in the freezer ( $-20 \pm 5$  °C) as determined in a full method validation study conducted at [REDACTED].

**Mortality:** All animals survived until the end of the treatment period.

**Clinical observations:** General daily clinical signs: No treatment-related clinical signs were observed in any animals of any dose groups throughout the study period.

Detailed clinical observations: No treatment-related clinical observations were present during the weekly assessments during weeks 1 to 3. In a few animals, miosis was noted during week 2 and 3 in both eyes of one male (no. 19) at 12000 ppm, in two females (nos. 33 and 35) at 6000 ppm in week 3 and weeks 1 to 3, respectively, and in two females (nos. 36 and 37) at 12000 ppm during weeks 2 to 3 or weeks 1 to 3, respectively. This observation is considered to represent an incidental clinical sign, as it was of low incidence, there was no consistency in the onset and duration, it could not be confirmed in female nos. 35, 36 and 37 during the week 4 FOB examination, and there was not a clear dose response-relationship present. In addition, it was not accompanied by any other findings in the daily clinical observations, the detailed clinical observations, the week 4 FOB examination, or in ophthalmic or microscopic examinations. Therefore, this transient, incidental observation was not related to treatment with the test item.

FOB investigations: No treatment-related clinical observations were present during the FOB evaluations performed during week 4. During week 4, miosis was noted in both eyes of two males (nos. 17 and 19) at 12000 ppm, and in one female (no. 33) at 6000 ppm. These three isolated occurrences in the FOB were considered incidental to treatment, as the incidence of miosis did not show a dose response, was variable between weeks and was not accompanied by any other findings in the daily clinical observations, detailed clinical observations, the FOB (out of 43 different parameters evaluated) or the histopathology examination of the eyes. Grip strength in the fore- and hindlimbs and locomotor activity testing performed during week 4 revealed no treatment-related effects.

**Bodyweight and weight gain:** Body weights were not affected by treatment with CSAA798670. Cumulative body weight gain was higher than control values for females at 12000 ppm on days 8 and 28, whereas there were no differences from the respective control values in any male groups. Young animals can exhibit large variability in weight gain in a study of this duration, and considering the direction of change in body weight gain in 12000 ppm females; this is considered a normal variation and not an effect of treatment.

**Food consumption and compound intake:** Food consumption was not affected by treatment with CSAA798670.

The slight increase in food consumption for all groups including group 1 (controls) during treatment week 3 (days 15 - 21) represents an artifact produced by the temporary removal of the grid insert in the food hoppers. The grid insert was restored during treatment week 4 and the values for food consumption returned to normal.

Dose rates (based on nominal dietary levels of CSAA798670) were calculated in terms of mg CSAA798670/kg body weight. Overall mean values of doses received over treatment are presented in Table 6.8.1-6.



**Table 6.8.1-6: Mean Dose Received (mg/kg bw/day)**

Group	Time intervals	Dietary concentration	Males	Females
			Nominal test item intake	Nominal test item intake
Nos.	Week	(ppm)	(mg/kg bw/day)	(mg/kg bw/day)
1	Overall (1 - 4)	0	---	---
2		2000	167	175
3		6000	511	572
4		12000	1007	1043

**Food utilisation:** Food utilisation was not affected by treatment with CSAA798670.

**Ophthalmoscopic examination:** Ophthalmoscopic examinations revealed no treatment-related effects.

Corneal opacity, persistent hyaloid vessel in vitreous body and persistent pupil membrane in lens were noted in individual animals of the control or high dose groups. These observations represent typical background findings in rats of this strain and age.

**Haematology:** There were no differences in haematological parameters which were considered to be related to treatment.

Slight differences from control values in some hematology parameters were noted in the intermediate dose groups. There were not treatment-related effects, because the values were within the reference range, they represent only minor differences and they did not reveal any dose response-relationship.

**Blood clinical chemistry:** There were no differences in blood clinical biochemistry parameters which were considered to be related to treatment.

**Urinalysis:** There were no differences in urinalysis parameters which were considered to be related to treatment.

#### **Sacrifice and pathology:**

**Organ weights:** No test item-related effects on absolute or adjusted organ weights were evident in males and females.

**Macroscopic findings:** There were no relevant treatment-related macroscopic findings present in any of the treated female groups. Minor findings in males were restricted to an enlarged liver in two males at 2000 ppm and in one male at 6000 ppm. The liver in one male of the control group (0 ppm) was enlarged, whereas the livers in males at 12000 ppm were not enlarged. These findings are not considered to be treatment-related changes as there was no clear dose response-relationship present and the alterations were of minor incidence and/or severity and, therefore, these gross lesions do not distinguish treated rats from controls.

Furthermore, an isolated and reddish focus with a diameter of 1 mm was observed in the thymus of one female of the control group (0 ppm). An isolated and dark red focus with a diameter of 1 mm was observed in the thymus of one male at 6000 ppm, and several dark red foci with a diameter of 1 mm were present in the thymus of one male at 12000 ppm. In addition, several dark red foci with a size of 1x2 mm were recorded in the stomach of one male at 2000 ppm. The presence of a focus or foci in the thymus or stomach of these males is considered to be a background finding in rats of this strain and age as they occurred at similar incidence also in control animals and there was no dose-response relationship present.

**Microscopic findings:** In the liver, centrilobular hepatocellular hypertrophy was noted in 2 males and one female at 6000 ppm and in 2 males and 3 females at 12000 ppm. One female at 12000 ppm had diffuse hepatocellular hypertrophy accompanied by hepatocellular pigment. In addition, in high dose animals

(12000 ppm) the hypertrophy was associated with a decrease in the incidence of hepatocellular glycogen deposition, which is a common finding in control rats of this age.

**Table 6.8.1-7: Intergroup comparison of selected microscopic findings in the liver**

Parameter	Dietary Concentration of CSAA798670 (ppm)							
	Males				Females			
	0	2000	6000	12000	0	2000	6000	12000
<b>Centrilobular Hypertrophy</b>								
Minimal	0	0	2	2	0	0	1	3
<b>Diffuse Hypertrophy</b>								
Minimal	0	0	0	0	0	0	0	1
<b>Deposition of Glycogen</b>								
Minimal	1	2	3	0	3	5	3	0
Slight	0	0	0	0	2	0	0	0
<b>Pigmented Hepatocytes</b>								
Slight	0	0	0	0	0	0	0	1

A small number of other spontaneous lesions were observed, none of which was related to treatment. The following findings were considered to be incidental changes as they are known to occur spontaneously, did not show a clear difference between treated groups and controls, and are commonly diagnosed in rats of this strain and age:

- Tangible body macrophages (minimal grade) in the mesenteric lymph node were noted in one male and one female at 12000 ppm.
- Lymphocytolysis in the thymus occurred in one female at 0 and two females at 12000 ppm.
- Minimal tubular degeneration was noted in the testes in one male at 0 ppm (control) and two males at 120000 ppm.

## CONCLUSION:

In a GLP and OECD compliant 28-day study in Wistar rats, in which animals were given dietary concentrations of 0, 2000, 6000 and 12000 ppm CSAA798670 (0, 167/175, 511/572 and 1007/1043 mg/kg bw/d in M/F), there were no mortalities, no clinical signs of toxicity and no effects on FOB and MA parameters. There were also no effects on hematology, urinalysis or clinical biochemistry parameters, and no treatment-related effects on food consumption, body weights or organ weights were recorded. Hepatocellular hypertrophy was noted in livers of males and females at 6000 and 12000 ppm, and this was accompanied by a lower incidence of glycogen deposition at 12000 ppm. Considering the absence of any effect on liver weight or clinical biochemistry parameters related to liver function, the observed minimal hypertrophy was regarded an adaptive response.

As a result, HSE agrees with the EU evaluation, that CSAA798670 did not cause any adverse effects in this study and the **NOAEL is the top dose of 12000 ppm (approx. 1000 mg/kg bw/d), the limit dose for this test.**

(██████████, 2010)

90-day oral toxicity study in rats

<b>Report:</b>	K-CA 5.8.1/06 [REDACTED], [REDACTED], [REDACTED], [REDACTED], [REDACTED] (2009). Reg.No. 5069089 (metabolite of BAS 700 F): Repeated Dose 90-day Oral Toxicity Study in Wistar Rats - Administration in the Diet. [REDACTED] Report Number 50S0451/07119. Report issue date 8 October 2009. Syngenta File No. R958945_11273.
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**Guidelines:** According to OECD 408; EPA 870.3100; Council Regulation 440/2008/EC B.26.

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable. The analytical method for determination of pydiflumetofen in rodent diet has been assessed and validated (see DAR Vol. 3 B. 5).

## EXECUTIVE SUMMARY

Reg.No. 5069089 (Metabolite of BAS 700 F; Batch: L80-68, Purity: 99.2%) = M700F001 was administered to rats at nominal dose levels of 0, 100, 300 and 1000 mg/kg bw for at least 91 days. Dietary concentrations of the test-substance were adjusted weekly based on actual body weight and food consumption to meet the intended nominal dose levels. In this study no signs of systemic toxicity or any adverse changes in clinical pathology parameters or histopathological findings were noted.

**Based on the results of this study the no observed adverse effect level (NOAEL) was 1000 mg/kg bw.**

## MATERIAL AND METHODS

### Materials:

<b>Test Material:</b>	Reg.No. 5069089 (Metabolite of BAS 700 F) Synonym: M700F001
<b>Description:</b>	solid / pink
<b>Lot/Batch number:</b>	L80-68
<b>Purity:</b>	99.2%
<b>CAS#:</b>	176969-34-9
<b>Stability of test compound:</b>	The test substance was stable over the study period (Expiry date August 01, 2010).

**Vehicle and/or positive control: Rodent diet**

<b>Test Animals:</b>	
<b>Species:</b>	Rat
<b>Strain:</b>	WI (Han)
<b>Sex</b>	Male and female
<b>Age</b>	35 ± 1 day at delivery; approx. 42 ± 1 day at start of administration
<b>Weight</b>	only group means provided in the report
<b>Source:</b>	
<b>Housing:</b>	Group housing (5 animals/cage) housed in H-Temp (PSU) cages (TECNIPLAST Deutschland GmbH, Hohenpreißenberg, Germany), floor area about 2065 cm <sup>2</sup> with Lignocel FS 14 dust free bedding (SSNIFF, Soest).
<b>Environmental enrichment:</b>	Wooden gnawing blocks (Typ NGM E-022; Abedd® Lab. and Vet. Service GmbH, Vienna, Austria) Motor activity measurements were conducted in separate Polycarbonate cages
<b>Acclimatisation period:</b>	7 days
<b>Diet:</b>	maintenance diet for mouse/rats “GLP”, , <i>ad libitum</i>
<b>Water:</b>	Tap water in bottles, <i>ad libitum</i>
<b>Environmental conditions:</b>	Temperature: 20 - 24 °C Humidity: 30 - 70 % Air changes: 10/hour Photoperiod: 12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

**STUDY DESIGN**

**Dates of experimental work:** 13-Jan-2009 - 05-Aug-2009

(In life dates: 20-Jan-2009 (start of administration) to 23-Apr-2009 (necropsy))

**Animal assignment and treatment:**

M700F001 was administered in the diet to groups of 10 male and 10 female rats at nominal dose levels of 0, 100 (low dose), 300 (mid dose) and 1000 mg/kg (high dose) for at least 90 days. The animals were assigned to the treatment groups by means of computer generated randomization list based on body weights.

**Test substance preparation and analysis:**

The diets were prepared by mixing weighed amounts of test substance with a small amount of food. Subsequently, appropriate amounts of food were added to obtain the intended dietary concentrations and mixed for approx. 10 minutes in a laboratory mixer. Diet preparations were performed weekly. Dietary concentrations were adjusted to the body weight and food consumption data of the preceding week in order to meet as close as possible the nominal dose levels. Dietary concentrations for the first week were based on historical control food consumption and body weight data. The following equation was used to calculate the required dietary concentrations:

$$\frac{BW_x \times D}{FC_x} = \text{ppm}$$

with  $BW_x$  = mean body weight on day x [g], D = nominal dose,  $FC_x$  mean daily food consumption on day x [g] and ppm = dietary concentration of test substance in food for the week/period following day x.

Analyses performed prior to the start of the administration period revealed that the test-substance was stable in the diet for a period up to 12 days at room temperature.

Homogeneity and concentration analyses of the diet preparations were performed at the beginning and the end of the administration period for all concentrations. Three samples were taken from the top, middle and bottom of the storage container and analyzed. No test substance was determined in control diets.

**Statistics:**

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

### Statistics of clinical examinations

Parameter	Statistical test
body weight, body weight change	A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means
feces, rearing, grip strength forelimbs, grip strength hindlimbs, foot-splay test, motor activity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pairwise comparison of each dose group with the control group was performed using Wilcoxon-test (two-sided) for the equal medians

### Statistics of clinical pathology

Parameter	Statistical test
Clinical pathology parameters, urine volume, urine specific gravity	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using Wilcoxon-test (two-sided) for the equal medians
Urinalysis, except volume, color, turbidity and specific gravity	Pairwise comparison of each dose group with the control group using FISHER's exact test for the hypothesis of equal proportions

### Statistics of pathology

Parameter	Statistical test
Weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test for the hypothesis of equal medians

## METHODS

### Observations:

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Observation for clinical signs of toxicity was performed at least once daily.

Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. For this the animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable. The clinical examination included, but was not limited, to the following parameters/organs:

1. abnormal behavior during handling	10. abnormal movements
2. fur	11. impairment of gait
3. skin	12. lacrimation
4. body posture	13. palpebral closure
5. salivation	14. exophthalmus
6. respiration	15. feces (appearance/consistency)
7. activity/arousal level	16. urine
8. tremors	17. pupil size
9. convulsions	

### Body weight:

The body weight of the animals was determined before the start of the administration period (in order to randomize the animals), at the start of the treatment (day 0), and once weekly thereafter.

#### Food consumption, food efficiency and compound intake:

Food consumption was determined once weekly on a cage basis and calculated as mean food consumption in grams per animal and day.

Food efficiency was calculated based upon individual values for body weight and mean weekly food consumption per cage-group of animals:

$$\text{Food efficiency at day } x = \frac{BW_x - BW_y}{FC_{y \text{ to } x}} \times 100$$

with  $BW_x$  and  $BW_y$  body weight [g] at day x and day y (last weighing date before day x),  $FC_{y \text{ to } x}$  as the mean food consumption (in g) from day y to day x, calculated as mean daily food consumption on day x multiplied with the number of days from day y to day x.

The mean daily intake of test substance (group means) was calculated based upon individual values for body weight and food consumption.

$$\text{Substance intake for day } x = \frac{FC_x \times C}{BW_x}$$

with  $FC_x$  as the mean daily food consumption (in g/day) on day x, C as the dose in ppm and  $BW_x$  as body weight on day x of the study (in g).

#### Water consumption:

Water consumption was observed daily by visual inspection of the water bottles for overt volume changes. No water consumption values were recorded.

#### Ophthalmoscopy:

Prior to the start of the administration period the eyes of all animals, and on day 91 the eyes of the control and high dose animals were examined for any changes using an ophthalmoscope (after administration of a mydriatic).

#### Functional observation battery (FOB):

A functional observational battery was performed in all animals at the end of the administration period starting at about 10.00 a.m. The FOB started with passive observations without disturbing the animals (home cage observations), followed by removal from the home cage and open field observations in a standard arena. Thereafter, sensorimotor tests and reflex tests were conducted. The findings were ranked according to the degree of severity, if applicable (for details see Part III of the report).

During the home cage observation special attention was paid to posture, tremors, convulsions, abnormal movements, impairment of gait and - if applicable - other findings.

For open field observation the animals were transferred to a standard arena (50 x 50 cm with sides of 25 cm high) and observed for at least 2 minutes. The following parameters were assessed:

1. behavior when removed from cage	10. respiration
2. fur	11. tremors
3. skin	12. convulsions
4. salivation	13. abnormal movements
5. nose discharge	14. impairment of gait
6. lacrimation	15. activity/arousal level

7. eyes/pupil size	16. feces (number of fecal pellets/appearance/ consistency) within two minutes
8. posture	17. urine (appearance/quantity) within two minutes
9. palpebral closure	18. number of rearings within two minutes

For sensorimotor tests and reflexes the animals were removed from the open field. The following tests were performed:

1. approach response	8. behavior during "handling"
2. touch response	9. vocalization
3. vision ("visual placing response")	10. pain perception ("tail pinch")
4. pupillary reflex	11. grip strength of forelimbs
5. pinna reflex	12. grip strength of hind limbs
6. audition ("startle response")	13. landing foot-splay test
7. coordination of movements ("righting response")	14. other findings

**Motor activity measurement:**

Motor activity was measured at the same day the FOB was performed in a randomized order. The examinations were performed using the TSE Labmaster System supplied by TSE Systems GmbH, Bad Homburg, Germany. For this purpose, the animals were placed in new clean polycarbonate cages for the time of measurement. 18 beams were allocated per cage. During the measurement the animals were kept in clean Polycarbonate cages with absorbent material. Motor activity measurements were started at approx. 2 p.m. The number of beam interrupts was counted over twelve 5 minute intervals. Measurement started when the first beam was interrupted. Measurements ended exactly 60 minutes thereafter. The room was darkened after the transfer of the last animal in its cage. During the measurements the animals received no food and no water.

**Hematology and clinical chemistry:**

Blood was drawn in the morning from fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.

The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		White blood cells		Clotting Potential
✓	Erythrocyte count (RBC)	✓	Total leukocyte count (WBC)	✓	Prothrombine time
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)	✓	Thrombocyte count
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		Activated partial thromboplastin time (APPT)
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells		
Clinical chemistry:					
	Electrolytes		Metabolites and Proteins		Enzymes:
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Chloride	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
✓	Magnesium	✓	Cholesterol	✓	Alkaline phosphatase (ALP)
✓	Phosphorus (inorganic)	✓	Creatinine	✓	$\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT)
✓	Potassium	✓	Globulin (by calculation)		
✓	Sodium	✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

**Urinalysis:**

The following parameters were determined for all animals from urine collected overnight in metabolism cages (withdrawal of food and water):

Urinalysis					
	Quantitative parameters:		Semiquantitative parameters		
✓	Urine volume	✓	Bilirubin	✓	Protein
✓	Specific gravity	✓	Blood	✓	pH-value
		✓	Color and turbidity	✓	Urobilirubin
		✓	Glucose	✓	Sediment (microscopical exam.)
		✓	Ketones		

**Sacrifice and pathology:**

The animals were sacrificed by decapitation under Isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically:

Pathology:											
The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose).											
C	W	H		C	W	H		C	W	H	
✓	✓	#	adrenals	✓	✓	#	kidneys <sup>&amp;</sup>	✓		#	seminal vesicles
✓		#	aorta	✓			lachrymal glands <sup>%</sup>	✓		#	skin
✓		#	bone marrow <sup>§</sup>	✓		#	larynx	✓		#	spinal cord (3 levels) <sup>@</sup>
✓	✓	#	brain	✓	✓	#	liver	✓	✓	#	spleen
✓		#	caecum	✓		#	lung	✓			sternum w. marrow
✓		#	colon	✓		#	lymph nodes <sup>#</sup>	✓		#	stomach (fore- & glandular)
✓		#	duodenum	✓		#	mammary gland (♀)	✓	✓	#	testes
✓	✓	#	epididymides	✓			muscle, skeletal	✓	✓	#	thymus
✓		#	esophagus	✓		#	nerve, peripheral (sciatic n.)	✓	✓	#	thyroid/parathyroid
✓		#	eyes (with optic nerve)	✓		#	nose/nasal cavity <sup>‡</sup>	✓		#	trachea
✓			femur (with joint)	✓	✓	#	ovaries and oviduct <sup>**</sup>	✓		#	urinary bladder
			gall bladder	✓		#	pancreas	✓	✓	#	uterus
✓		✓	gross lesions	✓		#	pharynx	✓		#	vagina
			Harderian gland	✓		#	pituitary				
✓	✓	#	heart	✓		#	prostate		✓		body (anesthetized animals)
✓		#	ileum	✓		#	rectum				
✓		#	jejunum (w. Payer's patches)	✓		#	salivary glands <sup>*</sup>				

<sup>§</sup> from femur; <sup>#</sup> axillary and mesenteric; <sup>@</sup> cervical, thoracic, lumbar; <sup>\*</sup>mandibular and sublingual, <sup>\*\*</sup> oviduct not weighed; <sup>%</sup> extraorbital, <sup>‡</sup> histopathology at level III, <sup>&</sup> histopathological evaluation of all treatment groups for females only

<sup>§</sup> from femur; <sup>#</sup> axillary and mesenteric; <sup>@</sup> cervical, thoracic, lumbar; <sup>\*</sup>mandibular and sublingual; <sup>\*\*</sup> oviduct not weighed; <sup>%</sup> extraorbital; <sup>‡</sup> histopathology at level III; <sup>&</sup> histopathological evaluation of all treatment groups for females only

The organs or tissues were fixed in 4% formaldehyde.

The immunorelevant organs and tissues were evaluated according to the following parameters:

Thymus:	
•	Increased/decreased grade of cortico-medullary ratio (related only to area)
•	Increase of starry sky cells
•	Changes of cellular density in the cortex
•	Changes of cellular density in the medulla
Spleen:	
•	Changes of the cellularity of PALS, lymphoid follicles, marginal zone, red pulp
•	Altered cellular composition of follicles
•	Altered number of germinal centers
Lymph nodes (mesenteric and axillary lymph nodes):	
•	Changes in the cellularity of follicles, interfollicular area, paracortical area, medulla
•	Altered cellular composition of paracortex
•	Altered number of germinal centers
•	Hyperplasia of high endothelial venules



Peyer's patches (of the jejunum):
• Changes of the cellularity of follicles (including mantle zone and germinal centers)
• Changes of the cellularity of interfollicular area
Bone marrow:
• Changes of the cellularity
• Changes of the myeloid/erythropoid ratio

## RESULTS

**Diet analysis:** Relative standard deviations of the homogeneity samples in the range of 0.6 to 6.9% indicate the homogenous distribution of M700F001 in the diet preparations. The actual (mean) average test-substance concentrations were in the range of 96.8 to 105.3% of the calculated nominal concentrations.

### Observations

#### Clinical signs of toxicity

The only clinical observation was cornea opacity in one high dose male (1000 mg/kg) at the end of the study (see Ophthalmoscopy below).

#### Mortality

No mortality was observed in this study.

#### Ophthalmoscopy

No treatment-related ophthalmoscopical findings were noted. Cornea opacity was observed in one high dose male (1000 mg/kg) at the end of the study. Due to its isolated occurrence this observation was considered as not treatment related.

#### FOB and motor activity

Neither home cage nor open field observations revealed any indication of treatment-related effects. The same holds true for the sensimotor tests and reflexes.

All deviations from (rank) "zero values" were equally distributed between treated groups and controls or occurred in single animals and thus were considered to be incidental.

No statistically significant differences of overall motor activity between control and treated animals were observed. Considering individual observation intervals, the only statistically significant difference was observed for low dose males (100 mg/kg) at interval 10. Due to the isolated occurrence and the lack of a dose-response relationship this difference was considered to be incidental.

#### Body weight and body weight gain

Body weight development was not affected by treatment. At termination of treatment, no treatment related differences of absolute body weights or cumulative body weight gain were observed (see Table 6.8.1-8, Figure 6.8.1-1). The numerical decrease of body weight gain in mid dose male (300 mg/kg) displayed no dose-response relationship and was therefore considered to be incidental.

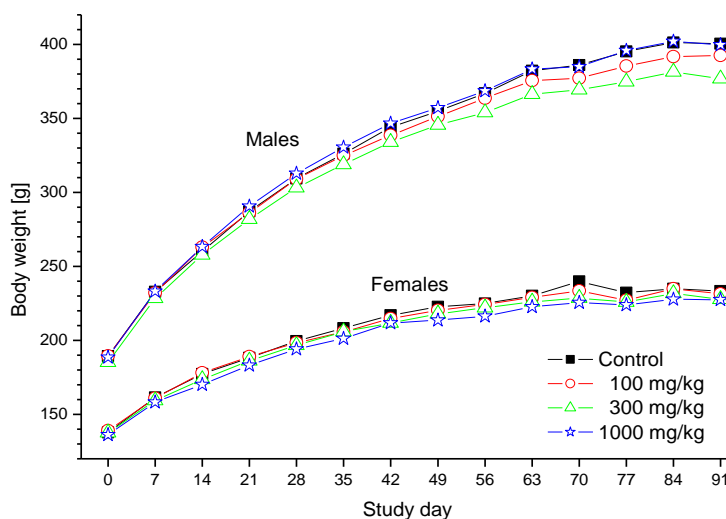
**Table 6.8.1-8: Mean body weight of rats administered M700F001 for at least 91 days**

Dose level [mg/kg]	Males				Females			
	0	100	300	1000	0	100	300	1000
Body weight [g]								
- Day 0	189.3	189.6	185.2	188.6	137.7	139.1	137.6	136.2
- Day 91	400.5	392.5	376.9	399.7	233.3	231.4	227.7	227.5
% (compared to control) #		-2.0	-5.9	-0.2		-0.8	-2.4	-2.5
Overall body weight gain [g]	211.2	202.9	191.7	211.0	95.6	92.3	90.1	91.4
% (compared to control) #		-3.9	-9.2	-0.1		-3.5	-5.8	-4.4

	Males				Females			
Dose level [mg/kg]	0	100	300	1000	0	100	300	1000

# Values may not calculate exactly due to rounding of mean values

**Figure 6.8.1-1: Body weight development of rats administered M700F001 for at least 91 days**



### Food consumption, food efficiency and compound intake

No treatment-related effects on food consumption were observed. There were no statistically significant differences between treated and control animals. Cumulative food consumption likewise did not indicate any effects of treatment (see Table 6.8.1-9)

**Table 6.8.1-9: Cumulative food consumption of rats administered M700F001 for at least 91 days**

	Males				Females			
Dose level [mg/kg]	0	100	300	1000	0	100	300	1000
Cumulative food consumption [g/animal]								
Day 0 to 91 <sup>#</sup>	1711	1707	1673	1865	1259	1221	1177	1174
% (compared to control) <sup>#</sup>		-0.2	-2.2	9.0		-3.1	-6.6	-6.8

# Values were calculated based on mean individual daily food consumption. Values may not calculate exactly due to rounding of mean values

Differences of food efficiency between control and treated groups were observed on several occasions throughout the study. However, due to their inconsistency they were considered incidental.

The actual mean daily test substance intake determined on the basis of actual food intakes was calculated to have been 94.6, 285.7 and 953.6 mg/kg bw/day in males and 98.8, 295.1 and 983.1 mg/kg bw/day in females.

### Water consumption

No treatment-related effects on water consumption were observed.

### Blood analysis

### Hematological findings

No treatment-related effects on hematological parameters were noted.

A statistically significant increase of prothrombin time was observed in mid dose females (see Table 6.8.1-10). Since the increase was not dose dependent and was within the historical control range (26.3 - 37.9 sec), the observed alteration was not considered to be related to treatment.

A statistically significant decrease of total eosinophil counts was noted in mid and high dose females. This observation was considered unrelated to treatment since the magnitude of change was marginal, the values were within the historical control range (0.06 to 0.12 x 10<sup>9</sup>/l) and the total white blood cell count was not affected.

**Table 6.8.1-10: Selected hematological findings in rats administered M700F001 for at least 90 days (group means)**

Sex	Males				Females			
Dose [mg/kg/day]	0	100	300	1000	0	100	300	1000
Eosinophils [10 <sup>9</sup> /l]	0.11	0.11	0.10	0.12	0.09	0.09	0.08*	0.08*
Eosinophils [%]	2.2	2.2	2.2	2.2	2.6	2.8	2.5	2.3
WBC [10 <sup>9</sup> /l]	4.94	5.07	4.87	5.87	3.67	3.43	3.43	3.46
Prothrombin time [sec]	35.2	35.7	36.0	35.3	32.6	32.0	34.6**	33.4

\* p ≤ 0.05; \*\* p ≤ 0.01 (Kruskal-Wallis and Wilcoxon-test, two sided)

### Clinical chemistry findings

No adverse changes of clinical chemistry parameters were noted.

The significantly increased triglyceride value observed in the males at the high dose (see Table 6.8.1-11) was regarded as test substance related, yet not adverse, since it is the only altered clinical pathology parameter. Furthermore, no treatment-related histopathological changes in the liver were observed.

**Table 6.8.1-11: Serum triglyceride levels in rats administered M700F001 for at least 91 days (group means)**

Sex	Males				Females			
Dose [mg/kg/day]	0	100	300	1000	0	100	300	1000
Triglycerides [mmol/l]	0.92	1.09	1.10	1.45**	0.62	0.61	0.72	0.61

\* p ≤ 0.05; \*\* p ≤ 0.01 (Kruskal-Wallis and Wilcoxon-test, two sided)

### Urinalysis

No treatment related, adverse changes of urinary parameters were observed.

A significantly decrease urine volume as well as an increased specific gravity was observed in the mid dose females (see Table 6.8.1-12). The increase of the specific gravity is a physiological, adaptive reaction of the kidneys during reduced urine flow and is therefore not regarded adverse. Furthermore, the change was not dose dependent and thus not considered treatment-related.

The urine of the males treated with the top dose and one male of the mid dose displayed a deep yellow discoloration (daffodil-like) distinctively different from the normal yellowish color of urine. This may have been due to the excretion of the pink colored M700F001 and its beige colored metabolite M700F002. Since in addition no other changes of urinary parameters were found clinical pathology this finding was considered as treatment related, yet not adverse.

**Table 6.8.1-12: Selected urine analysis findings in rats administered M700F001 for at least 91 days (group means)**

Sex	Males				Females			
Dose [mg/kg/day]	0	100	300	1000	0	100	300	1000
Volume [mL]	3.6	3.4	2.6	3.0	2.3	2.2	1.2**	2.2
Specific gravity [g/L]	1060	1059	1079	1062	1063	1071	1093**	1064

Sex	Males				Females			
Dose [mg/kg/day]	0	100	300	1000	0	100	300	1000

\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$  (Kruskal-Wallis and Wilcoxon-test, two sided)

## Necropsy

### Organ weights

No treatment-related effects on terminal body weights and absolute and relative organ weights were observed.

A statistically significant decrease of absolute and relative thymus weights was observed in mid dose males (see Table 6.8.1-13). In absence of a dose-response relationship and any corroborative histopathological findings this was considered to be incidental.

**Table 6.8.1-13: Selected mean absolute and relative organ weights of rats administered M700F001 for at least 91 days**

Sex	Males					Females			
Organ weight [mg]	Dose [mg/kg]	Absolute weight [mg]	□ %	Relative weight [% of b.w.]	□ %	Absolute weight [mg]	□ %	Relative weight [% of b.w.]	□ %
Terminal weight [g]	0	376,9				219,77			
	100	368,49	(-2.2)			219,05	(-0,3)		
	300	353,4	(-6.2)			214,5	(-2,4)		
	1000	377,1	(0.1)			213,82	(-2,7)		
Thymus	0	241.5		0.064		299.9		0.136	
	100	249.4	(3.3)	0.068	(5.8)	277.2	(-7.6)	0.126	(-7.6)
	300	191.5**	(-20.7)	0.054**	(-14.9)	260.4	(-13.2)	0.121	(-11.0)
	1000	247.8	(2.6)	0.065	(2.3)	254.6	(-15.1)	0.119	(-12.9)

\*  $p \leq 0.05$ ; \*\*  $p \leq 0.01$  (Kruskal-Wallis and Wilcoxon-test, two sided)

Values may not calculate exactly due to rounding of figures

## Gross and histopathology

No treatment-related gross necropsy and histopathological findings were noted.

A number of macropathological findings were noted in this study. These findings consisted of a single case of a reduced epididymis and testis size in a mid dose male, an epididymal abscess in each one low and high dose male, focal necrosis of adipose tissue in one low dose male, unilateral cornea cloudiness and focal renal cyst in one high dose male, focal retraction of the kidney in one high dose female and finally, multiple dark red foci in the pancreas in another high dose female.

All observations were noted in single animals only and often displayed no dose-response relationship. Thus these findings were not considered to be related to treatment.

A variety of histopathological changes were recorded in control and high dose animals. Neither the type, incidence nor severity of the findings indicated a relation to treatment. The findings were within the range of histopathological lesions expected for rats of this age and strain.

The only somewhat untypical histopathological finding was a moderate (Grade 3) diffuse atypical Islet cell hyperplasia characterized by elongated/fusiform Islet cells and multifocal presence of angiectasis (dilation of blood vessels) in the pancreas of one high dose females. This finding corroborated to the macroscopically observed dark red foci noted in this animal at gross necropsy. Due to the isolated occurrence and the lack of other histomorphological changes in the pancreas, this finding was considered to be incidental rather than related to treatment.

## CONCLUSION:

In a GLP and OECD compliant 90-day study in Wistar rats, in which animals were given dietary concentrations of CSAA798670 at nominal dose levels of 0, 100, 300 and 1000 mg/kg bw/d, there were no treatment-related mortalities, no clinical signs of toxicity and no effects on ophthalmoscopy, FOB and MA parameters. There were also no adverse effects on hematology, urinalysis or clinical biochemistry parameters, and no treatment-related effects on food consumption, water consumption, body weights or organ weights were recorded. The increase in triglycerides seen in top dose males was considered treatment-related, but in isolation, and taking into account the lack of any liver pathology, not adverse. Yellow discoloration of urine seen in top dose males was also considered treatment-related but not adverse. There were no effects on histopathology.

As a result, HSE agrees with the EU evaluation, that CSAA798670 did not cause any adverse effects in this 90-day study up to the top dose. A **NOAEL of 1000 mg/kg bw/d, the limit dose for this test, can be identified.**

(██████████, 2009)

#### PNDT study in rabbits

<b>Report:</b>	K-CA 5.8.1/07 ██████████, ██████████ and ██████████ (2009). Reg.No. 5069089 (metabolite of BAS 700 F) Prenatal Developmental Toxicity Study in New Zealand White Rabbits – Oral Administration (gavage), ██████████ ██████████ Laboratory Report No. 40R0451/07118. ██████████ DocID 2009/1072507. 14 October 2009. Syngenta File No. NOA449410_10005.
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**Guidelines:** OECD 414, Council Regulation 440/2008/EC B.17; EPA 870.3700

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

#### **EXECUTIVE SUMMARY**

The BAS 700 F metabolite M700F001 = Reg.No. 5069089 (batch L80-68; purity 99.2%) was administered to groups of 31 (treated groups) or 32 (control group) presumably pregnant ██████████:KBL (NZW) New Zealand White rabbits by oral gavage at daily dose levels of 0, 40, 100 and 250 mg/kg bw during days 6 to 28 of gestation.

No effects on maternal food consumption and body weight development were observed. Likewise, no treatment related necropsy findings were noted. Even though the conception rate of the time mated rabbits was relatively low (72, 87, 90 and 84% at 0, 40, 100 and 250 mg/kg bw/day, respectively), a sufficient number of litters for evaluation was available (20, 25, 26 and 25 at 0, 40, 100 and 250 mg/kg bw/day, respectively). No treatment-related effects on gestational parameters (number of corpora lutea, implantation sites, pre- and post-implantation loss, early and late resorptions, number of dead and viable fetuses, placental or foetal weights, or the sex ratio were observed.

Foetal examination did not reveal any treatment-related external, visceral or skeletal malformations, variations of unclassified observations.

**Based on the results of this study the NOAEL for maternal and developmental effects was 250 mg/kg bw/day.**

### Materials:

<b>Test Material:</b>	Reg.No. 5069089 (Metabolite of BAS 700 F) Synonym: Metabolite M700F001
<b>Description:</b>	solid / pink
<b>Lot/Batch number:</b>	L80-68
<b>Purity:</b>	99.2% (tolerance $\pm$ 1.0%)
<b>CAS#:</b>	176969-34-9
<b>Stability of test compound:</b>	The test substance was stable over the study period under the storage conditions. The Expiry Date was 01-Aug-2010.

<b>Test Animals:</b>	
<b>Species:</b>	Rabbit
<b>Strain:</b>	■■■■ KBL[NZW]
<b>Sex:</b>	Female
<b>Age:</b>	13-15 weeks at time of mating
<b>Weight:</b>	2744 ± 206.5 g (2133-3302g)
<b>Source:</b>	■■■■■
<b>Housing:</b>	Individual housing type 12.2395.C stainless steel wire mesh cages (Draht Bremer GmbH, Marktheidenfeld, Germany), floor area about 3000 cm². Underneath the cages, waste trays containing absorbent material (type 3/4 dust free embedding; supplied by Sniff, Soest, Germany) collected excreta. For enrichment, wooden gnawing blocks (Typ KNH E-041), supplied by Abedd® Lab. and Vet. Service GmbH, Vienna, Austria, was added.
<b>Environmental enrichment:</b>	Wooden gnawing blocks (Typ NGM E-022; Abedd® Lab. and Vet. Service GmbH, Vienna, Austria)  Motor activity measurements were conducted in separate Polycarbonate cages
<b>Acclimatisation period:</b>	At least 5 days
<b>Diet:</b>	■■■■■ maintenance diet for rabbits & guinea pigs, GLP, ■■■■■ ■■■■■, <i>ad libitum</i>
<b>Water:</b>	Tap water in bottles, <i>ad libitum</i>
<b>Environmental conditions:</b>	Temperature: 20 - 24 °C Humidity: 30 - 70 % Air changes: 15/hour Photoperiod: 12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

**Dates of experimental work:** 03-Mar-2009 to 04-Sep-2009  
(In-life dates: 08-Mar-2008 (Start of treatment at GD 6 of the first cohort) to 09-Apr-2009 (Sacrifice of the last cohort))

Reg.No. 5069089 (Metabolite of BAS 700 F) was administered to groups of 31 to 32 presumably pregnant rabbits by gavage at dose levels of 0, 40, 100, and 250 mg/kg bw during days 6 to 28 of gestation. During the acclimatization period the animals were assigned to the treatment groups based on a randomization plan as well as body weight. For technical reasons the study was performed in 4 cohorts.

The dose selection for this study was based on two maternal toxicity studies in pregnant rabbits. The first range-finding study was conducted in the Himalayan rabbit to determine maternal toxicity. Due to a change

in the rabbit strain in the conducting laboratory, a second range-finding study was conducted in the New Zealand White rabbit. No foetal examination was conducted in both studies.

Study N°1 Himalayan rabbits		Study N°2 NZW rabbits	
Tested doses (mg/kg bw/d)	Observed effects	Tested doses (mg/kg bw/d)	Observed effects
1000	Mortality in 1/5 doe, abortion in 1/5 doe Reduced defecation and poor general state in 2/5 animals Significantly reduced mean body weight gain during gestation (about 35% below controls) Stomach erosions/ulcerations in 2/does	1000	Mortality in 1/5 animal, abortion in 1/5 doe No defecation in 1/5 doe Cessation of food consumption in 1/5 doe before abortion Stomach erosions in 1/5 animal, empty intestines in 2/5 animals
300	Significantly reduced mean body weight gain during gestation (about 39% below controls)	500	Mortality in 2/10 does Stomach erosions in 1/10 doe, fluid-filled stomach and empty intestine in 1/10 doe
		250	Reduced food consumption in 2/10 does towards the end of gestation
100	No test substance-related adverse effects	100	No test substance-related adverse effects

Based on the results of the two maternal toxicity studies dose levels  $\geq 500$  mg/kg bw (see Table above) were considered to result in excessive toxicity. Thus, trying to avoid excessive maternal toxicity the following doses were chosen for the main prenatal developmental toxicity study in New Zealand White rabbits:

- 40 mg/kg body weight/day: as low-dose level (expected NOAEL)
- 100 mg/kg body weight/day: as mid-dose level with possible effects on mean body weight gain
- 250 mg/kg body weight/day: as high-dose level with effects on mean food consumption

#### Animal numbers and treatment groups

Test group	Dose (mg/kg bw/day)	Concentration of the dose preparation (mg/100 mL)	Volume (mL/kg)	No of animals
0	0	0	10	32
1	40	400	10	31
2	100	1000	10	31
3	250	2500	10	31

#### Test substance preparation and analysis:

Prior to study initiation the test substance was shown to be stable in the vehicle 1% CMC at a concentration of 10 mg/100 ml for up to 7 days. Thus, test substance preparations were performed at intervals of up to 7 days.

Samples of test preparations were analysed for achieved concentration twice during the study period. Homogeneity was performed on the first sampling period only for low and high dose groups. Three samples (1x top, 1 x middle, 1 x bottom) were taken from the beaker during stirring (magnetic stirrer) for analysis.

#### Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

**Statistical analysis**

Parameter	Statistical test
<b>Statistics for clinical and foetal examinations</b>	
Food consumption <sup>a)</sup> , body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of postimplantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean foetal body weight, litter mean placental weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with foetal findings	Pair-wise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pair-wise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal medians

<sup>a)</sup> For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pretreatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

**Methods****Insemination/Pairing**

The does were paired by the breeder (time-mated animals). The day of pairing was designated as gestation day 0 (GD 0). The presumably pregnant rabbits were supplied on the day after mating (GD 1). At the day of arrival in the laboratory (GD 1) the animals were assigned to the treatment groups according to a randomization table and on the basis of their body weights.

**Treatment**

The test substance was administered by gavage once a day from implantation to one day prior the expected day of parturition (GD 6 - 28). The volume administered each day was 10 ml/kg body weight. The calculation of the volume administered was based on the most recent individual body weight. Control animals received the vehicle (1% aqueous CMC).

**Clinical Observation for mortality and clinical signs**

The animals were examined for mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily or more often when clinical signs of toxicity were elicited.

**Body weight**

All animals were weighed on gestation days 1, 4, 6, 9, 11, 14, 16, 19, 21, 23, 25, 28, and 29. The body weight change of the animals was calculated from these results. In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 29 minus weight of the unopened uterus minus body weight on GD 6).

**Food consumption**

Food consumption was determined daily from GD 2 to GD 29.

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on GD 29 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.



For the above reasons the following females were excluded from the above-mentioned calculations:

- Control females #4, 6, 7, 13, 20, 22, 27, 28, and 32 (not pregnant) and #5 and 24 (died/sacrificed after gavage error)
- Low dose (40 mg/kg bw) females #34, 44, 55, and 55 (not pregnant), #52 (sacrificed after abortion), and #54 (died after gavage error)
- Mid dose (100 mg/kg bw) females #69, 80, and 92 (not pregnant), #67 (died intercurrently), and #84 (sacrificed moribund)
- High dose (250 mg/kg bw) females #96, 97, 98, 109, and 117 (not pregnant), #79 (death after gavage error) and #125 (died after gavage error)

### Sacrifice

On GD 29, the dams were sacrificed in randomized order by intravenous injection of pentobarbital (Narcoren®; dose: 2 ml/animal) and fetuses were removed from the uterus. Dams were subsequently assessed by gross pathology in randomized order to minimize bias.

The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Number of corpora lutea
- Number and distribution of implantation sites classified as
  - live fetuses or
  - dead implantations
    - a. early resorptions (only decidual or placental tissues visible or positive staining according to SALEWSKI of uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    - b. late resorptions (embryonic or foetal tissue in addition to placental tissue visible)
    - c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based in the above the following parameters were calculated:

- Conception rate [%]:

$$\frac{\text{number of pregnant animals}}{\text{number of fertilized animals}} \times 100 \%$$

- Preimplantation loss [%]:

$$\frac{\text{number of corpora lutea} - \text{number of implantations}}{\text{number of corpora lutea}} \times 100$$

- Postimplantation loss [%]:

$$\frac{\text{number of implantations} - \text{number of live fetuses}}{\text{number of implantations}} \times 100$$

### Examination of foetuses

At necropsy each fetus was weighed and examined macroscopically for any external findings. Furthermore, the viability of the fetuses and the condition of the placentae, the umbilical cords, the foetal membranes,

and fluids were examined. Individual placental weights were recorded. Subsequently the fetuses were sacrificed by s.c. injection of pentobarbital (Narcoren<sup>®</sup>, 0.2 ml/fetus).

After sacrifice the abdomen and thorax were opened in order to be able to examine the organs in situ before they were removed. The heart and the kidneys were sectioned in order to assess the internal structure. The sex of the foetuses was determined by internal examination of the gonads.

The heads of approximately one half of the fetuses per dam (and the heads of those fetuses, which revealed severe findings (e.g. anophthalmia, microphthalmia or hydrocephalus) already during the external examination) were severed from the trunk. These heads were fixed in BOUIN's solution and processed and assessed according to WILSON's method subsequently. About 10 transverse sections were prepared per head.

After skinning, all foetuses (including those without heads) were fixed for 1 to 5 days in ethyl alcohol. After that a cross section of the heads from all intact fetuses was made in the parietal bone area using a scalpel. The two halves of the heads were carefully bent to allow a thorough examination of the brain. Subsequently, the fetuses were placed back into the fixative for further fixation. After final fixation the skeletons (with the possible exception of the skulls) were double stained according to a modified method of KIMMEL and TRAMMELL and evaluated.

### Evaluation criteria for assessing the foetuses

Foetal morphology findings were described using the glossary of **Wise *et al.*, (1997)<sup>8</sup>** as far as possible. Exceptions were made for special findings not listed in the glossary.

<b>Malformation</b>	A permanent structural change that is likely to adversely affect the survival or health
<b>Variation</b>	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term "unclassified observation" was used for those foetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

## RESULTS

### Observations

#### Clinical signs of toxicity

The animals did not show any test substance related clinical signs of toxicity. Mid dose doe #79 had blood in bedding on GD 29 but uterus weight and litter size were in the expected range and only one early resorption was noted for this female.

**Table 6.8.1-14: Clinical observations in rabbits administered M700F001 during days 6 to 28 of gestation**

Dose level [mg/kg bw]	Animal #	Gestation Day	Observation
Control	5, 24	15	Died or sacrificed after gavage error
40 mg/kg bw	52	21	Abortion - sacrificed
	54	21	Died after gavage error

<sup>8</sup> Wise D, Beck S, Beltrame D, Beyer B, Chahoud I, Clark RL, Clark R, Druga, A, Feuston M, Guittin P, Henwood S, Kimmel C, Lindstrom P, Palmer A, Petrere J, Solomon H, Yasuda M and York R. Terminology of developmental abnormalities in common laboratory mammals (Version 1) Teratology 55, 249 - 292 (1997).

Dose level [mg/kg bw]	Animal #	Gestation Day	Observation
100 mg/kg bw	67	14	Intercurrent death
	75	29	Blood in bedding
	84	14	Accidental fracture of hindlimb, sacrificed moribund
250 mg/kg bw	125	12	Died after gavage error

### Mortality

Low dose doe #52 was sacrificed after abortion on GD 21. The animal displayed a loss in body weight from GD 16 and markedly reduced food consumption from GD 18 onwards. The fact that spontaneous abortions are also observed in control does and the absence of a dose-response relationship indicated that this abortion was of incidental nature.

Mid dose doe #67 died intercurrently on GD 14 without preceding clinical signs. There were no necropsy observations, which could help to elucidate the cause of death. Because of the isolated and non-dose dependent incidence of mortality, this death was considered to be of spontaneous origin and not related to treatment.

Four does (control does #5 and 24, low dose doe #54, high dose doe #125) died after gavage errors as indicated by blood or test substance filled thoracic cavity or congested lungs.

Mid dose female #84 had to be sacrificed in moribund state on GD12 after an accidental fracture of the hindlimb. None of these deaths was compound related.

### Food consumption

Mean food consumption was comparable between all test groups during the entire study period. Statistically significant differences between control and treated groups were observed between GD 5-6 (pre-treatment) in low and mid dose groups and between GD 7-8 for the low-dose group. In absence of a dose-dependency these changes were considered to be incidental.

### Body weight and body weight gain

Body weight development in all dose groups was comparable to the control animals. Observed differences of body weight gain were neither statistically significant nor considered biologically relevant.

### Necropsy Observations

#### Corrected (net) body weight gain

No treatment-related effects on uterus, carcass, and corrected (net) body weight gain were observed at any dose level.

#### Gross necropsy observations

A number of gross necropsy findings were noted in all groups including control at caesarian section. Neither the incidence nor the type of observations indicated a relation to treatment.

**Table 6.8.1-15: Gross necropsy findings in rabbits administered M700F001 during days 6 to 28 of gestation**

Dose group	Animal #	Observation
Control	1	Empty stomach
	5 <sup>b</sup>	Thoracic cavity filled with blood, congested lungs
	22	Absent lobus inferior medialis

Dose group	Animal #	Observation
	24 <sup>b</sup>	Thoracic cavity filled with blood, congested lungs
	28 <sup>NP</sup>	Bilobed gallbladder
40 mg/kg bw	54 <sup>b</sup>	Thoracic cavity filled with test substance
100 mg/kg bw	66	Lungs with petechiae
	71	Empty stomach
	74	Absent lobus inferior medialis
	84 <sup>a</sup>	Fracture of right hindlimb
250 mg/kg bw	125 <sup>b</sup>	Thoracic cavity filled with blood

mg/kg bw/d = milligram per kilogram body weight per day; NP = not pregnant

<sup>a)</sup> sacrificed moribund; <sup>b)</sup> died after gavage error

### Caesaren section data

Twenty-three, 27, 28 and 26 does were pregnant at 0, 40, 100 and 250 mg/kg bw. As already indicated above, one low dose female aborted. This abortion was not considered to be treatment-related. In one control doe (#9) a total resorption was observed. However, this animal had only 1 corpora luteum and consequently only one implant, which was resorbed early.

The mean number of corpora lutea, implantation sites, pre-implantation losses, early and late resorptions and live fetuses were comparable between control and treated groups. Likewise the mean number and weight of live, male and female fetuses as well as the sex ratio of foetuses were not affected by treatment. Finally, no effects on placental weights were observed.

**Table 6.8.1-16: Pregnancy status and Caesarean section data of does administered M700F001 during days 6 to 28 of gestation**

Dose level [mg/kg bw/d]	0	40	100	250
<b>Pregnancy status</b>				
Females				
- mated [n]	32	31	31	31
- pregnant [n]	23	27	28	26
conception rate [%]	72	87	90	84
- aborted [n]	0	1	0	0
- premature birth [n]	0	0	0	0
- dams with viable fetuses [n]	20	25	26	25
- dams with all resorptions [n]	1	0	0	0
- mortality	2	2	2	1
- pregnant terminal sacrifice [n]	21	25	26	25
<b>Caesarean section data<sup>a</sup></b>				
- Corpora lutea [n]	10.1±3.60	9.9±2.43	9.7 ± 2.00	9.3±2.54
total number [n]	213	248	252	232
- Implantation sites [n]	9.4±3.90	9.2±2.75	8.7±2.45	8.6±2.80
total number [n]	197	229	226	216
- Pre-implantation loss [%]	8.4±17.73	8.9±13.34	11.1±17.43	7.3±15.40
- Post-implantation loss [%]	10.3±23.09	8.7±15.67	7.9±12.23	8.2±12.49
- Resorptions [n]	0.6±0.97	0.7±0.90	0.7±1.12	0.6±0.99
total number [n]	13	17	19	16
- Early resorptions [%]	6.2±21.79	6.7±15.76	6.3±12.27	5.4±9.22
number [n]	0.2±0.40	0.5±0.87	0.6±1.10	0.4±0.65
total number [n]	4	12	15	10
- Late resorptions [%]	4.1±9.98	2.0±4.22	1.5±3.68	2.8±5.26
number [n]	0.4±0.93	0.2±0.41	0.2±0.37	0.2±0.44
total number [n]	9	5	4	6
- Dead fetuses [n]	0	0	0	0
- Dams with viable fetuses [n]	20	25	26	25
- Live fetuses	9.2±3.47	8.5±2.90	8.0±2.46	8.0±2.94
total number [n]	184	212	207	200
- Total live female fetuses [n]	4.3±2.62	4.3±2.42	3.3±1.55	3.4±2.31
total number [n]	87	107	87	86
Mean [%]	44.3±20.23	46.14±22.60	37.7±14.65	37.7±22.19
- Total live male fetuses [n]	4.8±2.50	4.2±1.78	4.6±1.65	4.6±2.27
total number [n]	97	105	120	114
Mean [%]	49.9±17.62	45.3±18.11	54.5±17.11	54.1±21.85
- Percent live females	47.3	50.5	42.0	43.0
- Percent live males	52.7	49.5	58.0	57.0
Placental weights [g]	5.3 ± 0.97	5.2 ± 0.99	5.2 ± 0.70	5.5 ± 0.81
- male fetuses [g]	5.5 ± 0.97	5.2 ± 1.04	5.2 ± 0.77	5.5 ± 0.89
- female fetuses [g]	5.2 ± 1.08	4.9 ± 0.81	5.1 ± 0.78	5.3 ± 0.65
Mean foetal weight [g]	37.9 ± 5.42	37.3 ± 5.16	38.5 ± 3.34	39.3 ± 5.13
- males [g]	38.7 ± 6.01	37.4 ± 5.36	38.7 ± 3.97	39.1 ± 5.87
- females [g]	37.7 ± 5.51	36.1 ± 4.41	37.9 ± 3.76	38.9 ± 3.79

<sup>a</sup> Mean ± SD on litter basis.**External, visceral and skeletal examination of foetuses****External examination**

External malformations were recorded for single animals of treated groups. Most of the observed malformations were observed in single animals only. The multiple external malformation of the head of one mid dose foetus was not dose dependent and the incidence of open eye in mid and top dose groups were within the historical control range. The umbilical hernias observed in one low dose fetus and two high dose fetuses from different litters did not display a dose-response relationship. Thus the observed incidences were not considered to be related to treatment.

The only external variation observed was paw hyperflexion, which was noted in each one low and high dose foetus. In absence of a dose-response relationship this finding was considered to be of spontaneous origin. Furthermore, the incidence was within the historical control database.

The only unclassified external observation noted was polyhydramnios in a low dose fetus. The missing dose-response indicated that there is no relation to treatment.

**Table 6.8.1-17: Incidence of external malformations and variations**

Dose level [mg/kg bw]	0	40	100	250
Litters Evaluated	20	25	26	25
Fetuses Evaluated	184	212	207	200
Live	184	212	207	200
Dead	0	0	0	0
<b>Total external malformations</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	2 (1.0)	3 (1.5)
- Litter incidence <sup>a</sup>	0 (0.0)	1 (4.0)	2 (7.7)	3 (12)
- Affected fetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.6 ± 2.18	1.2 ± 3.32
<b>Individual external malformations</b>				
- <b>Umbilical hernia</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	2(1.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	2(8.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.0 ± 0.00	0.9 ± 3.03
- <b>Multiple external malformations of the head</b>				
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	1 (0.5)	0(0.0)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	1 (3.8)	0(0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 1.51	0.0 ± 0.00
- <b>Open eye</b>				
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	1 (0.5)	1(0.5)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	1 (3.8)	1(4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.0 ± 0.00	0.3 ± 1.63	0.3 ± 1.54
<b>Total external variations</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	1(0.5)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	1(4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.5 ± 2.50	0.0 ± 0.00	0.5 ± 2.50
<b>Individual external variations</b>				
- <b>Paw hyperflexion</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	1(0.5)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	1(4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.5 ± 2.50	0.0 ± 0.00	0.5 ± 2.50

### Visceral examination

A variety of visceral (soft tissue) malformations were observed in all groups including control. The highest number of malformation was observed in the low dose group. As no statistically significant differences were observed, most of the malformations occurred only singly or without dose-response relationship or at incidences comparable to the historical control range, none of these visceral malformations were considered to be related to treatment.

All visceral variations occurred at incidences comparable with the historical control or displayed neither statistical significance nor dose-response relationship. Thus these variations were considered to be of spontaneous origin.

Unclassified visceral observations were fluid filled abdomen in one low dose animal and infarct of liver in one low dose animal. Additionally, blood coagulum around urinary bladder was found in all dose groups (4, 3, 1, and 2 at 0, 40, 100 and 250 mg/kg bw). These incidences did not indicate a relation to treatment.

**Table 6.8.1-18: Incidence of visceral (soft tissue) malformations**

Dose level [mg/kg bw]	0	40	100	250
Litters Evaluated	20	25	26	25
Foetuses Evaluated	184	212	207	200
Live	184	212	207	200
Dead	0	0	0	0
<b>Total visceral malformations</b>				
- Foetal incidence [No. (%)]	2 (1.1)	7 (3.3)	5 (2.4)	2 (1.0)
- Litter incidence	2 (10)	6 (24)	4 (15)	2 (8.0)
- Affected foetuses/litter (Mean ± SD) [%]	1.1 ± 3.42	4.3 ± 8.87	2.1 ± 5.38	0.8 ± 2.81
<b>Individual visceral malformations</b>				
<b>- Aortic arc atresia</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	1 (4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.5 ± 2.50	0.0 ± 0.00	0.4 ± 1.82
<b>- Malpositioned aorta origin</b>				
- Foetal incidence [N (%)]	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.6 ± 2.48	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
<b>- Absent subclavian</b>				
- Foetal incidence [N (%)]	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.6 ± 2.48	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
<b>- Persistent truncus arteriosus</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.3 ± 1.54	0.0 ± 0.00	0.0 ± 0.00
<b>- Retroesophageal aortic arch</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.0 ± 0.00	0.0 ± 0.00
<b>- Heart: muscular ventricular septum defect</b>				
- Foetal incidence [N (%)]	0 (0.0)	2 (0.9)	0 (0.0)	1 (0.5)
- Litter incidence [N (%)]	0 (0.0)	2 (8.0)	0 (0.0)	1 (4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	2.1 ± 7.63	0.0 ± 0.00	0.4 ± 1.82
<b>- Cardiomegaly</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	1.3 ± 6.67	0.0 ± 0.00	0.0 ± 0.00
<b>- Diaphragmatic hernia</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	2 (1.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	1 (3.8)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.9 ± 4.36	0.0 ± 0.00
<b>- Small spleen</b>				
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.4 ± 2.22
<b>- Malpositioned kidney</b>				
- Foetal incidence [N (%)]	0 (0.0)	2 (0.9)	0 (0.0)	1 (0.5)
- Litter incidence [N (%)]	0 (0.0)	2 (8.0)	0 (0.0)	1 (4.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.9 ± 3.08	0.0 ± 0.00	0.4 ± 1.82
<b>- Absent kidney</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.0 ± 0.00	0.0 ± 0.00
<b>- Hydroureter</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.0 ± 0.00	0.0 ± 0.00
<b>- Absent ureter</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean ± SD) [%]	0.0 ± 0.00	0.4 ± 2.22	0.0 ± 0.00	0.0 ± 0.00

**Table 6.8.1-19: Incidence of visceral (soft tissue) variations**

Dose level [mg/kg bw]	0	40	100	250
Litters Evaluated	20	25	26	25
Foetuses Evaluated	184	212	207	200
Live	184	212	207	200
Dead	0	0	0	0
<b>Total visceral variations</b>				
- Foetal incidence [N (%)]	5 (2.7)	6 (2.8)	10 (4.8)	4 (2.0)
- Litter incidence [N (%)]	4 (20)	5 (20)	7 (27)	4 (16)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	2.7 $\pm$ 6.05	6.4 $\pm$ 20.32	6.4 $\pm$ 14.50	1.9 $\pm$ 4.61
<b>Individual visceral variations</b>				
<b>- Dilated cerebral ventricle</b>				
- Foetal incidence [N (%)]	0 (0.0)	2 (0.9)	2 (1.0)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	1 (3.8)	0 (0.0)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	0.0 $\pm$ 0.00	0.6 $\pm$ 3.08	2.6 $\pm$ 13.07	0.0 $\pm$ 0.00
<b>- Narrowed carotid</b>				
- Foetal incidence [N (%)]	1 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
- Litter incidence [N (%)]	1 (5.0)	0 (0.0)	0 (0.0)	0 (0.0)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	0.4 $\pm$ 1.86	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00
<b>- Malpositioned carotid branch<sup>s</sup></b>				
- Foetal incidence [N (%)]	2 (1.1)	1 (0.5)	4 (1.9)	3 (1.5)
- Litter incidence [N (%)]	2 (10)	1 (4.0)	3 (12)	3 (12)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	1.1 $\pm$ 3.61	4.0 $\pm$ 20.00	1.7 $\pm$ 4.93	1.5 $\pm$ 4.20
<b>-Narrowed pulmonary trunk</b>				
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	1 (0.5)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	1 (3.8)	0 (0.0)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	0.0 $\pm$ 0.00	0.0 $\pm$ 0.00	0.3 $\pm$ 1.51	0.0 $\pm$ 0.00
<b>- Dilated aortic arch</b>				
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	1 (0.5)	0 (0.0)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	1 (3.8)	0 (0.0)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	0.0 $\pm$ 0.00	4.0 $\pm$ 20.00	0.3 $\pm$ 1.51	0.0 $\pm$ 0.00
<b>- Absent lung lobe (L. inferior medialis)<sup>s</sup></b>				
- Foetal incidence [N (%)]	2 (1.1)	2 (0.9)	3 (1.4)	1 (0.5)
- Litter incidence [N (%)]	1 (5.0)	2 (8.0)	3 (12)	1 (4.0)
- Affected foetuses/litter (Mean $\pm$ SD) [%]	1.1 $\pm$ 4.97	0.9 $\pm$ 3.27	1.9 $\pm$ 5.44	0.4 $\pm$ 2.22

<sup>s</sup> within the historical control range

## Skeletal examination

A small number of skeletal malformations were observed in all groups including controls. As no statistically significant differences were observed between control and treated groups and the malformations were observed either singly, without dose response-relationship or at incidences comparable to the historical control range, none of these skeletal malformations were considered to be related to treatment.

For all test groups skeletal variations were noted in various parts of the foetal skeletons. These variations were seen in all litters of all groups and in 97.1 to 98.4% of the foetuses. No relation to dosing was evident. The only statistically significant specific variation was "supernumerary 13<sup>th</sup> rib with present cartilage" at the high dose when viewed on an 'affected fetus per litter' basis. This increase, however, was not reflected for the litter incidence which is lower for the treated groups than for the control. Additionally, there was no dose-response relationship for this finding. Finally, slight changes of the ossification process occur frequently in gestation day 29 foetuses of this rabbit strain and are thus present in the historical control data (mean: 38.7%, range 0.0 to 59.7%). Therefore, this finding was not considered treatment related.

Eight different skeletal unclassified observations were observed in this study. Six were observed either with single or low (up to 3) foetal incidences. The most abundant unclassified observation was 'cartilaginous part of ribs not connected with sternum', which was observed in 11, 11, 19 and 9 foetuses of 6, 8, 9 and 5 litters at 0, 40, 100 and 250 mg/kg bw, respectively. The other finding with higher foetal incidences was bipartite processes xiphoideus where the highest foetal incidence of 12 occurred in the control group. The



total percentage of affected fetuses per litter was  $14.1 \pm 18.18$ ,  $13.0 \pm 15.73$ ,  $14.6 \pm 18.78$  and  $8.2 \pm 14.05$  at dose levels of 0, 40, 100 and 250 mg/kg bw. The observed findings did not indicate a relation to treatment.

**EFSA Request for additional information (February 2018), Question 37:** Applicant to provide further assessment of the external and visceral findings against the historical control data, and a summary table for the skeletal examination.

Further assessment of the external and visceral findings against the historical control data has been provided by the applicant. This assessment can be found in [REDACTED] and [REDACTED] (2018) report summary presented below. A table for the skeletal examination has been provided with in the assessment. There were no external or visceral malformations or variants which were considered related to administration of NOA449410.

<b>Report:</b>	K-CA 5.8.1/08 [REDACTED] [REDACTED] (2018). NOA449410 - Additional Historical Control Data to Support the Prenatal Developmental Toxicity Study in the Rabbit, [REDACTED] [REDACTED]. Syngenta File No. NOA449410_10013
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**Guidelines:** Not applicable.

**GLP:** This report contains an assessment of data from completed studies and additional scientific information and is not a study per se, a Good Laboratory Practice (GLP) statement as defined by OECD is not appropriate.

## EXECUTIVE SUMMARY

A prenatal developmental toxicity study in the rabbit was conducted on Reg.No. 5069089 (Metabolite of BAS 700 F) at [REDACTED], [REDACTED] in 2009, using the New Zealand White (NZW) rabbit ([REDACTED], 2009). The compound is known as NOA449410 in Syngenta. A limited set of historical control data (HCD) was included in the original study report from 5 studies in the NZW rabbit conducted between 2000 and 2009. Additional HCD has been obtained from the conducting laboratory in the NZW rabbit for 2009 to 2016 (Appendix 4). This document provides an assessment of the external and visceral findings against the additional HCD in this strain of rabbit from the same conducting laboratory between 2009 and 2016 and an evaluation of the skeletal findings including a comparison with HCD.

## RESULTS AND DISCUSSION

### An assessment of the external and visceral findings against historical control and relevant external literature

There were no external or visceral malformations or variants which were considered related to administration of the test item. A comparison of external and visceral malformations and variants observed in high dose fetuses with HCD are provided in Tables 5.8.1-21 to 5.8.1-23.

There were no statistically significant increases in any external or visceral malformation or variant or in the total number of external malformations or variants. A small number of external or visceral malformations and variants occurred in the high dose group (1000 mg/kg/day) which were not observed in the concurrent control group, however, they were observed as a spontaneous malformations in control fetuses in other studies from 2000 to 2009 and/or 2009 to 2016.

Umbilical hernia (also can be referred to as omphalocele (Makris *et al.*, 2009) was observed in 2 fetuses from 2 litters at the high dose, and in 1 fetus in 1 litter in the low dose (Schneider, 2009), this malformation has been observed within in the presented HCD as an incidence of 1 fetus in 1 litter (Appendix 4). In the absence of the observation in the mid-dose group, no clear dose-response relationship and in the absence of any other effects in the fetuses following administration of the test item, it is considered that the incidence of umbilical hernia is of spontaneous origin.

Umbilical hernia is a failure of the ventral abdominal wall to close completely at the umbilical ring, allowing a varying amount of the intestines to protrude from the umbilical area. There are no effects on the development of the intestines and they loop normally into the body cavity. The protruding intestines are covered by skin and may present superficially just as a bulge or protrusion in the umbilical region.

Umbilical hernia and omphalocele are embryonically comparable (Wakarny, 1971). Both defects are characterised by the protrusion of intestine through the umbilicus.

Omphalocele is the result of failure of return of the intestinal loops into the body cavity, such that some of the intestines remain protruding through a defect in the ventral abdominal wall, usually at the umbilicus. The protruding intestines are covered by a thin transparent membrane.

Since both defects are characterized by the protrusion of intestine through the umbilicus, omphalocele might sometimes be mistakenly described as umbilical hernia and *vice versa*.

In a typical study using twenty or so pregnant rabbits per group, there are likely to be only a few fetuses with malformations, and the incidence of each specific malformation even lower. It is not uncommon in the conduct of developmental toxicity studies to observe one or two instances of a malformation in a single group. If these occur in the control then it is obvious that they are attributable to spontaneous background, but if they occur in a treatment group the interpretation is more difficult. Therefore, it is customary to rely on historical control datasets to aid in the interpretation (Daston and Beehuijzen, 2018). Contract laboratories and large industry laboratories compile their own historical control data. However, even very active laboratories are limited in the number of studies they can draw from to compile these data, which is clearly the significant issue with the current HCD at [REDACTED] which only contains 24 rabbit developmental toxicity studies in the New Zealand White Rabbit from 2000 to 2016.

While the HCD within a specific laboratory are useful, they still represent a small sample size for reliably estimating the incidence of relatively rare spontaneous events. Recognizing this limitation, the Middle Atlantic Reproduction and Teratology Association (MARTA) compiled a large data set from a survey of 21 member organizations for the period of 1989 to 1992. This database consisted of 2776 NZW rabbit litters and 20,071 fetuses (MARTA, 1996), which is large enough to provide reasonable estimates for the rates of many individual malformations. Omphalocele was the most common external malformation in NZW rabbits in the MARTA database. Umbilical hernia was reported separately in that database, and when combined with omphalocele the incidence was more than twice as high as any other external malformation. The cumulative incidence of omphalocele (including umbilical hernia) was 0.64% (litter incidence) and 0.11% (foetal incidence) (MARTA, 1996).

The rate of omphalocele or umbilical hernia in NZW rabbit fetuses was recently determined by Daston and Beehuijzen (2018). A compilation of external malformation data from NZW rabbit developmental toxicity studies published in three peer-reviewed journals (Fundamental and Applied Toxicology (FAAT), Birth Defects Research B: Developmental and Reproductive Toxicology (BDRB), and Reproductive Toxicology (RT)) resulted in a data set with 4905 litters and 36,977 individual fetuses. Omphalocele and umbilical hernia were considered together and was one of the most common external malformation in this data set. Omphalocele or umbilical hernia occurred in 59 fetuses in 54 litters; the cumulative litter incidence was 1.10 % and the foetal incidence 0.16 %. It occurred in 18 fetuses in control groups from 14 studies, and occurred at a maximum incidence of 2/16 litters (12.5%) affecting 2/137 fetuses (14.6%). Daston and Beehuijzen (2018) evaluated incidences in control and treated groups and concluded that given the relatively high rate of omphalocele in controls and lack of dose-responsiveness, it appears that this malformation has a high background incidence, confirming this to be a common spontaneous finding in the NZW rabbit.

The detailed evaluation of the occurrence of umbilical hernia/omphalocele in the NZW rabbit (MARTA, 1996 and Daston and Beehuijzen, 2018) supports that the incidence of umbilical hernia (omphalocele) in the high dose group after administration of NOA449410 is of spontaneous origin and is not related to test item administration.

### Evaluation of the skeletal findings and an assessment against historical control data and relevant external literature

There were no skeletal malformations or variants which were considered related to administration of the test item. Skeletal malformations observed in high dose fetuses or litters at a higher incidence than concurrent controls are compared in Table 6.8.1-24. For skeletal variants a comparison is made with HCD if the foetal and/or litter incidence in the high dose group is higher than the concurrent control (Table 6.8.1-25).

A small number of skeletal malformations were observed in all groups including controls. There were no statistically significant differences were observed between control and treated groups and the malformations which were observed were either single isolated incidences, without a dose response-relationship or at incidences comparable to the HCD, none of these skeletal malformations were considered to be related to treatment.

For all test groups skeletal variations were noted in various parts of the foetal skeletons. These variations were seen in all litters of all groups and in 97.1% to 98.4% of the fetuses and were within the HCD range (Table 6.8.1-25). No dose-relationship was evident. The only statistically significant specific variation was "supernumerary 13th rib with present cartilage" at the high dose when viewed on an 'affected fetus per litter' basis (Table 6.8.1-20). This increase, however, was not reflected for the litter incidence which is lower for the treated groups than for the control. Additionally, there was no dose-response relationship for this finding and the incidence was comparable with range reported in the HCD. Finally, slight changes of the ossification process occur frequently in gestation day 29 fetuses of this rabbit strain and are thus present in the HCD (mean: 38.7%, range 0.0 to 59.7%). Therefore, this finding was considered not treatment related.

**Table 6.8.1-20: Occurrence of statistically significantly increased foetal skeletal variations (expressed as mean percentage of affected fetuses/litter)**

Dose level [mg/kg bw/day]	0	40	100	250	HCD 2000-2009	HCD 2009-2016
Litters Evaluated	20	25	26	25	114	431
Fetuses Evaluated	184	212	207	200	934	3642
<b>Supernumery rib (13<sup>th</sup>); cartilage present</b>						
- Foetal incidence [N (%)]	98 (53)	142 (67)	129 (62)	149 (75)	380 (60.4)	2273 (73.7)
- Litter incidence [N (%)]	20 (100)	24 (96)	24 (92)	24 (96)	77 (97.6)	409 (100)
- <b>Affected fetuses/litter (mean %)</b>	53.5	68.0	60.1	72.9*	38.7 (0-59.7)	68.1 (50-73.9)

\* =  $p \leq 0.05$  (Wilcoxon-Test [one-sided]).

HCD 2000-2009 were provided in the original study report (n=5 studies).

HCD 2009-2016 additional data provided after the original study (n=19 studies)

For the HCD the maximum per study % value is presented.

In addition, these findings do not represent malformations, but rather, are variations in skeletal and cartilage development (Solecki *et al.*, 2001; Carney and Kimmel, 2007; Kimmel *et al.*, 2014), variations are not considered adverse, have no detrimental effect on survival, development, growth, or health postnatally, and are not mechanistic precursors to malformations; therefore, these endpoints should not be used to identify adverse limit doses (i.e. NOAEL's and LOAEL's) (Chahoud and Paumgarten, 2009).

A variation is defined as an alternative structure occurring regularly in control populations which may be permanent or transient and have no impact on the survival, growth, development or function of the developing neonate (Makris *et al.*, 2009).

Furthermore, this is an isolated incidental finding with no associated changes in any other rib parameters such as incomplete or absent ossification. The increased incidence of this skeletal variant does not demonstrate a dose-response relationship and therefore, this apparent increase cannot be considered to be treatment-related and is incidental to treatment with NOA449410.

This is further supported by the absence of major foetal abnormalities attributed to administration of NOA449410, or any effects on the number of live foetuses, foetal weight or sex ratio. There was also no difference from control in the overall foetal and litter incidences of minor abnormalities.

**Table 6.8.1-21: Incidence of External Malformations and Variations**

Dose level [mg/kg bw]	0	40	100	250	HCD 2000-2009	HCD 2009-2016
Litters Evaluated	20	25	26	25	1135	3642
Foetuses Evaluated	184	212	207	200	135	431
<b>Total external malformations</b>						
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	2 (1.0)	3 (1.5)	10 (1.3)	15 (1.1)
- Litter incidence	0 (0.0)	1 (4.0)	2 (7.7)	3 (12)	8 (8.3)	14 (9.1)
<b>Individual external malformations</b>						
<b>- Umbilical hernia</b>						
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	2 (1.0)		1 (0.04)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	2 (8.0)		1 (3.8)
<b>- Open eye</b>						
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	1 (0.5)	1 (0.5)	1 (0.5)	1 (0.4)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	1 (3.8)	1 (4.0)	1 (4.2)	1 (4.5)
<b>Total external variations</b>						
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	3 (0.6)	8 (1.1)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	1 (4.0)	3 (4.9)	7 (5.0)
<b>Individual external variations</b>						
<b>- Paw hyperflexion</b>						
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)	3 (0.6)	8 (1.1)
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	1 (4.0)	3 (4.9)	7 (5.0)

HCD 2000-2009 were provided in the original study report (n=5 studies)

HCD 2009-2016 additional data provided after the original study (n=19 studies)

For the HCD the maximum per study % value is presented.

**Table 6.8.1-22: Incidence of Visceral (Soft Tissue) Malformations**

Dose level [mg/kg bw]	0	40	100	250	HCD 2000-2009	HCD 2009-2016
Litters Evaluated	20	25	26	25	135	431
Foetuses Evaluated	184	212	207	200	1135	3642
<b>Total visceral malformations</b>						
- Foetal incidence [No. (%)]	2 (1.1)	7 (3.3)	5 (2.4)	2 (1.0)	15 (2.4)	22 (1.7)
- Litter incidence	2 (10)	6 (24)	4 (15)	2 (8.0)	14 (16.7)	21 (14.3)
<b>Individual visceral malformations</b>						
<b>- Aortic arch atresia</b>						
- Foetal incidence [N (%)]	0 (0.0)	1 (0.5)	0 (0.0)	1 (0.5)		
- Litter incidence [N (%)]	0 (0.0)	1 (4.0)	0 (0.0)	1 (4.0)		
<b>- Heart: muscular ventricular septum defect</b>						
- Foetal incidence [N (%)]	0 (0.0)	2 (0.9)	0 (0.0)	1 (0.5)		1 (0.5)
- Litter incidence [N (%)]	0 (0.0)	2 (8.0)	0 (0.0)	1 (4.0)		1 (4.3)
<b>- Small spleen</b>						
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)	2 (0.9)	1 (0.7)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)	1 (4.2)	1 (6.3)
<b>- Malpositioned kidney</b>						
- Foetal incidence [N (%)]	0 (0.0)	2 (0.9)	0 (0.0)	1 (0.5)		2 (1.1)
- Litter incidence [N (%)]	0 (0.0)	2 (8.0)	0 (0.0)	1 (4.0)		2 (9.1)

HCD 2000-2009 were provided in the original study report (n=5 studies)

HCD 2009-2016 additional data provided after the original study (n=19 studies)

For the HCD the maximum per study % value is presented.

**Table 6.8.1-23 Incidence of Visceral (Soft Tissue) Variations**

Dose level [mg/kg bw]	0	40	100	250	HCD 2000-2009	HCD 2009-2016
Litters Evaluated	20	25	26	25	135	431
Foetuses Evaluated	184	212	207	200	1135	3642
<b>Total visceral variations</b>						
- Foetal incidence [N (%)]	5 (2.7)	6 (2.8)	10 (4.8)	4 (2.0)	47 (6.3)	31 (3.7)
- Litter incidence [N (%)]	4 (20)	5 (20)	7 (27)	4 (16)	37 (38.1)	
<b>Individual visceral variations</b>						
<b>- Malpositioned carotid branch</b>						
- Foetal incidence [N (%)]	2 (1.1)	1 (0.5)	4 (1.9)	3 (1.5)	18 (5.0)	31 (3.7)
- Litter incidence [N (%)]	2 (10)	1 (4.0)	3 (12)	3 (12)	15 (28.6)	27 (31.3)
<b>- Absent lung lobe (L. inferior medialis)</b>						
- Foetal incidence [N (%)]	2 (1.1)	2 (0.9)	3 (1.4)	1 (0.5)	21 (2.3)	45 (4.5)
- Litter incidence [N (%)]	1 (5.0)	2 (8.0)	3 (12)	1 (4.0)	19 (38.1)	33 (18.8)

HCD 2000-2009 were provided in the original study report (n=5 studies)

HCD 2009-2016 additional data provided after the original study (n=19 studies)

For the HCD the maximum per study % value is presented.

**Table 6.8.1-24 Incidence of Skeletal Malformations**

Dose level [mg/kg bw]	0	40	100	250	HCD 2000- 2009	HCD 2009- 2016
Litters Evaluated	20	25	26	25	934	3642
Foetuses Evaluated	184	212	207	200	114	431
<b>Total skeletal malformations</b>						
- Foetal incidence [No. (%)]	8 (4.3)	8 (3.8)	8 (3.9)	7 (3.5)	21 (5.2)	43 (3.8)
- Litter incidence	5 (25)	7 (28)	8 (31)	6 (24)	17 (33.3)	39 (27.3)
<b>Individual skeletal malformations</b>						
<b>- Misshapen interparietal</b>						
- Foetal incidence [N (%)]	3 (1.6)	1 (0.5)	2 (1.0)	2 (1.0)	1 (0.3)	5 (0.9)
- Litter incidence [N (%)]	3 (15)	1 (4.0)	2 (7.7)	1 (4.0)	1 (2.4)	5 (7.7)
<b>- Absent lumbar vertebra</b>						
- Foetal incidence [N (%)]	0 (0)	0 (0)	1 (0.5)	1 (0.5)	2 (0.6)	2 (0.5)
- Litter incidence [N (%)]	0 (0)	0 (0)	1 (3.8)	1 (4.0)	2 (4.8)	2 (4.5)
<b>- Severely malformed sternum</b>						
- Foetal incidence [N (%)]	1 (0.5)	2 (0.9)	0 (0.0)	2 (1.0)	2 (0.5)	3 (0.6)
- Litter incidence [N (%)]	1 (5.0)	2 (8.0)	0 (0.0)	2 (8.0)	2 (4.2)	3 (5.0)
<b>- Sternebrae severely fused (bony plate); changed cartilage</b>						
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)		
<b>- Sternebrae severely fused (bony plate); unchanged cartilage</b>						1 (0.6)
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		1 (5.0)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)		
<b>- Cleft Sternum (split cartilage)</b>						
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		1 (0.5)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)		1 (4.5)
<b>- Branched rib; cartilage present</b>						
- Foetal incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.5)		7 (1.1)
- Litter incidence [N (%)]	0 (0.0)	0 (0.0)	0 (0.0)	1 (4.0)		6 (9.1)

HCD 2000-2009 were provided in the original study report (n=5 studies)

HCD 2009-2016 additional data provided after the original study (n=19 studies)

For the HCD the maximum per study % value is presented.

Table 6.8.1-25: Incidence of Skeletal Variations

Dose level [mg/kg bw]	0	40	100	250	HCD 2000-2009	HCD 2009-2016
Litters Evaluated	20	25	26	25	114	431
Foetuses Evaluated	184	212	207	200	934	3642
<b>Total skeletal variations</b>						
- Foetal incidence [N (%)]	180 (98)	208 (98)	200 (97)	197 (99)	887 (98.3)	3486 (100)
- Litter incidence [N (%)]	20 (100)	25 (100)	26 (100)	25 (100)	114 (100)	431 (100)
<b>Individual skeletal variations</b>						
<b>- Incomplete ossification of hyoid; cartilage present</b>						
- Foetal incidence [N (%)]	76 (41)	73 (34)	80 (39)	62 (31)	212 (38.2)	1208 (45.6)
- Litter incidence [N (%)]	19 (95)	23 (92)	24 (92)	23 (92)	69 (90.2)	373 (100)
<b>- Incomplete ossification of frontal; unchanged cartilage</b>						
- Foetal incidence [N (%)]	0 (0)	1 (0.5)	0 (0)	1 (0.5)		7 (1.2)
- Litter incidence [N (%)]	0 (0)	1 (4.0)	0 (0)	1 (4.0)		6 (5.0)
<b>- Hole in the palatine shelves</b>						
- Foetal incidence [N (%)]	0 (0)	0 (0)	0 (0)	1 (0.5)	1 (0.3)	1 (0.3)
- Litter incidence [N (%)]	0 (0)	0 (0)	0 (0)	1 (4.0)	1 (2.4)	1 (2.4)
<b>- Incomplete ossification of cervical centrum; unchanged cartilage</b>						
- Foetal incidence [N (%)]	51 (28)	58 (27)	53 (26)	82 (41)	138 (35.0)	647 (37.6)
- Litter incidence [N (%)]	18 (90)	22 (88)	19 (73)	21 (84)	45 (87.8)	255 (88.5)
<b>- Incomplete ossification of cervical arch; cartilage present</b>						
- Foetal incidence [N (%)]	0 (0)	2 (0.9)	0 (0)	1 (0.5)		5 (2.4)
- Litter incidence [N (%)]	0 (0)	2 (8.0)	0 (0)	1 (4.0)		2 (5.0)
<b>- Supernumerary thoracic vertebra</b>						
- Foetal incidence [N (%)]	19 (10)	44 (21)	42 (20)	50 (25)	232 (30.4)	776 (32.8)
- Litter incidence [N (%)]	11 (55)	14 (56)	14 (54)	16 (64)	80 (92.9)	267 (85.0)
<b>- Dumbell ossification of thoracic centrum; unchanged cartilage</b>						
- Foetal incidence [N (%)]	10 (5.4)	9 (4.2)	16 (7.7)	14 (7.0)	16 (3.7)	299 (21.2)
- Litter incidence [N (%)]	7 (35)	9 (36)	14 (54)	9 (36)	14 (29.3)	165 (72.7)

Table 6.8.1-25: Incidence of Skeletal Variations (continued)

Dose level [mg/kg bw]	0	40	100	250	HCD 2000-2009	HCD 2009-2016
<b>- Bipartite ossification of thoracic centrum; dumbbell-shaped cartilage of centrum</b>						
- Foetal incidence [N (%)]	0 (0)	0 (0)	0 (0)	1 (0.5)	4 (1.5)	3 (1.1)
- Litter incidence [N (%)]	0 (0)	0 (0)	0 (0)	1 (4.0)	4 (12.5)	3 (9.5)
<b>- Incomplete ossification of lumbar arch; cartilage present</b>						
- Foetal incidence [N (%)]	19 (10)	23 (11)	29 (14)	26 (13)	32 (9.1)	88 (12.7)
- Litter incidence [N (%)]	12 (60)	12 (48)	11 (42)	15 (60)	17 (41.5)	47 (56.5)
<b>- Misshapen sacral vertebrae</b>						
- Foetal incidence [N (%)]	15 (8.2)	13 (6.1)	15 (7.2)	12 (6.0)	41 (6.3)	217 (12.4)
- Litter incidence [N (%)]	9 (45)	8 (32)	7 (27)	10 (40)	33 (39.0)	159 (72.4)
<b>- Incomplete ossification of sternebra; unchanged cartilage</b>						
- Foetal incidence [N (%)]	81 (44)	97 (46)	79 (38)	73 (37)	263 (41.5)	1270 (51.8)
- Litter incidence [N (%)]	17 (85)	23 (92)	22 (85)	19 (76)	80 (95.8)	376 (100)
<b>- Misshapen sternebrae; unchanged cartilage</b>						
- Foetal incidence [N (%)]	34 (18)	22 (10)	21 (10)	16 (8.0)	99 (16.0)	424 (18.8)
- Litter incidence [N (%)]	11 (55)	13 (52)	13 (50)	12 (48)	54 (66.7)	229 (81.0)
<b>- Supernumery rib (13<sup>th</sup>); cartilage present</b>						
- Foetal incidence [N (%)]	98 (53)	142 (67)	129 (62)	149 (75)	380 (60.4)	2273 (73.7)
- Litter incidence [N (%)]	20 (100)	24 (96)	24 (92)	24 (96)	77 (97.6)	409 (100)
<b>- Supernumery rib (13<sup>th</sup>); cartilage not present</b>						
- Foetal incidence [N (%)]	59 (23)	57 (27)	53 (26)	45 (23)	171 (29.6)	968 (34.1)
- Litter incidence [N (%)]	16 (80)	20 (80)	20 (77)	22 (88)	69 (90.2)	364 (96.2)

HCD 2000-2009 were provided in the original study report (n=5 studies)

HCD 2009-2016 additional data provided after the original study (n=19 studies)

For the HCD the maximum per study % value is presented.

**CONCLUSION:** There were no external or visceral malformations or variants which were considered related to administration of NOA449410.

For any findings observed at a higher frequency in the high dose group on a foetal or litter incidence when compared to the concurrent control, a comparison with HCD was conducted. The % foetal or litter incidences were within or comparable with the relevant HCD collated at [REDACTED], [REDACTED] from 5 studies (2000 to 2009) and 19 studies (2009 to 2016) in the New Zealand White rabbit and consistent with further external literature regarding the interpretation of pre-natal rabbit developmental toxicity studies.

## REFERENCES:

- Carney E. and Kimmel C. (2007). *Interpretation of skeletal variations for human risk assessment: Delayed ossification and wavy ribs. Birth Defects Res B Dev Reprod Toxicol.* 80:473-496.
- Chahoud I. and Paumgartten J. (2009). *Dose-response relationships of rat foetal skeleton variations: Relevance for risk assessment. Environ Res.* 109: 922-929.
- Daston G.P. and Beekhuijzen M. (2018). *Is omphalocele a non-specific malformation in New Zealand White rabbits? Reproductive Toxicology.* 78:29-39.
- Kimmel C., Garry M., and DeSesso J. (2014). *Relationship between bent long bones, bent scapulae, and wavy ribs: Malformations or variations? Birth Defects Res B Develop Reprod Toxicol.* 101:379-392.
- Makris S., Solomon H., Clark R., Shiota K., Barbellion S., Buschmann J., Ema M., Fujiwara M., Grote K., Hazelden K., Hew K., Horimoto M., Ooshima Y., Parkinson M., and Wise D. (2009). *Terminology of developmental abnormalities in common laboratory mammals (Version 2). Birth Defects Research, Part B.* 86:227-327.
- MARTA (1996). *MARTA, Historical control data, in: R.D. Hood (Ed.), Handbook of Developmental Toxicology, CRC Press, London, United Kingdom, pp.713-733.*

██████████ (2009). Reg.No. 5069089 (Metabolite of BAS 700 F): Prenatal Developmental Toxicity Study in New Zealand White Rabbits Oral Administration (Gavage). ██████████, ██████████. ██████████. Laboratory Report No. 40R0451/07118. ██████████ DocID 2009/1072507. 14 October 2009. Syngenta File No. NOA449410\_10005.

Solecki R., Bürgin H., Buschmann J., Clark R., Duverger M., Fialkowski O., Guittin P., Hazelden K., Hellwig J., Hoffmann E., Hofmann T., Hübel U., Khalil S., Link W., Mantovani A., Moxon M., Müller S., Parkinson M., Paul M., Paumgarten F., Pfeil R., Platzek T., Rauch-Ernst M., Scheecelenbos A., Seed J., Talsness C., Yasuda M., Younes M., and Chahoud I. (2001). Harmonisation of rat fetal skeletal terminology and classification. Report of the third workshop on terminology in developmental toxicology. Berlin 14-16 September 2000. *Reprod. Toxicol.* 15: 713-721. Wakarny J., (1971). *Congenital malformations: notes and comments*. Year Book Medical Publishers, Chicago.

The applicant provided an assessment of the external and visceral findings against additional HCD in the NZW rabbit from the same conducting laboratory between 2009 and 2016 and an assessment of the skeletal findings including a comparison with HCD. For the majority of findings, the % foetal or litter incidences were within or comparable with the relevant HCD collated at ██████████, ██████████. ██████████ from 5 studies (2000 to 2009) and 19 studies (2009 to 2016) in the New Zealand White rabbit. According to HSE, two findings (supernumerary 13<sup>th</sup> rib and umbilical hernia) warrant more attention:

- Supernumerary 13<sup>th</sup> rib with present cartilage

A statistically significant specific variation "supernumerary 13th rib with present cartilage" was observed at the high dose only when viewed on an 'affected foetus per litter' basis (see above Table 6.8.1-20).

However, HSE agrees with the applicant that this finding can be considered not treatment related for the following reasons:

- The increase was not reflected for the litter incidence which is lower for the treated groups than for the control
- There was no dose- response relationship
- The incidence was comparable with range reported in the HCD: mean 68.1%, range 50-73.9%.

Thus, the NOAEL of the study is still maintaining at

- Umbilical hernia

Umbilical hernias were observed in 2 foetuses from 2 litters at the high dose, and in 1 foetus in 1 litter in the low dose (see Table 6.8.1-21 above). No clear dose-response relationship was present as no incidence was observed at the mid-dose. However, at the high dose, the increase incidence is above the presented HCD (2 foetuses from 2 litters versus only 1 foetus in one litter in the HCD). However, it should be noted that these HCD from the conducting laboratory were derived from only 29 prenatal developmental toxicity studies in rabbit (performed between 2009 and 2016) and according to the applicant this might be represent a too small sample size for reliability estimating of the incidence of relatively rare spontaneous events as umbilical hernia. Based on MARTA database, it seems indeed that umbilical hernias (when combined with omphaloceles) are the most common external malformations observed in NZW rabbits prenatal developmental toxicity studies: the cumulative incidence of these malformations being 0.64% (litter incidence) and 0.11% (foetal incidence). Unfortunately, the MARTA database compiles data set for only a period of 1989 to 1992, which is largely prior to the date of the study of ██████████ (2009), leading the interpretation of this finding difficult. HSE performed a QSAR analysis using Derek Nexus v.2.1.1 and no alerts (including reprotoxicity end-point) were highlighted.

HSE is of opinion that in the absence of clear dose-response relationship (no observation in the mid-dose group) and in the absence of any other effects in the foetuses following administration of the test item, it can be considered that the incidence of umbilical hernia observed at the high dose might be of spontaneous origin.

The NOAEL of the study is still proposed at 250 mg/kg bw/d.



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**CONCLUSION:**

In a GLP and OECD compliant PNDT study in NZW rabbits, in which pregnant females were given oral gavage doses of 0, 40, 100 and 250 mg/kg bw/d CSAA798670, there were no treatment-related effects on the maternal animals. Doses were selected on the basis of a range-finding study showing significant toxicity from 500 mg/kg bw/d. No treatment-related developmental effects were noted.

A statistically significant increase in the skeletal variation "supernumerary 13th rib with present cartilage" was observed at the high dose only when viewed on an 'affected foetus per litter' basis. However, the finding was considered not treatment related for the following reasons:

- The increase was not reflected for the litter incidence which was lower in the treated group compared to the control;
- There was no dose- response relationship;
- The incidence was comparable with the range reported in the laboratory HCD from 2000 to 2016: mean 68.1%, range 50-73.9%.

Umbilical hernias (a malformation) were observed in 2 foetuses from 2 litters at the high dose, and in 1 foetus in 1 litter in the low dose. No clear dose-response relationship was present as no incidence was observed at the mid-dose. However, at the high dose, the increased incidence was above the laboratory HCD (only 1 foetus in one litter). However, it should be noted that these HCD were derived from only 29 prenatal developmental toxicity studies (performed between 2009 and 2016) and according to the applicant this might be represent a too small sample size for reliably estimating the incidence of a relatively rare spontaneous event such as umbilical hernia. Based on the much larger MARTA HCD database, it seems that umbilical hernias are the most common external malformations observed in NZW rabbits prenatal developmental toxicity studies: the cumulative incidence of these malformations being 0.64% (litter incidence) and 0.11% (foetal incidence). Unfortunately, the MARTA database presented a data set for the period of 1989 to 1992, which is largely prior to the date of the study of [REDACTED] (2009), making the interpretation of this finding difficult. A QSAR analysis using Derek Nexus v.2.1.1 showed and no alerts for reprotoxicity end-points.

Overall, in the absence of clear dose-response relationship and in the absence of any other effects in the foetuses following administration of the test item, it can be considered that the incidence of umbilical hernia observed at the high dose might be of spontaneous origin.

As a result, HSE agrees with the EU evaluation, that CSAA798670 did not cause any maternal toxicity or developmental toxicity up to the top dose of 250 mg/kg bw/d. A **NOAEL of 250 mg/kg bw/d was therefore identified for maternal and developmental toxicity from the study.**

([REDACTED], 2018; [REDACTED], 2009)

**Summary of toxicity data on metabolite CSAA798670****Summary of toxicity studies and derivation of dietary reference values for metabolite CSAA798670 glucuronide/sulphate**

CSAA798670 is a common metabolite to a number of SDHI molecules and toxicity studies performed on this metabolite have been assessed during the peer-review of other pyrazole active substances (sedaxane, fluxapyroxade, benzovendiflupyr).

Metabolite CSAA798670 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, CSAA798670. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

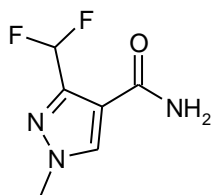
Overall, a number of GLP and OECD compliant toxicity studies (acute oral toxicity study, 28-d study, 90-d study, rabbit PNDT study) and standard in vitro genotoxicity assays are available on metabolite CSAA798670. The metabolite did not show any genotoxic potential in the standard three in vitro genotoxicity tests. It was of low acute oral toxicity ( $LD_{50} > 2000$  mg/kg bw) in the rat and did not show any adverse effects up to the limit dose of 1000 mg/kg bw/d in a 28-day and 90-day study in the rat. In addition, no maternal toxicity or developmental toxicity was seen in rabbits up to the top dose of 250 mg/kg bw/d. However, significant maternal toxicity was noted at doses of 500 mg/kg bw/d and above in a range-finding study in pregnant rabbits.

The table below summarises the available studies on CSAA798670:

Study & Acceptability	Result	Reference
Screening Acute Oral Toxicity Study in the SD Rat. GLP  <i>Acceptable</i>	$LD_{50} > 2000$ mg/kg bw	██████████ (2008). Report No. ██████████ 2364/0169. Syngenta File No. CA4312/0003
Ames test in bacteria GLP, OECD 471  <i>Acceptable</i>	Negative up to limit concentration	██████████ (2007). Report No. RCC 1077403. Syngenta File No. SYN520453/0096
Chromosome Abberation Test in Human Lymphocytes <i>In Vitro</i> . GLP, OECD 473  <i>Acceptable</i>	Negative up to limit concentration	██████████ (2009). Report No. Harlan 1266902. Syngenta File No. NOA449410_10001
Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells. GLP, OECD 476  <i>Acceptable</i>	Negative up to limit concentration	██████████ (2009). Report No. Harlan 1266901. Syngenta File No. NOA449410/10000
28-Day Oral (Dietary) Toxicity Study in the Wistar Rat. GLP, OECD 407  <i>Acceptable</i>	NOAEL = $>1000$ mg/kg/d in both sexes No adverse toxicological findings up to the top dose of 1000 mg/kg bw/d	██████████ (2010). Report No. ██████████ ██████████. Syngenta File No. NOA449410_10003
90-day Oral (dietary) Toxicity Study in Wistar Rats GLP, OECD 407  <i>Acceptable</i>	NOAEL = $>1000$ mg/kg/d No adverse toxicological findings up to the top dose of 1000 mg/kg bw/d	██████████ <i>et al.</i> , (2009). Report No. ██████████ 50S0451/07119. Syngenta File No. R958945_11273
Prenatal Developmental Toxicity Study in New Zealand White Rabbits GLP, OECD 414  <i>Acceptable</i>	Maternal & developmental NOAEL = 250 mg/kg bw/d (highest dose tested).	██████████ <i>et al.</i> , (2009). Report No. ██████████ 2009/1072507. Syngenta File No. NOA449410_10005

In conclusion, CSAA798670 is of significantly lower toxicity than the parent substance (parent 28-day rat NOAEL = 43/40 mg/kg bw/d based on liver effects and parent 90-day rat NOAEL = 18.6/21.6 mg/kg bw/d based on liver effects). From a toxicological point of view, CSAA798670 glucuronide/sulphate might not be needed to be included in the residue definition for risk assessment (RD-RA). Alternatively, if inclusion is required from a residue perspective, the parent dietary reference values could be used on a conservative basis.

At EU level, a specific ADI of 0.25 mg/kg bw/d was derived from the NOAEL of 250 mg/kg bw/d from the rabbit PNDT study with an UF of 1000 (extra assessment factor of 10 to account for the limited database, as no long-term, multigeneration or rat developmental toxicity studies are available). An ARfD was not established, but if required, it could be set at the same level of the ADI. HSE is of the view, that if this metabolite needs to be taken into account in the dietary risk assessment, then it would be more appropriate to include it in the RD-RA together with the parent (and applying the parent reference values) rather than setting a separate RD-RA and applying the metabolite-specific ADI of 0.25 mg/kg bw/d.

**B.6.8.1.2. Metabolite SYN508272 glucuronide/sulphate**

3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide (SYN508272 aglycon)

Metabolite SYN508272 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN508272. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

Toxicity (acute oral and 28-day study) and genotoxicity studies have been conducted with the metabolite SYN508272. The same metabolite has also been tested in studies undertaken by [REDACTED] under the test substance code M700F007 and hence the summaries for studies conducted by [REDACTED] (for which Syngenta has co-ownership) refer to the test substance as M700F007.

**Acute oral toxicity study**

<b>Report:</b>	K-CA 5.8.1/09 [REDACTED], (2009). Reg. No. 5621781 (Metabolite of BAS 700 F): Acute Oral Toxicity Study in Rats. [REDACTED] [REDACTED], Laboratory Report No. 2009/1084176, 02 October 2009. Unpublished. ([REDACTED]-Project No.: 10A0432/099058 [REDACTED]-Project No.: [REDACTED]) Syngenta File No. SYN508272_10903.
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**Guidelines:** Acute Oral Toxicity (rat): OECD Test Guideline 423 (2001); EPA OPPTS 870.1100 (2002); EC 440/2008 (2008)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

**HSE comment:** the study was considered acceptable

**EXECUTIVE SUMMARY**

In an acute oral toxicity study performed according to the Acute Toxic Class method, doses of 2000 and 500 mg/kg bw of the test item Reg. No. 5621781 (Metabolite of BAS 700 F) in 0.5% aqueous CMC water were administered to test groups of three fasted female Wistar rats (2000 mg/kg bw in 3 females, 500 mg/kg bw in 6 females) by gavage in a sequential manner.

All animals of the 2000 mg/kg bw test group were found dead within day 1 after the administration (two animals at hour 5 and one animal at study day 1).

No mortality occurred in the six females administered 500 mg/kg bw.

The combined acute oral LD<sub>50</sub> was calculated to be > 500 mg/kg bw < 2000 mg/kg bw.

Clinical observation in the 2000 mg/kg bw test group revealed impaired and poor general state, dyspnoea, ataxia, tremor, staggering, twitching, abdominal position and piloerection at hour 0 until hour 5 after the administration.

No clinical signs and findings were observed in the animals of the first 500 mg/kg bw group.

In contrast, one animal of the second 500 mg/kg bw group showed impaired general state, dyspnoea, piloerection, chromodacryorrhea and reduced feces from hour 4 until study day1.

The mean body weight of the surviving animals increased throughout the study period within the normal range.

There were no macroscopic pathological findings in the animals of the 500 mg/kg bw test groups sacrificed at the end of the observation period and in the animals which died after the administration of 2000 mg/kg bw.

**Under the conditions of this study, the acute oral median lethal dose (LD<sub>50</sub>) of the test item, Reg. No. 5621781 (Metabolite of BAS 700 F), was greater than 500 mg/kg bw and lower than 2000 mg/kg bw in female Wistar rats.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	Reg.No. 5621781 (Metabolite of BAS 700 F)
<b>Description:</b>	Solid/ white
<b>Lot/Batch number:</b>	L81-1 08
<b>Purity:</b>	99.4% (± 1.0%)
<b>CAS#:</b>	Not indicated
<b>Expiry date::</b>	Stability under storage conditions over the study period was guaranteed by the sponsor, and the sponsor holds the responsibility.

**Vehicle and/or positive control:** 0.5% cleaned sodium carboxymethylcellulose solution in doubly distilled water

<b>Test Animals:</b>	
<b>Species</b>	Rat (female)
<b>Strain</b>	Wistar / Cri:WI (Han)
<b>Age/weight at dosing</b>	Young adult rats, approximate 10 weeks old / 187-205 g
<b>Source</b>	
<b>Housing</b>	Individual caging
<b>Acclimatisation period</b>	At least 5 days
<b>Diet</b>	Animals received VRF1 (P); ) <i>ad libitum</i>
<b>Water</b>	Tap water <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 20 - 24 °C Humidity: 20 – 80 % Air changes: Not indicated. Photoperiod: 12 hours daily, from 6.00 a.m. to 6.00 p.m.

### Study Design and Methods:

<b>In-life dates:</b>	Experimental Starting Date	04 August 2009
	Experimental Completion Date	30 September 2009

### Animal assignment and treatment:

Feed was withdrawn from the animals at least 16 hours before test item administration, but water was available *ad libitum*. Animals were treated with a single oral (gavage) dose of Reg. No. 5621781 (Metabolite of BAS 700 F). The starting dose was 2000 mg/kg bw. Because all animals died the dose was lowered subsequently to 500 mg/kg bw. All animals survived, thus a second group of animals was dosed with 500 mg/kg bw. The dosing was followed by a 14 day observation period.

All surviving animals were observed individually after dosing several times a day on the day of dosing and at least once a day for 14 days thereafter.

The body weights were recorded on Days 0 (beginning of the experiment), 7 and 14.

All animals were euthanised at the end of the observation period by CO<sub>2</sub>-inhalation in a chamber with increasing concentrations over time. After examination of the external appearance necropsy with gross-pathology examination was performed on the last day of observation and of dead animals as soon as possible after death. No histological examinations were performed.

**Statistics:** Not performed.

## RESULTS

**Mortality:** All animals of the 2000 mg/kg bw test group were found dead within 1 day after the administration (two animals at hour 5 and one animal at study day 1).

No mortality occurred in the six females administered 500 mg/kg bw.

**Table 6.8.1-27: Acute oral toxicity of Reg. No. 5621781 (Metabolite of BAS 700 F) in the rat, application scheme and mortality data**

Animal Number	Dosage [mg/kg body weight]	Dose volume [mL/kg bw]	Viability/Mortality
R102	2000	5	Died 5 hours after dosing
R103	2000	5	Died 1 day after dosing
R104	2000	5	Died 5 hours after dosing
R218	500	5	Survived
R219	500	5	Survived
R220	500	5	Survived
R250	500	5	Survived
R251	500	5	Survived
R252	500	5	Survived

**Clinical observations:** Clinical observation in the 2000 mg/kg bw test group revealed impaired and poor general state, dyspnoea, ataxia, tremor, staggering, twitching, abdominal position and piloerection at hour 0 until hour 5 after the administration.

No clinical signs and findings were observed in the animals of the first 500 mg/kg bw group. In contrast, one animal of the second 500 mg/kg bw group showed impaired general state, dyspnoea, piloerection, chromodacryorrhea and reduced feces from hour 4 until study day 1.

**Bodyweight:** The mean body weight of the surviving animals increased throughout the study period within the normal range.

**Necropsy:** There were no macroscopic pathological findings in the animals of the 500 mg/kg bw test groups sacrificed at the end of the observation period and in the animals which died after the administration of 2000 mg/kg bw.

## CONCLUSION:

The potential acute oral toxicity of metabolite SYN508272 was investigated in a GLP and OECD (423) compliant study, according to the Acute Toxic Class method. Doses of 2000 and 500 mg/kg bw of the test

item were administered to groups of three female Wistar rats (2000 mg/kg bw in 3 females, 500 mg/kg bw in 6 females) by gavage in a sequential manner. All animals of the 2000 mg/kg bw test group were found dead within day 1 after the administration (two animals at hour 5 and one animal at study day 1). No mortality occurred in the six females administered 500 mg/kg bw.

Clinical observation in the 2000 mg/kg bw test group revealed impaired and poor general state, dyspnoea, ataxia, tremor, staggering, twitching, abdominal position and piloerection up to 5 hours after administration. No clinical signs and findings were observed in the animals of the first 500 mg/kg bw group. In contrast, one animal of the second 500 mg/kg bw group showed impaired general state, dyspnoea, piloerection, chromodacryorrhea and reduced faeces from 4 hours post-dosing until study day 1. The mean body weight of the surviving animals increased throughout the study period within the normal range. There were no macroscopic pathological findings in the animals of the 500 mg/kg bw test groups sacrificed at the end of the observation period and in the animals which died after the administration of 2000 mg/kg bw.

As a result, HSE agrees with the EU evaluation, that the **acute oral LD<sub>50</sub> of SYN508272 was > 500 mg/kg bw and < 2000 mg/kg bw, triggering classification in Category 4 under Reg 1272/2008**. Based on these results, SYN508272 appears more acutely toxic than parent (acute oral LD<sub>50</sub> > 5000 mg/kg bw).

(██████████, 2009)

#### Ames test

<b>Report:</b>	K-CA 5.8.1/10 ██████████ (2014a). SYN508272: Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1555901, issue date 13 March 2014. Unpublished. Syngenta File No. SYN508272_10908.
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**Guidelines:** Reverse Mutation Test Using Bacteria. OECD 471 (1997): OPPTS 870.5100 (1998): EC 440/2008 B.13/14 (2008)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

**EFSA Request for additional information (February 2018), Question 38:** Applicant to submit the summary tables of the results regarding the Ames test and the cell mutation assay in mammalian cells with SYN508272.

Additional summary tables have been included to the OECD summaries of the Ames test and the cell mutation assay in mammalian cells with SYN508272.

#### **EXECUTIVE SUMMARY**

This study was performed to investigate the potential of SYN508272 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA1535, TA1537, TA98, and TA100, and the Escherichia coli strains WP2 uvrA pKM101 and WP2 pKM101.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation in both independent experiments. No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN508272 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance of 2.0 fold over concurrent control. Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

**In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test substance did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, SYN508272 is considered to be non-mutagenic in this Salmonella typhimurium and Escherichia coli reverse mutation assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN508272
<b>Description:</b>	Pale brown
<b>Lot/Batch number:</b>	MES 114/2
<b>Purity</b>	94 % (estimated error $\pm$ 5 %)
<b>Cas#:</b>	Not reported
<b>Expiry date:</b>	30 June 2016

<b>Control Materials:</b>	
<b>Negative:</b>	Concurrent untreated and solvent controls were performed
<b>Solvent control (final concentration):</b>	100µl/plate
<b>Positive control:</b>	Nonactivation: Sodium azide 10 µg/plate TA100, TA1535 4-nitro-o-phenylene-diamine, 50 µg/plate TA 1537, 10 µg/plate TA98 methyl methane sulfonate 2 µL/plate WP2 (pKM101), WP2 <i>uvrA</i> (pKM101)
	Activation: 2-Aminoanthracene 2.5 µg/plate TA 1535, TA 1537, TA100, TA98 10 µg/plate WP2 (pKM101), WP2 <i>uvrA</i> (pKM101)

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

- 8 mM MgCl<sub>2</sub>
- 33 mM KCl
- 5 mM Glucose-6-phosphate
- 4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment the S9 mix was stored in an ice bath.

**Test organisms:***S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

*E. coli* strains

X	WP2 (pKM101)	X	WP2 <i>uvrA</i> (pKM101)						
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Properly maintained?

☒

Yes

☐

No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?☒

Yes

☐

No

**Test compound concentrations used**

The test item was tested at the following concentrations:

Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

**Study Design and Methods:****In-life dates:** Start: 20 March 2013 End: 16 April 2013**TEST PERFORMANCE****Preliminary Cytotoxicity Assay:** Not performed.

Type of Bacterial assay:

- X standard plate test (pre-experiment/experiment I; –S9, +S9)
- X pre-incubation (60 minutes) (second experiment ; –S9, +S9)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Protocol:**

For each strain and dose level including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL Test solution at each dose level, solvent (negative control) or reference mutagen solution (positive control),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL Bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution, 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspensions were mixed in a test tube and shaken at 37° C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45° C) was added to each tube. The mixture was poured on selective agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37° C in the dark .



\* Substitution buffer: 7 parts of the 100 mM sodium-ortho-phosphate-buffer pH 7.4 with 3 parts of KCl solution 0.15 M

**Statistical analysis:** None – see Evaluation Criteria below.

**Evaluation criteria:** A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control is observed (1).

A concentration dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (6).

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A concentration dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

## RESULTS

**Preliminary cytotoxicity assay:** Not performed.

**Mutagenicity assay:** The test substance SYN508272 was assessed for its potential to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and the *E. coli* strains WP2 *uvrA* pKM101 and WP2 pKM101.

The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test substance was tested at the following concentrations in:

Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

No precipitation of the test item occurred up to the highest investigated concentration.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN508272 at any concentration, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance of 2.0 fold over concurrent control.

The data of our historical control range were slightly exceeded in strain WP2pKM 101 in the absence of metabolic activation in experiment I (untreated control) and in experiment II (untreated and solvent control). Since this deviation is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies.

Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

**Table 6.8.1-28: Results Obtained in the Absence and Presence of Metabolic Activation:****Experiment 1**

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)						
			TA 1535	TA 1537	TA 98	TA 100	WP2 pkm 101	WP2 uvrA pKM101	WP2pKm 101*
Without Activation	DMSO		14 ± 2	9 ± 3	20 ± 7	88 ± 8	261 ± 13	421 ± 26	
	Untreated		12 ± 3	10 ± 5	27 ± 7	100 ± 8	310 ± 6	413 ± 33	
	SYN508272	3 µg	16 ± 3	8 ± 2	38 ± 5	88 ± 29	286 ± 19	400 ± 3	
		10 µg	16 ± 2	11 ± 4	19 ± 2	99 ± 10	279 ± 13	379 ± 17	
		33 µg	18 ± 5	9 ± 1	27 ± 4	96 ± 8	283 ± 5	398 ± 29	
		100 µg	12 ± 2	8 ± 3	21 ± 1	89 ± 17	246 ± 15	424 ± 13	
		333 µg	16 ± 0	8 ± 2	23 ± 4	91 ± 7	291 ± 22	371 ± 32	
		1000 µg	14 ± 6	11 ± 3	24 ± 2	80 ± 3	285 ± 9	397 ± 23	
		2500 µg	14 ± 2	7 ± 2	29 ± 4	96 ± 4	283 ± 20	395 ± 9	
		5000 µg	19 ± 6	7 ± 5	18 ± 4	93 ± 7	262 ± 1	373 ± 17	
	NaN3	10 µg	1925 ± 198			1841 ± 43			
	4-NOPD	10 µg			267 ± 3				
				57 ± 4					
	4-NOPD MMS	50 µg 2.0 µL					3580 ± 98	3925 ± 46	
With Activation	DMSO		16 ± 4	19 ± 5	40 ± 2	131 ± 6	303 ± 33	408 ± 18	243 ± 1
	Untreated		17 ± 4	15 ± 2	37 ± 10	145 ± 6	321 ± 14	397 ± 45	242 ± 2
	SYN508272	3 µg	16 ± 9	25 ± 3	39 ± 4	129 ± 11	305 ± 8	392 ± 25	238 ± 11
		10 µg	25 ± 5	18 ± 4	38 ± 11	126 ± 13	285 ± 24	433 ± 19	241 ± 17
		33 µg	17 ± 9	25 ± 3	32 ± 9	126 ± 21	305 ± 8	435 ± 4	241 ± 29
		100 µg	19 ± 3	22 ± 2	36 ± 3	137 ± 12	271 ± 17	420 ± 3	250 ± 3
		333 µg	19 ± 4	20 ± 5	41 ± 10	124 ± 6	287 ± 36	394 ± 6	230 ± 11
		1000 µg	19 ± 4	17 ± 3	45 ± 11	122 ± 9	298 ± 35	430 ± 22	216 ± 20
		2500 µg	20 ± 3	19 ± 4	45 ± 2	126 ± 8	310 ± 17	436 ± 16	218 ± 8
		5000 µg	18 ± 4	15 ± 4	43 ± 4	135 ± 16	301 ± 3	410 ± 10	231 ± 10
	2-AA	2.5 µg	458 ± 5	565 ± 7	2526 ± 178	4187 ± 268			484 ± 67
							560 ± 50	2704 ± 67	
	2-AA	10.0 µg							

## Key to Positive Controls

NaN3	Sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sultanate

\*Repeated WP2 pKM101 experiment, as the positive control fell slightly short of the positive response threshold of 2.0 fold solvent control (1.9)

**Table 6.8.1-29: Results Obtained in the Absence and Presence of Metabolic Activation:****Experiment 2**

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)						
			TA 1535	TA 1537	TA 98	TA 100	WP2 pkm 101	WP2 uvrA pKM101	
Without Activation	DMSO		20 ± 5	10 ± 2	34 ± 7	96 ± 4	304 ± 36	357 ± 41	
	Untreated		22 ± 4	13 ± 4	24 ± 4	120 ± 18	357 ± 21	374 ± 17	
	SYN508272	33 µg	17 ± 2	10 ± 5	24 ± 2	114 ± 11	261 ± 19	408 ± 17	
		100 µg	20 ± 1	7 ± 3	30 ± 5	104 ± 6	249 ± 19	360 ± 5	
		333 µg	23 ± 3	9 ± 2	24 ± 3	105 ± 9	274 ± 9	325 ± 32	
		1000 µg	17 ± 3	10 ± 3	27 ± 11	99 ± 8	247 ± 9	337 ± 33	
		2500 µg	19 ± 4	11 ± 3	34 ± 4	94 ± 24	245 ± 32	337 ± 23	

	NaN3	5000 µg 10 µg	18 ± 6 2783 ± 26	14 ± 1	26 ± 4	108 ± 21 1968 ± 125	256 ± 20	325 ± 13
	4-NOPD	10 µg			282 ± 61			
	4-NOPD MMS	50 µL 2.0 µL		63 ± 4			3422 ± 103	2752 ± 94
With	DMSO		18 ± 3	21 ± 2	43 ± 9	150 ± 15	259 ± 23	367 ± 4
Activation	Untreated		12 ± 6	30 ± 5	46 ± 3	154 ± 4	327 ± 39	290 ± 72
	SYN508272	33 µg	15 ± 4	16 ± 5	48 ± 10	141 ± 14	275 ± 7	405 ± 14
		100 µg	22 ± 5	19 ± 2	46 ± 3	158 ± 21	244 ± 23	363 ± 36
		333 µg	22 ± 11	18 ± 2	38 ± 8	149 ± 29	246 ± 6	334 ± 15
		1000 µg	19 ± 3	16 ± 6	52 ± 5	138 ± 15	258 ± 17	366 ± 38
		2500 µg	19 ± 2	21 ± 2	46 ± 3	140 ± 5	244 ± 39	359 ± 28
		5000 µg	22 ± 4	24 ± 4	55 ± 9	127 ± 8	280 ± 36	377 ± 12
	2-AA	2.5 µg	652 ± 39	502 ± 32	2551 ±17	3787 ± 271		
	2-AA	10.0 µg					2810 ± 8	1670 ± 246
Key to Positive Controls								
	NaN3	sodium azide						
	2-AA	2-aminoanthracene						
	4-NOPD	4-nitro-o-phenylene-diamine						
	MMS	methyl methane sulfonate						

## CONCLUSION:

In a GLP and OECD compliant Ames test, the potential of SYN508272 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strains WP2 uvrA (pKM 101), and WP2 (pKM 101), was investigated.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation in both independent experiments. No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN508272 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance of 2.0 fold over concurrent control. Appropriate reference mutagens were used as positive controls and they showed a distinct increase of revertant colonies.

As a result, HSE agrees with the EU evaluation, that **SYN508272 is not mutagenic in bacteria when tested up to the limit concentration.**

(██████████, 2014a)

### In vitro chromosome aberration test

<b>Report:</b>	K-CA 5.8.1/11 ██████████ (2013a). SYN508272: Chromosome Aberration Test in Human Lymphocytes <i>In Vitro</i> . Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1555902, issue date: 18 December, 2013. Unpublished. Syngenta File No. SYN508272_10904.
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**Guidelines:** Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (1997); OPPTS 870.5375 (1998); EC 440/2008 B10 (2008)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

This *in vitro* assay was performed to assess the potential of SYN508272 to induce structural chromosomal aberrations in cultured human lymphocytes in the absence and presence of an exogenous metabolic activation system (liver S9 mix from phenobarbital/ $\beta$ -naphthoflavone treated male rats).

In each experimental group two parallel cultures were analysed. Per culture at least 100 metaphases were evaluated for structural chromosomal aberrations, except for the positive controls in Experiment II without S9 mix, where only 50 metaphases were evaluated.

The highest applied concentration in this study (1860.0  $\mu\text{g/mL}$  of the test substance, approx. 10 mM) was chosen with regard to the molecular weight and the purity (94 %) of the test substance and with respect to the current OECD Guideline 473.

Concentration selection for the cytogenetic experiments was performed considering the toxicity data and test substance precipitation and in accordance with OECD Guideline 473.

In Experiment I in the absence and presence of S9 mix and in Experiment II in the presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In Experiment II in the absence of S9 mix, and under long exposure conditions, concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage. However, the mitotic index was markedly reduced to 53.9 % and 57.3 % of control at the two highest evaluated concentrations.

In Experiment I either with or without metabolic activation, no clastogenicity was observed at the concentrations evaluated. In Experiment II in the absence of S9 mix one single statistically significant increase in chromosomal aberrations (4.5 % aberrant cells, excluding gaps) was observed after treatment with 1062.9  $\mu\text{g/mL}$ . Results at this concentration clearly exceeded the historical control data range (0.0 – 2.5 % aberrant cells, excluding gaps). In Experiment II in the presence of S9 mix one single statistically significant increase in chromosomal aberrations (4.3 % aberrant cells, excluding gaps) was observed after treatment with 347.1  $\mu\text{g/mL}$ . Results at this concentration slightly exceeded the historical control data range (0.0 – 3.5 % aberrant cells, excluding gaps). However, in the presence of S9 mix the effect was not reproducible in the two independent experiments performed. The response seen at 347.1  $\mu\text{g/mL}$ , in the presence of S9 mix, was not part of a concentration-related response.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ( $p < 0.05$ ) in cells with structural chromosome aberrations.

**In conclusion, it can be stated that under the experimental conditions reported, the test substance induced structural chromosomal aberrations in human lymphocytes *in vitro*. Therefore, SYN508272**

is considered to be clastogenic in this chromosome aberration test, when tested up to precipitating or the highest evaluable concentrations.

## MATERIALS AND METHODS

### Materials:

Test Material:	SYN508272
Description:	-
Lot/Batch number:	MES 114/2
Purity:	94%, estimated error $\pm$ 5 %
CAS#:	Not reported
Expiry date:	June 30 2016

Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 770.0 µg/mL (Experiment I), 660.0 µg/ml (Experiment II)
	Presence of S9 mix: Cyclophosphamide 7.5 µg/mL (Experiment I), 2.5 µg/ml (Experiment II)

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM	MgCl <sub>2</sub>
33 mM	KCl
5 mM	glucose-6-phosphate
4 mM	NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment, the S9 mix was stored in an ice bath. The S9 mix preparation was performed according to Ames *et al.*

### Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No

Periodically checked for karyotype stability?		Yes		No
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X indicates those that apply

**Test compound concentrations used:**

Absence of S9 mix	Experiment 1 Experiment 2	607.3, 1062.9, 1860.0 µg/mL 347.1, 607.3, 1062.9, 1860.0 µg/mL
Presence of S9 mix	Experiment 1 Experiment 2	347.1, 607.3, 1062.9, 1860.0 µg/mL 198.3, 347.1, 607.3, 1062.9 µg/mL

**TEST PERFORMANCE**

**Preliminary Cytotoxicity Assay:** Not performed.

**Cytogenetic Assay:**

Cell exposure time:		Test Material	Solvent Control	Positive Control
- S9 mix	Experiment 1	4h	4h	4h
+ S9 mix		4h	4h	4h
- S9 mix	Experiment 2	22h	22h	22h
+ S9 mix		4h	4h	4h

<b>Spindle inhibition:</b>	
Inhibitor used/ concentration:	Colcemid 0.2 µg/mL
Administration time:	3 hours (before cell harvest)

Cell harvest time after termination of treatment:	Test Material	Solvent Control	Positive Control
- S9 mix (4 hour treatment)	18h	18h	18h
+ S9 mix (4 hour treatment)	18h	18h	18h
- S9 mix (22 hour treatment)	0h	0h	0h

**Details of slide preparation**

**Exposure time 4 hours:** About 48 h after seeding for each test group 2 blood cultures (10 mL each) were set up in parallel in 25 cm<sup>2</sup> cell culture flasks (Nunc GmbH & Co. KG, 65203 Wiesbaden, Germany). The culture medium was replaced with serum-free medium containing the test substance. For the treatment with metabolic activation 50 µL S9 mix per mL medium were used. Concurrent solvent and positive controls were performed. After 4 h the cells were spun down by gentle centrifugation for 5 minutes (approx. 900 x g). The supernatant with the dissolved test substance was discarded and the cells were re-suspended in "saline G". The washing procedure was repeated once as described.

The "saline G" solution was composed as follows (per litre):

NaCl      8000 mg  
 KCl       400 mg  
 glucose•H<sub>2</sub>O            1100 mg  
 Na<sub>2</sub>HPO<sub>4</sub>•2H<sub>2</sub>O        192 mg  
 KH<sub>2</sub>PO<sub>4</sub>   150 mg  
 pH was adjusted to 7.2

After washing the cells were re-suspended in complete culture medium and cultured until preparation.

**Exposure time 22 hours (without S9 mix):** About 48 h after seeding for each test group 2 blood cultures (10 mL each) were set up in parallel in 25 cm<sup>2</sup> cell culture flasks (Nunc GmbH & Co. KG, 65203 Wiesbaden, Germany). The culture medium was replaced with complete medium (with 10 % FBS) containing the test substance without S9 mix. The culture medium at continuous treatment was not changed until preparation of the cells. Concurrent solvent and positive controls were performed.

All cultures were incubated at 37 °C in a humidified atmosphere with 5.5 % CO<sub>2</sub> (94.5 % air).

Three hours before harvesting, colcemid (Fluka, 89203 Neu-Ulm, Germany) was added to the cultures (final concentration 0.2 µg/mL). The cultures were harvested by centrifugation for 5 minutes (approx. 900 x g) 22 h after beginning of treatment. The supernatant was discarded and the cells were re-suspended in approximately 5 mL hypotonic solution (0.0375 M KCl). The cell suspension was then allowed to stand at 37 °C for 20 minutes. After removal of the hypotonic solution by centrifugation for 5 minutes (approx. 900 x g) the cells were fixed with a mixture of methanol and glacial acetic acid (3 parts plus 1 part). At least two slides per experimental group were prepared by dropping the cell suspension onto a clean microscope slide. The cells for evaluation of cytogenetic damage were stained with Giemsa (MERCK, 64293 Darmstadt, Germany).

### Metaphase analysis

No. of cells examined per dose: At least 200				
Scored for structural?	X	Yes		No
Scored for numerical?		Yes (polyploidy noted if observed)	X	No
Coded prior to analysis?	X	Yes		No

X indicates those that apply

**Evaluation criteria:** The percentages of aberrant metaphases were calculated for each treatment scored, both including and excluding cells with only gap-type aberrations.

The Fisher Exact Probability Test (one-sided) was used to evaluate statistically the percentage of metaphases showing aberrations (excluding cells with only gap-type aberrations). Data from each treatment group, in the presence and absence of S9 mix, was compared with the respective solvent control group value. The data have been interpreted as follows:

- No statistically significant increase in the percentage of aberrant cells (at any concentration) above concurrent solvent control values - **NEGATIVE**.
- A statistically significant increase in the percentage of aberrant cells above concurrent solvent control values, which falls within the laboratory historical solvent control range - **NEGATIVE**.
- An increase in the percentage of aberrant cells, at least at one concentration, which is substantially greater than the laboratory historical solvent control values - **POSITIVE**.
- A statistically significant increase in the percentage of aberrant cells which is above concurrent solvent values and which is above the laboratory historical solvent control range upper value but below that described in (c) may require further evaluation.

**Statistical analysis:** Data were evaluated for statistical significance using the Fisher Exact Probability Test (one-sided).

## RESULTS

**Preliminary cytotoxicity assay:** Not performed.

**Cytogenetic assay:** In Experiment I in the absence and presence of S9 mix and in Experiment II in the presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In Experiment II in the absence of S9 mix concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage. In this experiment the mitotic index was markedly reduced to 53.9 % and 57.3 % of control at the two highest evaluated concentrations.

In Experiment I in the absence and presence of S9 mix, no statistically significant or biologically relevant increase in the percentage of aberrant cells was observed at the concentrations evaluated (Table 6.8.1-17). The aberration rates of the cells after treatment with the test substance (0.0 – 3.0 % aberrant cells, excluding gaps) did not exceed the solvent control values (2.0 – 3.0 % aberrant cells, excluding gaps) and were within the range of the laboratory historical solvent control data (see Appendix I).

In Experiment II in the absence of S9 mix, one single statistically significant increase in chromosomal aberrations (4.5 % aberrant cells, excluding gaps) was observed after treatment with 1062.9 µg/mL. This value clearly exceeded the historical control data range (0.0 – 2.5 % aberrant cells, excluding gaps). In Experiment II in the presence of S9 mix one single statistically significant increase in chromosomal aberrations (4.3 % aberrant cells, excluding gaps) was observed after treatment with 347.1 µg/mL. This value slightly exceeds the historical control data range (0.0 – 3.5 % aberrant cells, excluding gaps). However, in the presence of S9 mix the effect was not reproducible in the two independent experiments performed. The response seen at 347.1 µg/mL was not part of a concentration-response.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.

Either EMS (770.0 or 660.0 µg/mL) or CPA (7.5 or 2.5 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

**Table 6.8.1-30: Summary of results of the chromosomal aberration study with SYN508272**

Exp.	Preparation	Test item	Mitotic indices	Aberrant cells		
	interval	concentration	in %		in %	
		in µg/mL	of control	incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 hrs without S9 mix						
I	22 hrs	Solvent control <sup>1</sup>	100.0	3.0	3.0	0.0
		Positive control <sup>2</sup>	54.3	17.5	<b>16.0<sup>s</sup></b>	5.0
		607.3	74.5	2.0	1.5	0.0
		1062.9 <sup>p</sup>	75.2	3.0	3.0	0.0
		1860.0 <sup>p</sup>	84.2	0.0	0.0	0.0
Exposure period 22 hrs without S9 mix						
II	22 hrs	Solvent control <sup>1</sup>	100.0	1.0	0.5	0.0
		Positive control <sup>3#</sup>	34.5	40.0	<b>40.0<sup>s</sup></b>	7.0
		347.1	84.3	2.0	2.0	0.0
		607.3	53.9	2.0	2.0	0.0
		1062.9 <sup>##</sup>	57.3	5.3	<b>4.5<sup>s</sup></b>	0.0



Exp.	Preparation	Test item	Mitotic indices	Aberrant cells		
	interval	concentration	in %	in %		
		in µg/mL	of control	incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 hrs with S9 mix						
I	22 hrs	Solvent control <sup>1</sup>	100.0	2.5	2.0	0.0
		Positive control <sup>2</sup>	55.7	18.0	<b>17.5<sup>S</sup></b>	1.5
		347.1	108.2	0.5	0.5	0.0
		607.3	86.2	2.5	2.0	0.0
		1062.9 <sup>P</sup>	101.8	2.5	2.5	0.0
		1860.0 <sup>P</sup>	95.3	0.5	0.5	0.0
II	22 hrs	Solvent control <sup>1</sup>	100.0	1.5	1.0	0.0
		Positive control <sup>3</sup>	90.9	10.0	<b>9.5<sup>S</sup></b>	1.5
		198.3	114.0	1.5	1.0	0.0
		347.1 <sup>##</sup>	89.2	4.5	<b>4.3<sup>S</sup></b>	0.3
		607.3	106.5	1.5	1.5	0.0
		1062.9 <sup>P</sup>	99.5	3.0	3.0	0.5

\* Including cells carrying exchanges

## Evaluation of 200 metaphases per culture

<sup>P</sup> Precipitation occurred at the end of treatment

<sup>S</sup> Aberration frequency statistically significant higher than corresponding control values (p<0.05)

<sup>1</sup> DMSO 0.5% (v/v)

<sup>2</sup> CPA 7.5µg/mL

<sup>3</sup> CPA 2.5µg/mL

## CONCLUSION:

In a GLP and OECD compliant in vitro chromosome aberration test, the potential of SYN508272 to induce structural chromosomal aberrations in human lymphocytes in the absence and presence of an exogenous metabolic activation system was assessed.

In each experimental group two parallel cultures were analysed. Per culture 100 metaphase plates were scored for structural chromosomal aberrations. The highest applied concentration was 1860.0 µg/mL (approx. 10 mM), the limit concentration for the test. Exposure was for 4 hrs with and without S9 in Experiment I and for 4 hours with S9 and for 22 hrs without S9 in Experiment II.

In Experiment I in the absence and presence of S9 mix and in Experiment II in the presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In Experiment II in the absence of S9 mix, and under long exposure conditions (22 hrs), concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage. However, the mitotic index was markedly reduced to 53.9 % and 57.3 % of control at the two highest evaluated concentrations (607 and 1063 µg/mL). Precipitation was seen from the concentration of 1063 µg/mL with and without S9.

In Experiment I either with or without metabolic activation, no clastogenicity was observed at the concentrations evaluated. In Experiment II in the absence of S9 one single statistically significant increase in chromosomal aberrations (4.5 % aberrant cells, excluding gaps) was observed after treatment with the top concentration of 1062.9 µg/mL. Results at this concentration clearly exceeded the historical control data range (0.0 – 2.5 % aberrant cells, excluding gaps). In Experiment II in the presence of S9 mix (4 hrs) one single statistically significant increase in chromosomal aberrations (4.3 % aberrant cells, excluding gaps) was observed after treatment with 347.1 µg/mL. Results at this concentration slightly exceeded the historical control data range (0.0 – 3.5 % aberrant cells, excluding gaps). However, in the presence of S9 mix the effect was not reproducible in the two independent experiments performed. The response seen at 347.1 µg/mL, in the presence of S9 mix, was not part of a concentration-related response.

No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.

Appropriate mutagens were used as positive controls. They induced statistically significant increases ( $p < 0.05$ ) in cells with structural chromosome aberrations.

In conclusion, in this guideline study, SYN508272 induced structural chromosomal aberrations in human lymphocytes in vitro in the absence of S9 (following treatment for 22 hrs) at the top concentration of 1063 µg/ml, at which cytotoxicity and precipitation occurred. **Therefore, SYN508272 is considered to be clastogenic in this chromosome aberration test in vitro.**

(██████████, 2013a)

#### In vitro mouse lymphoma test

<b>Report:</b>	K-CA 5.8.1/12 ██████████ (2013a). SYN508272: Cell Mutation Assay at the Thymidine Kinase Locus (TK <sup>+/+</sup> ) in Mouse Lymphoma L5178Y Cells. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1555903, issue date: 18 December 2013. Unpublished. Syngenta File No. SYN508272_10906.
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**Guidelines:** *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997); OPPTS 870.5300 (1998):

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

#### **EXECUTIVE SUMMARY**

The highest concentration of the pre-experiment and both main experiments (1860 µg/mL) was equal to a molar concentration of approximately 10 mM.

The main experiments were evaluated at the following concentrations with and without metabolic activation:

116.25; 232.5; 465.0; 930.0; and 1860.0 µg/mL

No substantial or reproducible cytotoxic effect was noted up to the maximum concentration with and without metabolic activation.

No substantial or reproducible concentration dependent increase in mutant colony numbers was observed up to the maximum concentration with and without metabolic activation.

**In conclusion it can be stated that during the mutagenicity test described and under the experimental conditions reported SYN508272 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the presence and absence of metabolic activation. Therefore, SYN508272 is considered to be non-mutagenic in this mouse lymphoma assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN508272
<b>Description:</b>	Pale brown, solid
<b>Lot/Batch number:</b>	MES 114/2
<b>Molecular weight:</b>	175.14 g/mole
<b>Purity</b>	94% (estimated error $\pm 5$ %) Exposure concentrations adjusted to purity
<b>CAS#:</b>	Not reported
<b>Expiry date:</b>	June 30 2016
<b>Control Materials:</b>	
<b>Negative:</b>	-
<b>Solvent control (final concentration):</b>	DMSO (0.5%)
<b>Positive control:</b>	Absence of S9 mix: Methylmethanesulphonate, 19.5 $\mu\text{g/mL}$
	Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 $\mu\text{g/mL}$

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other $\beta$ -naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM  $\text{MgCl}_2$   
 33 mM  $\text{KCl}$   
 5 mM glucose-6-phosphate  
 4 mM  $\text{NADP}$

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

### Test cells: mammalian cells in culture

X	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media:</b> RPMI 1640			
Properly maintained?	X	Yes	No
Periodically checked for Mycoplasma contamination?	X	Yes	No
Periodically checked for karyotype stability?	X	Yes	No
Periodically "cleansed" against high spontaneous background?	X	Yes	No

X indicates those that apply

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na <sup>+</sup> /K <sup>+</sup> ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	Trifluorothymidine (TFT)				

X indicates those that apply

#### Test compound concentrations used:

Absence of S9 mix 116.25; 232.5; 465.0; 930.0; and 1860.0 µg/mL

Presence of S9 mix 116.25; 232.5; 465.0; 930.0; and 1860.0 µg/mL

#### Study Design and Methods:

**In-life dates:** Start: 22 July 2013 End: 02 September 2013

#### Test performance:

**Cell treatment:** Cells were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

After washing, cells were cultured for 2 days (expression period) before cell selection.

After expression, 10<sup>4</sup> cells/mL were dispensed, at 200 µL/well, into 96 well plates. The cells were cultured for 10-13 days in selection medium to determine numbers of mutants. Dilutions of the cultures to approximately 8 cells/mL were cultured for 10-13 days without selective agent to determine cloning efficiency.

Cell growth in individual microwell plates was assessed after 10-13 days using a dissecting microscope. The survival plates and viability plates were scored for the number of wells containing no cell growth (negative wells). The mutation plates were scored so that each well contained either a small colony (considered to be associated with clastogenic effects), a large colony (considered to be associated with gene mutation effects), or no colony.

#### Statistical Methods:

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT®11 (SYSTAT Software, Inc., 501, Canal Boulevard, Suite C, Richmond, CA 94804, USA) statistics software. The number of mutant colonies obtained for the groups treated with the test item was compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological relevance and statistical significance were considered together.

**Evaluation Criteria:** Each well of the mutation plates (those containing TFT) was scored as containing either, a small colony, a large colony or no colony according to the following criteria:

Colonies were counted manually. In accordance with their size the colonies were classified into two groups. The colony size distribution was determined in the controls and at all concentrations of the test item. Criteria to determine colony size were the absolute size of the colony (more than 1/3 of a well for large colonies) and the optical density of the colonies (the optical density of the small colonies is generally higher than the large colonies).

#### RESULTS

**Preliminary toxicity assay:**

The pre-experiment was performed in the presence and absence of metabolic activation with a treatment time of 4 hours. Test substance concentrations between 14.53 µg/mL and 1860 µg/mL were used. The highest concentration in the pre-experiment was equal to a molar concentration of approximately 10 mM considering the purity of the test item.

In the pre-experiment no cytotoxic effects leading to RSG values below 50% were observed up to the maximum concentration with and without metabolic activation.

The test medium was checked for precipitation at the end of the treatment period (4 hours) before the test substance was removed. No precipitation occurred up to the maximum concentration in the presence and absence of metabolic activation.

Both pH value and osmolarity were determined at the maximum concentration of the pre-experiment and in the solvent control without metabolic activation. No relevant shift of the pH value was observed (pH 7.49 of the solvent control versus pH 7.52 at 1860 µg/mL). The osmolarity was increased from 295 mOsm to 400 mOsm at 1860 µg/mL. Therefore, the osmolarity and pH was also measured at the next two lower concentrations (379 mOsm, pH 7.48 at 930 µg/mL, and 342 mOsm, pH 7.51 at 465 µg/mL).

The concentrations used in the main experiments were selected based on the results obtained in the pre-experiment.

To overcome problems with possible deviations of the solubility the main experiments were started with more than four concentrations. The individual concentrations were spaced by a factor of 2.

**Mutation assay:**

The study was performed to investigate the potential of SYN508272 to induce mutations at the mouse lymphoma thymidine kinase locus of the cell line L5178Y.

The assay was performed in two independent experiments, using two parallel cultures each. Experiments I and II were performed with and without liver microsomal activation and a treatment period of 4 hours.

The experimental part of the second experiment with metabolic activation was terminated prior to treatment as the available cell density was too low. This experimental part was repeated as experiment II.2. The data of the repeat experiment are reported as experiment II with metabolic activation.

The main experiments were evaluated at the following concentrations with and without metabolic activation: 116.25; 232.5; 465.0; 930.0; and 1860.0 µg/mL

No precipitation was noted up to the maximum concentration with and without metabolic activation. The osmolarity was moderately increased at the two highest concentrations of 930.0 and 1860.0 µg/mL (379 and 400 mOsm compared to a physiological level of approximately 300 mOsm). However, no unaccountable cytotoxic effect occurred up to the maximum concentration so, the moderately increased osmolarity had no impact on cell growth. Osmolarity values of approximately 400 mOsm are common whenever DMSO is used as solvent since the osmolarity is measured as freezing point depression.

No relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or a relative total growth (RTG) of less than 50% in both cultures were noted up to the maximum concentration with and without metabolic activation.

No substantial or reproducible concentration dependent increase of the mutation frequency exceeding the threshold of 126 above the corresponding solvent control was observed in the main experiments up to the maximum concentration tested with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible concentration-dependent increase of mutant frequencies using SYSTAT®11 statistics software. A significant concentration-dependent trend of the mutation frequency indicated by a probability value of <0.05 was detected in the second culture of the second experiment without metabolic activation and in both cultures of the second experiment with metabolic activation. As the mutation frequencies neither exceeded the historical range of solvent controls nor the global evaluation factor, the significant results are considered as biologically irrelevant fluctuations.

In this study the range of the solvent controls values was from 50 up to 116 mutant colonies per  $10^6$  cells; the range of the groups treated with the test substance was from 38 up to 193 mutant colonies per  $10^6$  cells. The viability of the solvent control of experiment I, culture I with metabolic activation and of the solvent control of experiment II, culture II without metabolic activation exceeded the upper limit of the acceptance criteria (124% and 131% versus an upper limit of 120%). This deviation was judged as irrelevant as it was rather minor and the viability of the parallel cultures remained within the acceptable range. Cloning efficiency values above 100% occasionally occur since even suspension cell cultures do not form an ideal solution in medium. The cells tend to form transient aggregates that are counted as single cells during determination of the cell density. The aggregation does not compromise the validity of the data however, since the absolute values of the cloning efficiency are used to calculate the mutation frequency.

MMS (19.5 µg/mL) and CPA (3.0 and 4.5 µg/mL) were used as positive controls and showed a distinct increase in induced total mutant colonies at acceptable levels of cytotoxicity with at least one of the concentrations of the controls.

**Table 6.8.1-31: Summary of results of the L5178Y study in the absence and presence of metabolic activation**

	conc.	S9	relative cloning efficiency	relative total growth	mutant colonies/ $10^6$ cells	threshold	relative cloning efficiency	relative total growth	mutant colonies/ $10^6$ cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
<b>Experiment I / 4 h treatment</b>			<b>culture I</b>				<b>culture II</b>			
Solv. control with DMSO		-	100.0	100.0	60	186	100.0	100.0	110	236
Pos. control with MMS	19.5	-	101.7	28.9	342	186	88.8	84.7	270	236
Test item	58.13	-	117.2	culture was not continued <sup>#</sup>			90.3	culture was not continued <sup>#</sup>		
Test item	116.25	-	95.2	54.4	102	186	93.3	112.4	111	236
Test item	232.5	-	92.2	82.0	88	186	86.0	152.6	77	236
Test item	465.0	-	98.4	59.4	102	186	94.9	129.7	92	236
Test item	930.0	-	108.9	50.9	102	186	98.3	55.3	193	236
Test item	1860.0	-	110.9	43.2	115	186	100.0	127.1	115	236
Solv. control with DMSO		+	100.0	100.0	90	216	100.0	100.0	62	188
Pos. control with CPA	3.0	+	69.1	18.3	676	216	31.7	48.6	306	188
Pos. control with CPA	4.5	+	50.3	8.8	1326	216	42.0	31.9	430	188
Test item	58.13	+	105.9	culture was not continued <sup>#</sup>			96.8	culture was not continued <sup>#</sup>		
Test item	116.25	+	77.4	61.7	126	216	105.2	116.4	49	188
Test item	232.5	+	123.0	63.0	141	216	79.2	87.6	62	188
Test item	465.0	+	109.1	60.6	120	216	76.9	88.5	50	188
Test item	930.0	+	73.1	46.4	152	216	85.4	152.6	38	188
Test item	1860.0	+	57.0	55.5	135	216	88.0	96.3	62	188

			relative	relative	mutant		relative	relative	mutant	
	conc.	S9	cloning	total	colonies/		cloning	total	colonies/	
	per mL	mix	efficiency 1	growth	10 <sup>6</sup> cells	threshold	efficiency 1	growth	10 <sup>6</sup> cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
<b>Experiment II/ 4 h treatment</b>			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	73	199	100.0	100.0	50	176
Pos. control with MMS	19.5	-	82.5	26.7	584	199	77.9	30.9	263	176
Test item	58.13	-	109.2	culture was not continued <sup>#</sup>			109.1	culture was not continued <sup>#</sup>		
Test item	116.25	-	120.1	119.4	122	199	113.1	142.5	48	176
Test item	232.5	-	107.2	104.4	58	199	98.3	72.3	56	176
Test item	465.0	-	105.3	103.3	59	199	130.0	147.7	55	176
Test item	930.0	-	100.0	117.3	83	199	107.1	114.9	51	176
Test item	1860.0	-	95.1	76.9	86	199	90.7	50.9	68	176
Solv. control with DMSO		+	100.0	100.0	92	218	100.0	100.0	116	242
Pos. control with CPA	3.0	+	64.5	61.4	544	218	57.5	61.8	508	242
Pos. control with CPA	4.5	+	45.8	21.3	1045	218	31.7	25.5	1272	242
Test item	58.13	+	103.7	culture was not continued <sup>#</sup>			115.5	culture was not continued <sup>#</sup>		
Test item	116.25	+	96.5	112.1	77	218	89.2	128.8	105	242
Test item	232.5	+	127.5	97.7	80	218	81.3	127.4	118	242
Test item	465.0	+	112.0	84.2	95	218	95.1	125.0	98	242
Test item	930.0	+	107.7	90.3	125	218	74.4	118.6	117	242
Test item	1860.0	+	80.6	63.1	148	218	86.4	102.5	164	242

threshold = number of mutant colonies per 10<sup>6</sup> cells of each solvent control plus 126

# culture was not continued as a minimum of only four analysable concentrations are required

## CONCLUSION:

In a GLP and OECD compliant mammalian cell gene mutation assay, the potential of SYN508272 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y was investigated. The assay was performed in two independent experiments, using two parallel cultures each. The main experiments were performed with and without liver microsomal activation and a treatment period of 4 hours. The cells were exposed at the following concentrations: 116.25; 232.5; 465.0; 930.0; and 1860.0 µg/mL.

The highest applied concentration (1860 µg/mL, approx. 10 mM) was the limit concentration for this test. No precipitation was noted. No reproducible cytotoxic effects occurred in both main experiments up to the maximum concentration in the absence and presence of metabolic activation. No substantial or reproducible concentration dependent increase of the mutation frequency exceeding the threshold of 126 above the corresponding solvent control was observed in the main experiments up to the maximum concentration tested with and without metabolic activation. A linear regression analysis was performed to assess a possible concentration-dependent increase of mutant frequencies. A significant concentration-dependent trend of the mutation frequency indicated by a probability value of <0.05 was detected in the second culture of the second experiment without metabolic activation and in both cultures of the second experiment with metabolic activation. As the mutation frequencies neither exceeded the historical range of solvent controls nor the global evaluation factor, these isolated significant trends were considered as biologically irrelevant fluctuations.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

As a result, HSE agrees with the EU evaluation, that **SYN508272 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation when tested up to the highest required concentration.**

(██████████, 2013a)

#### In vivo bone marrow micronucleus study in the rat

<b>Report:</b>	K-CA 5.8.1/13 ██████████ (2014a). SYN508272: Micronucleus Assay in Bone Marrow Cells of the Rat. ██████████ ██████████. Laboratory Report No.1602600, issue date: 24 June, 2014, Unpublished. Syngenta File No.SYN508272_10910.
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**Guidelines:** Rat bone marrow micronucleus test OECD 474 (1997); OPPTS 870.5395 (1998); 2000/32/EC 440/2008 B.12 (2008)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

### **EXECUTIVE SUMMARY**

This study was performed in order to investigate the potential of SYN508272 to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the rat. The test substance was suspended in sterile water, which was also used as the vehicle control. The volume administered orally was 10 mL/kg body weight (bw). At 24 h and 48 h after a single administration of the test substance, the bone marrow cells were collected for micronuclei analysis. Seven males per test group (except the control groups with 5 males only) were evaluated for the occurrence of micronuclei. At least 2000 polychromatic erythrocytes (PCEs) were scored per animal for micronuclei. To describe a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined per slide and reported as the number of PCEs per 2000 erythrocytes. The following dose levels of the test substance were investigated:

24 h preparation interval: 312.5, 625, and 1250 mg/kg bw.

48 h preparation interval: 1250 mg/kg bw.

The highest dose was estimated to be a suitable maximum tolerated dose based on a pre-experiment. After treatment with the test substance the number of PCEs per 2000 erythrocytes was not substantially decreased as compared to the mean value of PCEs per 2000 erythrocytes of the vehicle control, thus indicating that SYN508272 did not exert any significant cytotoxic effects in the bone marrow. In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of the test substance with any dose level used. The mean values of micronuclei observed after treatment with SYN508272 were below the value of the vehicle control group and within the historical vehicle control range. A dose of 20 mg/kg bw. cyclophosphamide administered orally was used as the positive control, which showed a substantial increase of induced micronucleus frequency. The volume of the positive control administered was 10 mL/kg bw.



In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the rat. Therefore, SYN508272 is considered to be non-mutagenic in this bone marrow micronucleus assay.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN508272
<b>Description:</b>	brown, solid
<b>Lot/Batch number:</b>	MES 114/2
<b>Purity:</b>	94 % w/w (Dose calculation was adjusted to purity. A correction factor of 1.06 was applied)
<b>CAS#:</b>	Not reported
<b>Expiry date:</b>	June 30 2016

<b>Control Materials:</b>			
<b>Negative control (if not vehicle) :</b>	N/A	<b>Final Volume:</b> N/A	<b>Route:</b> N/A
<b>Vehicle:</b>	Sterile water	<b>Final Volume:</b> 10mL/kg	<b>Route:</b> Oral
<b>Positive control :</b>	Cyclophosphamide	<b>Final Doses:</b> 20mg/kg	<b>Route:</b> Oral

<b>Test Animals:</b>	
<b>Species</b>	Rat (male)
<b>Strain</b>	Wistar
<b>Age/weight at dosing</b>	8 – 9 weeks (at start of experiment); mean value 273.7 g (Standard Deviation $\pm$ 9.5 g)
<b>Source</b>	
<b>Housing</b>	Group
<b>Acclimatisation period</b>	At least 5 days
<b>Diet</b>	Pelleted standard diet, <i>ad libitum</i>
<b>Water</b>	Tap water, <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 19-25°C Humidity: 23-65% % Air changes: 15/hour Photoperiod: 12hours dark/12 hours light

Test compound administration:			
	Dose Levels	Final Volume	Route
<b>Preliminary:</b>	800, 2000, 1250 mg/kg bw	10mL/kg bw	oral
<b>Main Study:</b>	312.5, 625, 1250 mg/kg bw	10mL/kg bw	oral

### Study Design and Methods:

**In-life dates:** Start: 05 February 2014      End: 12 March 2014

**Preliminary Toxicity Assay:** A maximum tolerated dose (MTD) was determined, based on patterns of lethality or severe toxicity observed over a two-day observation period following a single oral dose.

**Micronucleus Test:****Experimental design**

Treatment	Dose	Number of Animals /Time of kill	
		24 hours	48 hours
Vehicle control	10 ml/kg	5	5
Positive control	20 mg/kg	5	
Test substance	312.5 mg/kg	7	
Test substance	625 mg/kg	7	
Test substance	1250 mg/kg	7	7

**Slide Preparation:**

The animals were sacrificed using CO<sub>2</sub> followed by bleeding. The femora were removed, the epiphyses were cut off and the marrow was flushed out with fetal calf serum using a syringe. The nucleated cells were separated from the erythrocytes using the method of Romagna. The cell suspensions were passed through a column consisting of  $\alpha$ -Cellulose and Cellulose. The columns will then be washed with Hank's buffered saline. The cell suspension was centrifuged at 1500 rpm (390  $\times$  g) for 10 minutes and the supernatant was discarded. A small drop of the re-suspended cell pellet was spread on a slide. The smear was air-dried and then stained with May-Grünwald /Giemsa. Cover slips were mounted with EUKITT. At least one slide was made from each bone marrow sample.

**Slide Analysis:** Slides were coded and scored blind. Two thousand immature erythrocytes were examined for the presence of micronuclei for each animal. The slides were also examined for evidence of cytotoxicity, which may be manifest by alterations in the ratio of different cell types in the bone marrow. This was assessed by counting the ratio of immature to mature erythrocytes per 2000 erythrocytes.

**Evaluation of results**

A test substance is classified as mutagenic if it induces either a dose-related increase or a clear increase in the number of micronucleated polychromatic erythrocytes in a single dose group above the laboratory's historical solvent control data range. Statistical methods (nonparametric Mann-Whitney test) were used as an aid in evaluating the results. However, the primary point of consideration was the biological relevance of the results. A test substance that fails to produce a biologically relevant increase in the number of micronucleated polychromatic erythrocytes is considered non-mutagenic in this system. A test substance failing to meet the criteria for a positive or negative response may be judged equivocal in this assay and may be considered for further investigation.

**RESULTS****Preliminary toxicity assay:**

In the first pre-experiment 2 male and 2 female animals received a single oral dose of SYN508272 (800 mg/kg bw) suspended in sterile water (10 mL/kg bw). The animals treated with 800 mg/kg bw expressed signs of toxicity, ruffled fur (Table 6.8.1-32). In the second pre-experiment 2 male and 2 female animals received a single oral dose of SYN508272 (2000 mg/kg bw) suspended in sterile water (10 mL/kg bw). The animals treated with 2000 mg/kg bw expressed signs of toxicity, ruffled fur, reduced spontaneous activity, dyspnea, hunchback, sunken flanks, dehydration, porphyrin secretion, cold feeling upon touching (Table 6.8.1-33). In the third pre-experiment 2 male and 2 female animals received a single oral dose of SYN508272 (1250 mg/kg bw) suspended in sterile water (10 mL/kg bw). The animals treated with 1250 mg/kg bw expressed signs of toxicity, ruffled fur, reduced spontaneous activity, hunchback, tippy toe walk (Table 6.8.1-34).

On the basis of these data 1250 mg/kg bw was considered suitable for the use as highest dose level for the study. Although no mortality occurred at a dose of 2000 mg/kg bw, signs of toxicity were regarded as too

severe to include this dose level into the main study (i.e., dehydration, dyspnea, cold to the touch, reduced spontaneous activity during almost the whole course of the study and hunched posture), as the chance of seeing significant mortality was regarded as high.

No substantial gender specific differences in toxicity were observed. Thus, the main study was performed using male animals only, as permitted by the Guideline.

Table 6.8.1-32: 1<sup>st</sup> Pre-experiment for toxicity: 800 mg/kg bw SYN508272

Signs of Toxicity	hours post-treatment					
	male / female					
	0-1 h	2-4 h	5-6 h	24 h	30 h	48 h
Ruffled fur	0/0	0/0	2/2	2/2	2/0	2/0

Table 6.8.1-33: 2<sup>nd</sup> Pre-experiment for toxicity: 2000 mg/kg bw SYN508272

Signs of Toxicity	hours post-treatment					
	male / female					
	0-1 h	2-4 h	5-6 h	24 h	30 h	48 h
Ruffled fur	0/0	0/0	1/1	2/2	2/2	2/2
Reduction of spontaneous activity	0/0	2/2	2/2	2/2	2/2	0/1
Dyspnea	0/0	2/2	0/0	0/0	0/0	0/0
Hunchback	0/0	2/2	1/1	1/1	1/2	0/1
Sunken flanks	0/0	0/0	0/0	0/1	0/2	0/1
Dehydration	0/0	0/0	0/0	2/2	0/0	0/1
Porphyria secretion	0/0	0/0	0/0	0/0	0/0	0/1
Feels cold upon touching	0/0	0/0	0/0	0/0	0/1	0/0

Table 6.8.1-34: 3<sup>rd</sup> Pre-experiment for toxicity: 1250 mg/kg bw SYN508272

Signs of Toxicity	hours post-treatment					
	male / female					
	0-1 h	2-4 h	5-6 h	24 h	30 h	48 h
Ruffled fur	0/0	0/0	2/2	2/2	2/2	2/2
Reduction of spontaneous activity	0/0	1/2	2/2	0/0	0/0	0/0
Hunchback	0/1	2/2	1/2	1/1	1/1	0/1
Tippy-toe walk	0/0	0/1	0/0	0/0	0/0	0/0

### Micronucleus test:

The test substance SYN508272 was assessed in the micronucleus assay for its potential to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the rat. The test substance was suspended in sterile water, which was also used as vehicle (negative) control. The volume administered orally was 10 mL/kg bw. At 24 h and 48 h after a single administration of the test substance, the bone marrow cells were collected for micronuclei analysis. Seven males per test group (except the control groups with 5 males only) were evaluated for the occurrence of micronuclei. At least 2000 polychromatic erythrocytes (PCEs) were scored per animal for micronuclei. To describe a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 2000 erythrocytes. The following dose levels of the test substance were investigated:

24 h preparation interval: 312.5, 625, and 1250 mg/kg bw

48 h preparation interval: 1250 mg/kg bw

As estimated by a pre-experiment in male and female rats, 1250 mg/kg of SYN508272 was suitable as the highest dose. Since no obvious substantial gender-specific differences in the sensitivity to the test substance were observed, the main experiment was performed using male animals only. The mean number of PCEs was not substantially decreased after treatment with the test substance as compared to the mean value of PCEs of the vehicle control, indicating that SYN508272 did not have any significant cytotoxic properties in the bone marrow. In comparison to the corresponding vehicle control values there was no biologically relevant enhancement or statistically significant increase in the frequency of the detected micronuclei at any preparation interval or dose level after administration of the test substance. The mean values of micronuclei observed after treatment with SYN508272 were below the value of the respective vehicle control group and within the historical vehicle control range. Additionally no dose dependence was observed. A dose of 20 mg/kg bw cyclophosphamide administered orally was used as positive control which showed a statistically significant increase of induced micronucleus frequency. The volume of the positive control administered was 10 mL/kg bw (Table 6.8.1-35). Statistical significance at the five per cent level ( $p < 0.05$ ) for the incidence of micronuclei was evaluated by means of the non-parametric Mann-Whitney test Table 6.8.1-36).

**Table 6.8.1-35: Summary of micronucleus test**

test group	dose mg/kg b.w.	sampling time (h)	PCEs with micronuclei (%)	range	PCE per 2000 erythrocytes
vehicle control	0	24	0.280	2 - 11	1186
test substance	312.5	24	0.136	0 - 6	1070
test substance	625	24	0.164	1 - 7	1108
test substance	1250	24	0.250	1 - 11	1021
positive control	20	24	1.830	14 - 65	957
vehicle control	0	48	0.240	0 - 8	1023
test substance	1250	48	0.179	0 - 5	1001

**Table 6.8.1-36 : Biometry**

Vehicle control versus test group	Significance	p
312.5 mg SYN508272 /kg b.w.; 24 h	n.t.	-
625 mg SYN508272 /kg b.w.; 24 h	n.t.	-
1250 mg SYN508272 /kg b.w.; 24 h	n.t.	-
20 mg CPA/kg b.w.; 24 h	+	0.0040
1250 mg SYN508272 /kg b.w.; 48 h	n.t.	-

+ = significant

- = not significant

n.t. = not tested, as the mean micronucleus frequency was not above the vehicle control value

**EFSA Request for additional information (February 2018), Question 39:** Applicant to submit the analysis of blood samples taken from the original micronucleus study with SYN508272 as supporting evidence to demonstrate bone marrow exposure.

#### **Applicant Response:**

Blood samples taken from the original micronucleus study (■■■■■, 2014a) with SYN508272 have been analysed to demonstrate proof of exposure. The additional proof of exposure and validation work to support the rat bone marrow micronucleus test are provided in ■■■■■, 2018, summarised following the summary of

■■■■■, 2014a and K-CA 4.1.2, respectively. The work by ■■■■■, 2018 indicates there is clear evidence of exposure of SYN508272 in blood.

<b>Report:</b>	K-CA 5.8.1/14 ■■■■■ (2018) SYN508272 - The Determination of SYN508272 in Rat Blood:Water: (50:50) by LC-MS/MS in Samples Generated in Study 1602600 Alderley Analytical Ltd, The BioHub, Alderley Park, Alderley Edge, Cheshire, SK10 4TG, United Kingdom. Laboratory Report No. 0057/003. Issue date: 10 April 2018. Unpublished. Syngenta File No. SYN508272_10923
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**Guidelines:** Not applicable

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable. The analytical method for determination of pydiflumetofen in rat blood has been assessed and validated (see DAR Vol. 3 B. 5.1.2.2).

## EXECUTIVE SUMMARY

Analysis of SYN508272 in rat blood:water (50:50) samples generated from ■■■■■ Study Number 1602600 (a rat micronucleus study) was conducted using a validated high performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) method. The method was validated at Alderley Analytical prior to sample analysis.

The analytical procedure involved analysis of SYN508272 in rat blood:water (50:50) following the addition of acetonitrile and centrifugation. LC-MS/MS analysis was performed using a Waters Acquity I-Class UPLC chromatography system which was coupled with a Waters Xevo TQ-S Mass Spectrometer.

Calibration standards and quality control samples met the acceptance criteria described in the study plan, thus demonstrating acceptable performance of the methods during the analysis of the study samples.

The concentrations of SYN508272 in rat blood:water (50:50) was greater than the upper limit of quantification (ULOQ) in all except three samples; ranging from 138,000 to greater than 200,000 ng/mL.

**Blood:water (50:50) samples originating from ■■■■■ Study Number 1602600 were successfully assayed for SYN508272, and the data demonstrated exposure in blood. The results from calibration curve standards and quality control samples met the acceptance criteria for accuracy and precision, demonstrating acceptable performance of the method throughout the sample analysis period. Blood:Water (50:50) concentrations of SYN508272 were equal to or greater than 138,000 ng/mL in the 2, 8 and 24 h samples at all dose levels.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN508272
<b>Description:</b>	Beige Powder
<b>Lot/Batch number:</b>	MES114/2
<b>Purity:</b>	94 % w/w
<b>CAS#:</b>	Not available
<b>Retest date:</b>	31 August 2020
<b>Storage:</b>	<10°C
<b>Internal standard:</b>	Diazepam-D5
<b>Description:</b>	Liquid
<b>Supplier:</b>	Cerilliant
<b>Lot/Batch number:</b>	FE07061501
<b>Concentration:</b>	1 mg/mL
<b>CAS#:</b>	Not available
<b>Expiry date:</b>	1 July 2020
<b>Storage:</b>	Approx. -20°C

A purity correction factor of 1.06 was applied to the SYN508272 reference stock solution prepared.

### Study Design and Methods:

**In-life dates:** Start: 10 July 2017      End: 26 July 2017

### Study Sample Receipt, Storage and Analysis Dates

A total of thirty-six rat K3EDTA blood:water (50:50) samples from [REDACTED] Study Number 1602600 were received frozen on dry ice and in good condition from the Sponsor, on 24 May 2017 for analysis at Alderley Analytical. All samples were stored at a nominal temperature of  $\leq -70^{\circ}\text{C}$  until analysed and were returned to these storage conditions post analysis. Sample analysis was completed on 26 July 2017.

### Bioanalysis

Rat blood: water (50:50) extracts were analysed for SYN508272 by LC-MS/MS using the validated quantitative method detailed in 0057/002. Diazepam-D5 was added as an internal standard.

## RESULTS

A total of 36 rat blood:water (50:50) samples originating from [REDACTED] Study Number 1602600, from dose groups reportedly dosed at 800 mg/kg, 1250 mg/kg and 2000 mg/kg, were analysed in accordance with the Sample Analysis Method. Calibration standards and quality control samples met the acceptance criteria thus demonstrating acceptable performance of the method.

The concentrations of SYN508272 obtained in the rat blood:water (50:50) samples originating from [REDACTED] Study Number 1602600 were greater than the ULOQ; ranging from 138,000 to greater than 200,000 ng/mL, which accounts for a 40 fold dilution of the top standard (5000 ng/mL) (Table 6.8.1-37).

The samples were collection in February 2014 (as provided by the Sponsor, Syngenta Limited). The samples were analysed in July 2017, with the date of the last reportable blood:water (50:50) sample analysis on 26<sup>th</sup> July 2017.

Based on the number of days from the sample collection date and last sample analysis date, samples were stored at a nominal temperature of  $\leq -70^{\circ}\text{C}$  for a maximum of 1267 days. The samples were analysed using

a validated quantitative method and all, except 3, samples were above the validated range of 5000 ng/mL. As such the absence of long term storage analysis does not affect the purpose or validity of this study.

The 36 rat blood:water (50:50) samples were analysed within five analytical runs, one of which was rejected due to instrument issues, one run failed calibration line acceptance criteria and one run failed QC acceptance criteria. All samples concentration results were generated within acceptable Runs 1 and 5.

### Out of Range Samples

The lower limit of quantification (LLOQ) for SYN508272 was established at 10 ng/mL. All sample concentrations were above the LLOQ in rat blood:water (50:50) samples.

The upper limit of quantification (ULOQ) for SYN508272 in blood:water (50:50) was established at 5000 ng/mL. All samples, with the exception of three samples, had concentrations that were above the ULOQ (ALQ).

**Table 6.8.1-37 : Sample results for SYN508272 in rat blood :water (50:50)**

Dose level (mg/kg)	Subject Number/Sex	Time point (hours)	Concentration* ng/mL
800	1M	2	ATS
800	2M	2	ATS
800	3F	2	ATS
800	4F	2	ATS
800	1M	8	ATS
800	2M	8	ATS
800	3F	8	ATS
800	4F	8	ATS
800	1M	24	ATS
800	2M	24	ATS
800	3F	24	195000
800	4F	24	199000
1250	1M	2	ATS
1250	2M	2	ATS
1250	3F	2	ATS
1250	4F	2	ATS
1250	1M	8	ATS
1250	2M	8	ATS
1250	3F	8	ATS
1250	4F	8	ATS
1250	1M	24	138000
1250	2M	24	ATS
1250	3F	24	ATS
1250	4F	24	ATS
2000	1M	2	ATS
2000	2M	2	ATS
2000	3F	2	ATS
2000	4F	2	ATS
2000	1M	8	ATS
2000	2M	8	ATS
2000	3F	8	ATS
2000	4F	8	ATS
2000	1M	24	ATS
2000	2M	24	ATS
2000	3F	24	ATS
2000	4F	24	ATS

ATS – above top standard, concentration value greater than ULOQ of 5000 ng/mL, which equates to 200,000 ng/mL, corrected for 40 fold dilution

\* concentrations corrected for a 40 fold dilution factor

**CONCLUSION:** Blood:water (50:50) samples originating from [REDACTED] Study Number 1602600 were successfully assayed for SYN508272, and the data demonstrated exposure in blood. The results from calibration curve standards and quality control samples met the acceptance criteria for accuracy and precision, demonstrating acceptable performance of the method throughout the sample analysis period. Blood:Water (50:50) concentrations of SYN508272 were equal to or greater than 138,000 ng/mL in the 2, 8 and 24 h samples at all dose levels.

### REFERENCES

**Study Number 0057/002** Validation of a Bioanalytical Method for the Determination of SYN508272 in Rat Blood:Water 1:1 (v/v) by LC-MS/MS, Alderley Analytical Study Number 0057/002 (2018). Syngenta File No. SYN508272\_10924. Included in K-CA 4.1.2/17.

**Study Number 1602600** SYN508272: Micronucleus Assay in Bone Marrow Cells of the Rat. Unpublished. [REDACTED] Study Number 1602600 (2014). Syngenta File No. SYN508272\_10910

**CONCLUSION:**

In a GLP and OECD compliant rat bone marrow micronucleus study, SYN508272 suspended in sterile water, was given by gavage to groups of male Wistar rats (7 per each treatment group, and 5 for the negative and positive control groups). At 24 h and 48 h after administration, the bone marrow cells were collected for micronuclei analysis. At least 2000 polychromatic erythrocytes (PCEs) were scored per animal for micronuclei. To describe a cytotoxic effect due to the treatment with the test substance the ratio between polychromatic and normochromatic erythrocytes was determined per slide and reported as the number of PCEs per 2000 erythrocytes. The following dose levels of the test substance were investigated:

24 h preparation interval: 0, 312.5, 625, and 1250 mg/kg bw.

48 h preparation interval: 0, 1250 mg/kg bw.

The highest dose was estimated to be a suitable maximum tolerated dose based on three pre-experiments which showed minimal signs of toxicity at 800 mg/kg bw, moderate clinical signs of toxicity at 1250 mg/kg bw and severe clinical signs of toxicity at 2000 mg/kg bw. After treatment, the number of PCEs per 2000 erythrocytes was not substantially decreased as compared to the mean value of PCEs per 2000 erythrocytes of the vehicle control, thus indicating that SYN508272 did not exert any significant cytotoxic effects in the bone marrow. It is unclear from the study report whether any clinical signs of toxicity were observed in the main study in particular at the top dose. In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of the test substance at any dose level. The mean values of micronuclei observed after treatment with SYN508272 were below the value of the vehicle control group and within the historical vehicle control range. The positive control, cyclophosphamide (20 mg/kg bw) showed a substantial increase of induced micronucleus frequency.

**In conclusion, in this GLP and guideline compliant rat bone marrow micronucleus assay, SYN508272 was negative for clastogenicity and aneugenicity. Exposure of the bone marrow was demonstrated by subsequent blood analysis (██████████ 2018) of samples taken from the pre-experiment animals in the ██████████ (2014a) study (see below).**

Blood samples taken at 2, 8 and 24 hr after test substance administration from animals treated with 800, 1250 or 2000 mg/kg bw SYN508272 and analysed for the presence of the test item showed that the concentrations of SYN508272 were equal to or greater than 138,000 ng/mL at all dose levels.

(██████████, 2014a; ██████████, 2018)

28-day study in rats

<b>Report:</b>	K-CA 5.8.1/15 ██████████ (2015). SYN508272: A 28 Day Dietary Toxicity Study in Rats. ██████████ ██████████ Laboratory Report No. 35015. 13 November 2015. Unpublished. Syngenta File No. SYN508272_10919.
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**Guidelines:** OECD 407 (1995); OPPTS 870.3050 (2000); 96/54/EC B.7 (1996).

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable. The analytical method for determination of pydiflumetofen in rodent diet has been assessed and validated (see DAR Vol. 3 B. 5.1.2.2).



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## EXECUTIVE SUMMARY

The objective of the study was to assess the potential toxicity of SYN508272 when given orally by diet for 28 days to rats.

Four groups of 5 males and 5 females Han Wistar rats (■■■:WI(Han)) were fed diet containing 0, 100, 500 or 2000 ppm (males)/4000 ppm (females) of SYN508272 for a period of 28 consecutive days.

The animals were monitored regularly for viability and for signs of ill health or reaction to treatment. Clinical observations, body weights, food consumption, ophthalmic examinations, detailed functional observations battery (including motor activity) were assessed at pre-determined intervals from pretrial until study completion on all animals. Urine samples were collected during Week 4 for analysis. Blood samples were collected for haematology, coagulation and clinical chemistry investigations prior to termination.

After completion of 28 days of treatment, all animals were euthanised and subjected to a detailed necropsy examination, where selected organs were weighed and tissues were preserved. Tissues from all animals in the Control and High dose groups and gross lesions from animals in Low and Intermediate groups were processed and examined histologically.

The overall mean achieved dosages were 0, 7.3, 37.4 and 143.1 mg SYN508272/kg/day in males and 0, 7.8, 42.5 and 243.5 mg SYN508272/kg/day in females corresponding to dietary inclusions levels of 0, 100, 500 and 2000 ppm (males)/4000ppm (females).

There were no premature decedents or notable clinical observations during this study. Group mean body weight, cumulative body weight change and food consumption in females receiving 4000 ppm were lower throughout the treatment period when compared with controls. Cumulative body weight change was 77.8% lower than the control value at the end of this study and this difference was statistically significant. Cumulative body weight change and food consumption in males receiving 2000 ppm were also lower for first 12 days of the treatment when compared with controls. The cumulative weight gain after 12 days of feeding was 40.6% lower than the control value and this difference was statistically significant. Body weight for males at 2000 ppm recovered during the remainder of the study, and the cumulative body weight gain at the end of the study was only 5.5% lower than the control values.

There were no treatment related inter-group differences in the functional observation battery parameters following administration of SYN508272 at dietary inclusion level up to 2000 ppm in males and 4000 ppm in females.

Higher white blood cell (WBC) counts and higher values for the related white blood cells differential counts of neutrophil, lymphocytes, monocytes and basophil were noted in males at 2000 ppm and females at 4000 ppm. In the absence of any micropathology or signs of infection or disease these changes are considered not adverse.

Among clinical chemistry parameters, statistically significant differences were only seen in the high dose groups. Lower globulin values and corresponding higher albumin-globulin ratios were observed in 2000 ppm males and 4000 ppm females. Creatinine levels were higher in females at 4000 ppm, but they were unaffected in males at 2000 ppm. Urea levels were lower in males at 2000 ppm, but they were unaffected in females at 4000 ppm. In the absence of any changes in micropathology, and considering the direction of the changes (urea) and their presence in one sex only (urea and creatinine) these small differences are considered not adverse.

There were no other notable inter-group differences in functional observation battery, coagulation and urinalysis parameters or organ weights that were considered to be due to the consumption of diets containing SYN508272. There were no macroscopic or microscopic findings that could be related to the administration of SYN508272.

**Dietary administration of SYN508272 to rats at 2000 ppm (males) or 4000 ppm (females) for at least 28 days resulted in lower body weight, cumulative body weight gain and food consumption. At these**

dose levels, higher white blood cell counts and certain differential white blood cells counts were observed in males and females, along with changes in a limited number of clinical chemistry parameters. Changes in haematology and clinical chemistry were considered non-adverse. At 500 ppm and 100 ppm, there were no effects of treatment with SYN508272. Under the conditions of this study the No Observed Adverse Effect Level (NOAEL) following at least 28 consecutive days of dietary exposure to SYN508272 is 500 ppm equating to 37.4 mg/kg/day in males and 42.5 mg/kg/day in females.

## MATERIALS AND METHODS

### Materials:

Test Material:	SYN508272
Description:	Light brown solid
Lot/Batch number:	MES 114/2
Purity:	94%
CAS#:	Not Reported
Expiry date:	June 30 2016

**Vehicle and/or positive control:** The test substance was administered via Rat and Mouse No. 1 Expanded (Ground) Diet ( )

Test Animals:	
Species	Rat (male and female)
Strain	Han Wistar ( ):WI(Han))
Age/weight at dosing	8-9 weeks old and weighed 225 to 318 g (males) and 164 to 215 g (females)
Source	
Housing	Animals were housed by sex with 2-3 animals per cage. Animals were housed in suspended polycarbonate/polypropylene cages with stainless steel grid tops and solid bottoms. The cages were suspended on moveable racks.
Acclimatisation period	16 days
Diet	Rat and Mouse No.1 Expanded (Ground) Diet ( ) <i>ad libitum</i>
Water	Mains drinking water <i>ad libitum</i>
Environmental conditions	Temperature: 17-23 °C Humidity: 39-83% Air changes: 10 per hour Photoperiod: 12hours per day from 0700-1900 h

### Study Design and Methods:

**In-life dates:** Start: 15 November 2013      End: 20 December 2013

**Animal assignment:** Animals were allocated to dose groups and treated as outlined below. Animals were dosed continuously via the diet for 28 days. Five animals per sex were allocated into dose groups to receive either 100, 500, 2000 (males only) or 4000 (females only) ppm of SYN508272 via their diet. A control group of 5 males and 5 females was also included and these animals received a blank diet only.

### Study design

Test group	Dietary concentration (ppm)	Animal Numbers	
		Males	Females
Control	0	1-5	21-25
Low	100	6-10	26-30
Mid	500	11-15	31-35
High	2000	16-20	-

	4000	-	36-40
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**Diet preparation and analysis:**

Diet formulations were prepared by addition of the test compound to the diet and followed serial dilutions, as required, to achieve the required concentrations. Diet formulations were mixed until visibly homogenous.

The test compound was found to be stable in the diet for 18 days when prepared and stored at ambient temperature at concentrations used in the present study.

The concentration analysis and homogeneity analysis results were not reported in this study.

**Observations:** All animals were checked early morning and as late as possible each day for viability.

All animals were observed in their cage for reaction to treatment at least once each day. In addition, once each week, starting during the acclimatisation period, all animals received a detailed examination, including appearance, movement and behaviour patterns, skin and hair condition, eyes and mucous membranes, respiration and excreta.

**Bodyweight:** Body weights were recorded twice during the acclimatisation period and daily throughout the dosing period. The terminal body weight was also recorded.

**Food consumption and test substance intake:** The quantity of food consumed by each cage of animals was measured and recorded twice during acclimatisation and daily throughout the dosing period.

The group mean food consumption and overall achieved dose was calculated for all groups, including the controls.

**Water consumption:** Water consumption was monitored regularly by visual inspection of the water bottles throughout the study.

**Detailed Functional Observations:** These were completed during the pre-trial period and also at week 4.

Cage Side Observations: Posture/condition on first approach (animal undisturbed), (including Prostration, Lethargy, Writhing, Circling, Breathing abnormalities, Gait abnormalities, Tremor, Fasciculation, Convulsions, Biting (including cage components or self-mutilating), Vocalisations, Piloerection), Ease of removal from the cage, Body temperature, Condition of the eyes, Condition of the coat, Presence of salivation and Overall ease of handling.

Observations in a Standardised Area: Latency (time to first locomotory movement), Level of mobility, Rearing, Grooming, Urination/defecation, Arousal (level of alertness), Posture, tremor/convulsions, vocalisation, piloerection – recorded as for cage side observations, Palpebral closure, Gait abnormalities, Stereotypy (excessive repetition of behaviours) and/or unusual behaviours.

Functional Tests: Reaction to sudden sound, reaction to touch, grip strength, pain perception, foot splay landing and motor activity.

**Ophthalmoscopic examination:** The eyes were examined using an indirect ophthalmoscope following application of a mydriatic agent (1% Tropicamide, Mydriacyl®). The anterior, lenticular and fundic areas were examined from all animals during pre-trial period and Groups 1 and 4 during Week 4.

**Haematology:** Blood samples were taken via the orbital sinus under isoflurane anaesthesia prior to terminal kill. Animals were not fasted prior to terminal kill. 0.5 mL of whole blood was sampled into tubes containing EDTA and analysed for:

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Red blood cell count	White blood cell count
Haemoglobin	Neutrophils
Haematocrit	Lymphocytes
Mean cell volume	Monocytes
Mean cell haemoglobin concentration	Eosinophils
Mean cell haemoglobin	Basophils
Reticulocytes	Large unclassified cells
Reticulocyte count (absolute)	
Red blood cell distribution width	
Platelets	

**Coagulation:** Blood samples were taken via the orbital sinus under isoflurane anaesthesia prior to terminal kill. Animals were not fasted prior to terminal kill. Approximately 0.9 mL blood was transferred into tubes containing 3.8% (w/v) trisodium citrate (w/v) and assayed for:

Activated partial thromboplastin time	Prothrombin time
Fibrinogen	

**Clinical chemistry:** Blood samples were taken via the orbital sinus under isoflurane anaesthesia prior to terminal kill. Animals were not fasted prior to terminal kill. 1 mL of blood was sampled into tubes containing lithium heparin which were then centrifuged and the plasma separated and assayed for:

Aspartate aminotransferase	Lactate dehydrogenase
Alanine aminotransferase	Creatine phosphokinase
Alkaline phosphatase	Total protein
Cholesterol	Total bilirubin
Triglycerides	Sodium
Glucose	Potassium
Creatinine	Chloride
Albumin	Calcium
Globulin	Inorganic phosphate
Albumin/globulin ratio	Urea

**Urinalysis:** Urine samples were collected at week 4 from animals which were individually housed in metabolism cages over a 4 hour period. The animals were not allowed access to food and water over the collection period.

Microscopic evaluation of spun deposit	Glucose
Colour	Bilirubin
Turbidity	Ketones
Specific gravity	Leukocytes
Volume	Blood Pigments
pH	Urobilinogen
Protein	

**Investigations *post mortem*:** All animals were euthanized on day 29 by rotating across dose groups, such that similar numbers of animals from each group, including controls, were necropsied throughout the day, by exposure to a rising concentration of carbon dioxide. The terminal body weight of each animal was recorded followed by exsanguination of major blood vessels.

**Macroscopic examination:** All animals were examined *post mortem*. This involved an external observation and an internal examination of all organs and structures.

**Organ weights:** From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed (paired organs were weighed together):

Adrenal glands	Ovaries
Brain	Spleen
Epididymies	Testis
Heart	Thymus
Kidneys	Uterus (with cervix)
Liver	Pituitary glands
Thyroid Gland	Lungs

**Tissue submission:** The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

Animal identification	Nerve, optic
Gross lesions including masses	Oesophagus
Adrenal gland	Ovaries
Aorta	Oviduct
Brain (forebrain, midbrain, cerebrum, cerebellum and brainstem)	Peyer's patches
Bone marrow (smear)	Pancreas
Bone marrow (sternum)	Parathyroid gland
Bone marrow (femur)	Pharynx
Caecum	Pituitary gland
Colon	Prostate gland
Duodenum	Salivary gland
Epididymis	Seminal vesicle
Eyes	Skeletal muscle
Femur (including femorotibial joint)	Spinal cord (cervical, thoracic, lumbar)
Harderian gland	Skin
Heart	Spleen
Ileum	Sternum
Jejunum	Stomach
Kidney	Testis
Lacrimal gland	Thymus
Larynx	Thyroid gland
Liver	Tongue
Lung	Trachea
Lymph node - cervical	Ureter
Lymph node- mandibular	Urinary bladder
Lymph node - mesenteric	Uterus (with cervix)
Mammary gland	Vagina
<b>Nerve - sciatic</b>	

**Microscopic examination:** Tissues listed above were embedded in paraffin wax blocks, sectioned, mounted on glass slides, and stained with haematoxylin and eosin. Histopathological evaluation was performed by a veterinary pathologist with training and experience in laboratory animal pathology. Histopathological evaluation of all tissues was undertaken for all control and high dose animals and gross lesions from low and intermediate dose animals.

**Statistics:** Data were processed to give mean values and standard deviations, where appropriate. Body weights, cumulative body weight gain, selected functional observation battery & motor activity, absolute organ weights, haematology, coagulation, clinical chemistry and selected urinalysis parameters were analysed initially by a one-way analysis of variance (ANOVA). Organ weights were also analysed by analysis of covariance (ANCOVA) on terminal body weight. For all of the parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test was used to compare the control and treated groups, based on the error mean square in the ANOVA or ANCOVA. The Dunnett's test was performed for all continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant, and statistical flags are presented in the tables of results in the report. Micropathology incidence data was analysed using Fisher's Exact Test.

## RESULTS AND DISCUSSION

**Mortality:** There were no premature decedents during this study.

**Clinical observations:** There were no notable clinical observations, throughout the treatment period that were considered to be related to treatment with SYN508272.

**Bodyweight and weight gain:** Group mean body weight and cumulative body weight change in females receiving 4000 ppm were lower throughout the treatment period when compared with controls, with statistical significance being achieved for cumulative body weight change. At the end of 28 days, the mean cumulative body weight gain in 4000 ppm females was 77.8% lower than the control value.

Group mean body weight and cumulative body weight in males receiving 2000 ppm were slightly lower for the first 12 days of treatment when compared with controls, with statistical significance being achieved for cumulative body weight change in most of the occasions. The cumulative body weight gain after 12 days of feeding was 40.6% lower than the control value. Body weight for males at 2000 ppm recovered during the remainder of the study, and the cumulative body weight gain on the end of the study was only 5.5% lower than the control value and not statistically significant.

Body weight and cumulative body weight change in animals receiving 100 or 500 ppm were considered to be similar to that of the controls.

**Table 6.8.1-37: Intergroup comparison of cumulative body weight gain (g) (selected time points)**

	Dietary Concentration of SYN508272 (ppm)							
	Males				Females			
days	0	100	500	2000	0	100	500	4000
0-7	24	25	27	13*	9	11	11	-3**
0-12	37	40	49	22*	18	19	25	-2**
0-14	45	44	54	32	20	21	24	1**
0-21	63	67	77	59	30	30	40	8**
0-28	73	74	82	69	36	37	43	8**

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett's Test)

\*\* Statistically significant difference from control group mean,  $p < 0.01$  (Dunnett's Test)

**Food consumption, utilisation and compound intake:** Food consumption was lower throughout the treatment period in females receiving 4000 ppm when compared with controls.

Food consumption was also slightly lower for first 8 days in males receiving 2000 ppm when compared with controls, after which it was considered similar to that of the controls.

Food consumption was considered to be unaffected in males and females receiving 100 or 500 ppm.

No effect on food utilisation was discussed in the study.

Dose rates (based on nominal dietary levels of SYN508272) were calculated in terms of mg SYN508272/kg body weight. Mean values are shown below:

**Table 6.8.1-38: Mean Dose Received (mg/kg/day)**

SYN508272 (ppm)	100	500	2000	4000
Males	7.3	37.4	143.1	-
Females	7.8	42.5	-	243.5

**Water consumption:** There was no effect on water consumption.

**Ophthalmoscopic examination:** There were no inter-group differences.

**Functional observations:** There were no treatment-related inter-group differences in the quantitative functional observation parameters following administration of SYN508272 at dietary inclusion levels up to 2000 ppm in males and up to 4000 ppm in females.

There was a longer reaction time to the tail flick (pain) in females at 4000 ppm in Week 4 of the treatment, with statistical significance being achieved. However, during pretrial, there was also a longer reaction time to the tail flick in females at 4000 ppm which was not statistically significant, but was a similar magnitude when compared to controls as the difference following 28 days of treatment. Therefore, these observations were considered to be incidental to treatment with SYN508272.

**Motor activity:** There were no treatment-related inter-group differences in motor activity following administration of SYN508272 at dietary inclusion levels up to 2000 ppm in males and up to 4000 ppm in females.

Isolated instances of statistically significant differences were observed within a small number of 5-minute intervals, but the total values for basic and fine movements, and for X and Y ambulation were similar across all groups. All control and treated groups showed evidence of habituation during the 1-hour motor activity session.

**Haematology:** White blood cell (WBC) counts were statistically significantly higher than the control values in 2000 ppm males ( $p < 0.05$ ) and 4000 ppm females ( $p < 0.01$ ). At these same dose levels, the following differential WBC counts were higher than the control values:

- 2000 ppm males: neutrophil ( $p < 0.01$ ), lymphocyte (not significant), monocyte ( $p < 0.05$ ) and basophil ( $p < 0.01$ ) counts
- 4000 ppm females: neutrophil ( $p < 0.01$ ), lymphocyte ( $p < 0.01$ ), monocyte (not significant) and basophil ( $p < 0.01$ ) counts

Mean values were outside the range of historical control mean values for males. For females mean values, with the exception of neutrophils, were within the range of historical control mean values. The overall pattern of effects on these WBC parameters was considered to reflect a treatment-related increase in high dose males and females.

Statistically significantly higher reticulocytes (%) and reticulocyte counts were observed in the 2000 ppm males and 4000 ppm females. However, the mean values for reticulocyte counts in 2000 ppm males and 4000 ppm females were within the range of historic control mean values from a limited number (5) of available 28-day rat studies. Considering this normal variability in reticulocyte counts and the lack of any effects on RBC-related parameters in males or females, these differences are considered not an effect of treatment.

At 500 ppm and 100 ppm, there were no effects of treatment on haematology parameters in males or in females.

There were no other notable inter-group differences in haematology or coagulation parameters that were considered to be due to the consumption of diets containing SYN508272 at dietary inclusion levels up to 2000 ppm in males and up to 4000 ppm in females.

**Table 6.8.1-39: Intergroup comparison of selected haematology parameters**

Parameter	Dietary Concentration of SYN508272 (ppm)									
	Males					Females				
	0	100	500	2000	Historical controls	0	100	500	4000	Historical controls
WBC	8.34	7.73	10.60	<b>12.11*</b>	4.28-10.63	4.03	4.54	4.45	<b>7.54**</b>	1.27-9.30
Neutrophil	1.28	1.26	1.60	<b>2.38**</b>	0.31-1.94	0.77	0.89	0.76	<b>1.41**</b>	0.32-1.75
Lymphocyte	6.67	6.18	8.42	9.15	3.82-9.28	3.06	3.42	3.47	<b>5.83**</b>	0.67-7.81
Monocyte (10 <sup>9</sup> /L)	0.16	0.11	0.21	<b>0.27*</b>	0.05-0.26	0.09	0.09	0.09	0.14	0.04-0.35
Basophil	0.05	0.05	<b>0.11*</b>	<b>0.12**</b>	0-0.08	0.02	0.02	0.03	<b>0.05**</b>	0-0.11
Reticulocyte %	2.2	2.3	2.4	<b>2.9**</b>	1.3-2.3	2.3	2.7	2.8	<b>3.2**</b>	1.1-2.4
Reticulocyte count (10 <sup>9</sup> /L)	187	194	205	<b>233*</b>	113-263	168	205	211	<b>247**</b>	90-298
RBC (10 <sup>12</sup> /L)	8.63	8.39	8.62	8.22	-	7.49	7.63	7.70	7.78	-

Historical controls were collected for all parameters between 2010-2012 except for reticulocyte % which were collected in 2010

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett's test)

\*\* Statistically significant difference from control group mean,  $p < 0.01$  (Dunnett's test)

**Blood clinical chemistry:** For clinical chemistry parameters, statistically significant differences from the control group were observed only in the high dose groups. Lower globulin levels and corresponding higher albumin / globulin ratios in plasma were observed in males at 2000 ppm ( $p < 0.01$ ) and females at 4000 ppm ( $p < 0.05$  and  $p < 0.01$ , respectively) when compared to controls. Creatinine level was higher in females at 4000 ppm ( $p < 0.01$ ). The mean values were outside of the range of historic control means, and were considered to be treatment-related effects.

Urea values were lower in 2000 ppm males ( $p < 0.01$ ) compared to the control group, and this value at the high dose was outside the range of the historic control means. There was no effect on urea levels in 4000 ppm females.

Aspartate aminotransferase (AST) activity was higher in males at 2000 ppm ( $p < 0.01$ ) and females at 4000 ppm ( $p < 0.01$ ) when compared to controls. These mean values at the high dose were within the range of historic control group means for male and female rats. Considering the small differences observed and the absence of correlating findings in liver weights and histopathology, these differences were considered to be incidental to treatment with SYN508272.

Phosphate level in plasma was higher in males at 2000 ppm ( $p < 0.01$ ) and females at 4000 ppm ( $p < 0.01$ ) when compared to controls. These mean values at the high dose were within the range of historic control group means for male and female rats. Therefore, the small differences from the concurrent control values in phosphate levels were considered to reflect normal variability and were not treatment-related effects.

Any other minor differences observed in clinical chemistry parameters were attributed to normal biological variation, as they were small in magnitude, not statistically significant and lacked a dose-response. At 500 ppm and 100 ppm, there were no effects of treatment on clinical chemistry parameters in males or in females.



**Table 6.8.1-40: Intergroup comparison of selected clinical chemistry parameters**

Parameter	Dietary Concentration of SYN508272 (ppm)									
	Males					Females				
	0	100	500	2000	Historical controls	0	100	500	4000	Historical controls
Globulin (g/L)	19	19	17	14**	14-20	15	15	16	12*	12-19
Albumin:Globulin ratio	2.3	2.2	2.6	3.1**	2-3	3.1	3.2	3.2	4.0**	2.4-3.8
Creatinine (μmol/L)	34	34	34	32	19-33	32	33	36	38**	21-35
AST (U/L)	66	65	68	82**	56-169	60	62	62	80**	55-165
Phosphate (mmol/L)	1.50	1.40	1.55	1.95**	1.08-2.41	1.25	1.40	1.44	1.69**	0.87-2.55
Urea (mmol/L)	6.4	5.8	5.8	4.7**	4.6-7.3	5.8	5.2	5.6	5.7	4.5-7.8

Historical controls were collected for all parameters between 2010-2012

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett's test)

\*\* Statistically significant difference from control group mean,  $p < 0.01$  (Dunnett's test)

**Urinalysis:** There were no notable inter-group differences in urinalysis parameters that were considered to be due to the consumption of diets containing SYN508272 at dietary inclusion levels up to 2000 ppm in males and up to 4000 ppm in females.

Specific gravity was statistically significantly lower than control values in females at 500 and 4000 ppm, whereas there were no significant differences from control in the male groups. The mean values for females at 500 ppm (1.016) and 4000 ppm (1.018) were outside the range of the historic control group means (1.026 – 1.044), but only three studies of this duration were available for comparison. These small differences in specific gravity are not considered treatment-related, based on:

- The small number of urine samples available for analysis in control and treated groups (2-5 per group).
- No effect of treatment on specific gravity was observed in males, and all of the mean values for treated and control group males (1.025 – 1.031) were consistent with available individual animal value range from historic control data (1.024 – 1.080).
- The individual animal values for the 500 ppm and 4000 ppm females (1.009 – 1.026) were consistent with the individual animal value range from historic control data (1.012 – 1.046)

**Table 6.8.1-41: Intergroup comparison of selected urinalysis parameters**

Parameter	Dietary Concentration of SYN508272 (ppm)									
	Males					Females				
	0	100	500	2000	Historical controls	0	100	500	4000	Historical controls
Specific gravity	1.031	1.031	1.025	1.025	1.024-1.080	1.034	1.026	1.016*	1.018*	1.012-1.046

Historical controls were collected for all parameters between 2010-2012

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett's test)

\*\* Statistically significant difference from control group mean,  $p < 0.01$  (Dunnett's test)

### Sacrifice and pathology:

### Organ weights:

The absolute thymus weights were statistically significantly higher than control values in 500 ppm males (0.704 g) and in 2000 ppm males (0.661 g), and these mean values were higher than the historic control

range of mean values (0.462 – 0.619 g). However, the control group absolute thymus weight (0.441 g) was lower than this control range, and thymus weights are known to vary greatly in young rats depending on age and associated rapid increases in body weight (Rhouma and Sakly, 1994). The thymus weights adjusted for body weights by covariance analysis in 500 ppm males and 2000 ppm males were statistically significantly higher than the concurrent control value; however, the relative thymus weights (% of body weight) of 500 ppm males (0.1963) and 2000 ppm males (0.1922) were within the range of the historic control means (0.1531 – 0.1971).

Absolute thymus weights were statistically significantly higher than control in 500 ppm females (0.568 g) and numerically higher though not statistically significant in 4000 ppm females (0.510 g). These mean values were higher than the range of historic control means (0.423 – 0.508 g). The mean thymus weight adjusted for body weights by covariance analysis in 500 ppm females (0.554) was statistically significantly higher than the concurrent control value (0.417), but the mean adjusted weight in 4000 ppm females (0.567) was not statistically significant. The relative thymus weights (% of body weight) were within the range of historic controls (0.2089 – 0.2699) for both the 500 ppm females (0.2535) and 4000 ppm females (0.2662), whereas the concurrent control group value (0.1954) was below this reference range.

Overall, the higher thymus weights at 500 ppm and/or 2000/4000 ppm in males and females were within the range of historic control ranges as % of body weight. Considering the normal variation in thymus weights in young rats, comparisons to historic controls, and the lack of any micropathology changes in the thymus, these differences were considered not to be treatment-related effects.

In 2000 ppm males the absolute weight of the adrenals (0.0740 g) was statistically significantly higher than the control value (0.0609 g), and this mean value was outside of the historic control range of mean values (0.518-0.0613 g); however, when adjusted for body weight by covariate analysis, the difference between groups was not statistically significant. Considering the lack of statistical significance after adjustment for body weight, and the lack of any effect on adrenal weights in 4000 ppm females, these differences in 2000 ppm males are considered not to be an effect of treatment.

No other notable differences in absolute or relative organ weights were observed. While, there were isolated organ weight values that were different from their respective controls, there were no patterns, trends, or correlating data to suggest these values were toxicologically relevant. Thus, the organ weight differences observed were considered incidental and unrelated to administration of SYN508272 when given orally by diet for 28 days.

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**Table 6.8.1-42: Intergroup comparison of organ weights (g)**

Organ weights		Dietary Concentration (ppm)									
		Males					Females				
		0	100	500	2000	HCD (range of mean values) #	0	100	500	4000	HCD (range of mean values) #
Adrenals	Absolute	0.0609	0.0622	0.0625	<b>0.0740<sup>a</sup></b>	0.0518-0.0613	0.0699	0.0665	0.0804	0.0667	0.0621-0.0775
	Covariate	0.0612	0.0621	0.0622	0.0741	-	0.0689	0.0627	0.0788	0.0731	-
	Relative (%BW)	0.01789	0.01786	0.01781	0.02131	0.01722-0.02098	0.03156	0.02901	0.03635	0.03461	0.03312-0.03750
Brain	Absolute	2.10	2.12	2.02	2.11		1.95	1.99	1.91	1.90	
	Covariate	2.11	2.11	2.01	2.11		1.94	1.96	1.90	1.95	
	Relative (%BW)	0.615	0.605	0.572	0.611		0.892	0.865	0.863	0.992	
Epididymides	Absolute	1.0930	1.1647	1.1201	1.1534		-	-	-	-	
	Covariate	1.0940	1.1645	1.1191	1.1537		-	-	-	-	
	Relative (%BW)	0.32261	0.33284	0.31962	0.33256						
Heart	Absolute	0.97	1.05	1.04	0.95		0.72	0.75	0.74	0.59	
	Covariate	0.99	1.04	1.02	0.95		0.71	0.71	0.72	0.65	
	Relative (%BW)	0.284	0.300	0.291	0.274		0.328	0.324	0.333	0.309	
Kidneys	Absolute	2.11	2.15	2.27	2.34	2.03-2.21	1.51	1.57	1.51	1.26 <sup>a</sup>	1.30-1.58
	Covariate	2.16	2.14	2.23	2.36 <sup>a</sup>	-	1.49	1.51	1.48	1.36	-
	Relative (%BW)	0.620	0.613	0.639	0.676	0.662-0.702	0.689	0.681	0.677	0.656	0.654-0.761
Liver	Absolute	11.71	13.21	12.93	11.62	10.88-14.00	7.96	7.93	8.10	6.68	6.61-8.37
	Covariate	12.07	13.12	12.55	11.72	-	7.79	7.33	7.85	7.70	-
	Relative (%BW)	3.428	3.760	3.615	3.342	3.622-4.450	3.603	3.443	3.644	3.481	3.551-3.939
Lung	Absolute	1.49	1.59	2.02	1.87		1.39	1.14	1.44	1.57	
	Covariate	1.55	1.57	1.96 <sup>a</sup>	1.88		1.36	1.04	1.40	1.73	
	Relative (%BW)	0.435	0.454	0.562	0.537		0.635	0.497	0.645	0.808	
Pituitary	Absolute	0.009	0.009	0.009	0.010	0.007-0.008	0.012	0.012	0.013	0.009 <sup>a</sup>	0.010-0.010
	Covariate	0.009	0.009	0.008	0.010	-	0.012	0.012	0.013	0.009	-
	Relative (%BW)	0.00251	0.00245	0.00242	0.00284	0.00252-0.00281	0.00548	0.00540	0.00602	0.00459	0.00463-0.00528
Prostate	Absolute	0.335	0.407	0.472 <sup>b</sup>	0.411		-	-	-	-	
	Covariate	0.341	0.406	0.466 <sup>b</sup>	0.413		-	-	-	-	
	Relative (%BW)	0.0982	0.1160	0.1333	0.1187						

Spleen	Absolute	0.58	0.61	0.63	0.60		0.43	0.50	0.46	0.43	
	Covariate	0.59	0.60	0.61	0.61		0.42	0.44	0.43	0.52	
	Relative (%BW)	0.169	0.173	0.176	0.174		0.195	0.216	0.204	0.224	
Testes	Absolute	3.08	3.36	3.39	3.48		-	-	-	-	
	Covariate	3.11	3.35	3.36	3.48		-	-	-	-	
	Relative (%BW)	0.908	0.960	0.961	1.004						
Thymus	Absolute	0.441	0.458	0.704 <sup>a</sup>	0.661 <sup>a</sup>	0.462-0.619	0.427	0.411	0.568 <sup>a</sup>	0.510	0.423-0.508
	Covariate	0.456	0.454	0.688 <sup>a</sup>	0.665 <sup>a</sup>	-	0.417	0.377	0.554 <sup>a</sup>	0.567	-
	Relative (%BW)	0.1288	0.1304	0.1963	0.1922	0.1531-0.1971	0.1954	0.1778	0.2535	0.2662	0.2089-0.2699
Thyroid	Absolute	0.0170	0.0154	0.0189	0.0203		0.0153	0.0150	0.0154	0.0174	
	Covariate	0.0174	0.0153	0.0185	0.0204		0.0151	0.0146	0.0152	0.0181	
	Relative (%BW)	0.00499	0.00439	0.00526	0.00587		0.00697	0.00651	0.00693	0.00909	
Ovaries	Absolute	-	-	-	-		0.090	0.090	0.094	0.063 <sup>a</sup>	0.079-0.101
	Covariate	-	-	-	-		0.089	0.084	0.092	0.073	-
	Relative (%BW)						0.0411	0.0392	0.0423	0.0327	0.0427-0.0479
Uterus	Absolute	-	-	-	-		0.56	0.72	0.65	0.44	
	Covariate	-	-	-	-		0.56	0.70	0.64	0.47	
	Relative (%BW)						0.261	0.309	0.291	0.229	

<sup>a</sup> Statistically significant difference from control group mean,  $p < 0.05$

<sup>b</sup> Statistically significant difference from control group mean,  $p < 0.01$

<sup>#</sup> Historical control range of mean values (N=5 animals) compiled from five Han Wistar rats-28 days dietary studies performed by the conducting laboratory between 2010 and 2012

**Macroscopic findings:** No test substance-related gross findings were noted. The gross findings observed were considered incidental, of the nature commonly observed in this strain and age of rats, and/or were of similar incidence in control and treated animals and, therefore, were considered unrelated to administration of SYN508272 when given orally by diet for 28 days.

**Table 6.8.1-43: Intergroup comparison of macroscopic findings**

Necropsy findings <sup>a</sup>		Dietary Concentration (ppm)							
		Males				Females			
		0	100	500	2000	0	100	500	4000
Number of animals necropsied		5	5	5	5	5	5	5	5
Cervix	Small								1
Epididymis	Foci, pale, right			1					
Lung	Foci, dark		1		2				
	Foci, pale			1			1		
	Spongy								
	Area, dark	1							
	Discoloration, mottled				1			1	1
	Discoloration, dark			1	1	1			1
Lymph Node (Mandibular)	Discoloration, dark		2	1	1				
Lymph Node (Mesenteric)	Discoloration, dark		1						
Thymus	Discoloration, mottled							1	
Trachea	Fluid accumulation			1		1		1	2
Uterus	Dilation, one/both horns					1	1	1	1
	Small, both horns								1

a: The absence of a numeral digit indicates that the lesion specified was not identified

**Microscopic findings:** No test substance-related microscopic findings were noted. The microscopic findings observed were considered incidental, of the nature commonly observed in this strain and age of rat, and/or were of similar incidence and severity in control and treated animals and, therefore, were considered unrelated to administration of SYN508272 when given orally by diet for 28 days.

Table 6.8.1-44: Intergroup comparison of microscopic findings

Histological findings <sup>a</sup>		Dietary Concentration (ppm)							
		Males				Females			
		0	100	500	2000	0	100	500	4000
Number of animals from which the tissue was examined microscopically		5			5	5			5
Epididymis	Sperm granuloma	0		1*	0	-			-
	Inflammatory cell infiltration	1		0	2	-			-
Harderian Gland	Pigmentation	1			2	1			4
	Inflammatory cell infiltration	1			0	0			2
Kidney	Tubular dilation	1			0	0			1
	Inflammatory cell infiltration	1		1*	1	-			-
Liver	Inflammatory cell infiltration	1			1	2			0
Lymph Node (mandibular)	Erythrocytosis/erythrophagocytosis	0	2*	1*	1	0			1
Lymph Node (mesenteric)	Erythrocytosis/erythrophagocytosis	0	1*		0	-			-
Prostate	Inflammatory cell infiltration	2			0	-			-
Sternum	Fat increased	-			-	0			1
Stomach	Inflammatory cell infiltration, submucosal	2			1	-			-
Thyroid gland	Inflammatory cell infiltration	-			-	1			0
Urinary bladder	Proteinaceous plug	2			1	-			-
Uterus	Oestrus dilation	-			-	1	1*	1*	1
Vagina	Oestrous cycle: dioestrus					2			2
	Oestrous cycle: metoestrus					1			0
	Oestrous cycle: oestrus					0			2
	Oestrous cycle: proestrus					2			1

<sup>a</sup> For the purposes of tabulation only tissues that presented with abnormalities have been presented; therefore, if an organ is not presented no abnormality was detected.

\* These tissues were further examined microscopically due to the lesions identified during macroscopic examination

**Discussion:** The overall mean achieved dosages were 0, 7.3, 37.4 and 143.1 mg SYN508272/kg/day in males and 0, 7.8, 42.5 and 243.5 mg SYN508272/kg/day in females corresponding to dietary inclusions levels of 0, 100, 500 and 2000 ppm (males)/4000 ppm (females). There were no premature decedents or notable clinical observations during this study. Group mean body weights, cumulative body weight change and food consumption in females receiving 4000 ppm were lower throughout the treatment period when compared with controls, with statistical significance being achieved for cumulative body weight change. At the end of 28 days, the mean cumulative body weight gain in 4000 ppm females was 77.8% lower than

the control value. Cumulative body weight change and food consumption in males receiving 2000 ppm were also lower for first 12 days of the treatment when compared with controls, what impacted on mean body weights in this period. The cumulative weight gain after 12 days of feeding was 40.6% lower than the control value and this difference was statistically significant. Body weight for males at 2000 ppm recovered during the remainder of the study, and the cumulative body weight gain at the end of the study was only 5.5% lower than the control values. There were no treatment related inter-group differences in the functional observation battery parameters following administration of SYN508272 at dietary inclusion levels up to 2000 ppm in males and 4000 ppm in females.

Higher white blood cell (WBC) counts and higher values for the related WBC differential counts of neutrophils, lymphocytes, monocytes and basophils were noted in males at 2000 ppm and females at 4000 ppm. In the absence of any micropathology or signs of infection or disease these changes are considered not adverse. Among clinical chemistry parameters, statistically significant differences were only seen in the high dose groups. Lower globulin values and corresponding higher albumin-globulin ratios were observed in 2000 ppm males and 4000 ppm females. Creatinine levels were higher in females at 4000 ppm, but they were unaffected in males at 2000 ppm. Urea levels were lower in males at 2000 ppm, but they were unaffected in females at 4000 ppm. In the absence of any changes in micropathology, and considering the direction of the changes (urea) and their presence in one sex only (urea and creatinine), these small differences are considered not adverse. There were no treatment-related changes in urinalysis. The small number of organ weight changes that were observed, were considered to be within the range of normal variability in rats of this age and strain, and not an effect of treatment. There were no macroscopic or microscopic findings that could be related to the administration of SYN508272.

## CONCLUSION:

In a GLP and OECD compliant 28-day study in Han Wistar rats, animals (5/group) were given dietary concentrations of SYN508272 at 0, 100, 500 and 2000 (males)/4000 (females) ppm, equivalent to 0, 7.3/7.8, 37.4/42.5 and 143.1/243.5 mg/kg bw/d in males/females. There were no mortalities or clinical observations during the study. Group mean body weights, cumulative body weight gain and food consumption in females receiving 4000 ppm were lower throughout the treatment period when compared with controls, with statistical significance being achieved for cumulative body weight gain. At the end of 28 days, the mean cumulative body weight gain in 4000 ppm females was reduced by 22.2%. Cumulative body weight gain and food consumption in males receiving 2000 ppm were also lower for first 12 days of the treatment when compared with controls. The cumulative weight gain after 12 days of feeding was reduced by 59.4% and was statistically significant. Body weight for males at 2000 ppm recovered during the remainder of the study, and the cumulative body weight gain at the end of the study was only 5.5% lower than the control values. There were no treatment related effects on the FOB and MA parameters.

Higher white blood cell (WBC) counts and higher values for the related WBC differential counts of neutrophils, lymphocytes, monocytes and basophils were noted in males at 2000 ppm and females at 4000 ppm. In the absence of any micropathology or signs of infection or disease, these changes were considered not adverse. Among clinical chemistry parameters, statistically significant differences were only seen in the high dose groups. Lower globulin values and corresponding higher albumin-globulin ratios were observed in 2000 ppm males and 4000 ppm females. Creatinine levels were higher in females at 4000 ppm, but they were unaffected in males at 2000 ppm. Urea levels were lower in males at 2000 ppm, but they were unaffected in females at 4000 ppm. In the absence of any changes in micropathology, and considering the direction of the changes (urea) and their presence in one sex only (urea and creatinine), these small differences were considered not adverse. There were no treatment-related changes in urinalysis. The small number of organ weight changes that were observed, were considered to be within the range of normal variability in rats of this age and strain, and not an effect of treatment. There were no macroscopic or microscopic findings that could be related to the administration of SYN508272.

At 500 ppm and 100 ppm, there were no effects of treatment. As a result, HSE agrees with the EU evaluation, that **a NOAEL of 500 ppm (37.4/42.5 mg/kg bw/d in males/females) can be identified from this study based on effects on body weights and food consumption at the next dose level of 2000/4000 ppm (143.1/243.5 mg/kg bw/d in males/females).**



(██████████, 2015)

**Summary of toxicity data on metabolite SYN508272****Summary of toxicity studies and derivation of dietary reference values for metabolite SYN508272 glucuronide/sulphate**

Metabolite SYN508272 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN508272. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

Overall, several GLP and OECD compliant toxicity studies (acute oral toxicity study and 28-d study) and standard in vitro and in vivo genotoxicity assays are available on metabolite SYN508272. The metabolite was positive in the in vitro chromosome aberration test, but this result was not confirmed in vivo in a valid rat bone marrow micronucleus study. It was of moderate acute oral toxicity ( $500 < LD_{50} < 2000$  mg/kg bw) in the rat and a NOAEL of 500 ppm (37.4/42.5 mg/kg bw/d in males/females) was identified from a 28-day study in the rat based on effects on body weights and food consumption at the next dose level of 2000/4000 ppm (143.1/243.5 mg/kg bw/d in males/females).

The table below summarises the available studies on SYN508272:

Study & Acceptability	Result	Reference
Acute Oral Toxicity Study in the Han Wistar Rat. GLP, OECD 423  <i>Acceptable</i>	$LD_{50} > 500 < 2000$ mg/kg bw	██████████ (2009). Report No. 2009/1084176. Syngenta File No. SYN508272_10918
Ames test in bacteria GLP, OECD 471  <i>Acceptable</i>	Negative up to limit concentration	██████████ (2014a). Report No. Harlan 1555901. Syngenta File No. SYN508272_10908
Chromosome Abberation Test in Human Lymphocytes <i>In Vitro</i> . GLP, OECD 473  <i>Acceptable</i>	Positive – S9 (22 hr)	██████████ (2013a). Report No. Harlan 1555902. Syngenta File No. SYN508272_10904
Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells. GLP, OECD 476  <i>Acceptable</i>	Negative up to limit concentration	██████████ (2013a). Report No. Harlan 1555903. Syngenta File No. SYN508272_10906
Micronucleus Assay in bone marrow of the Wistar Rat GLP, OECD 474  <i>Acceptable</i>	Negative*	██████████ (2014a). Report No. ██████████ 1602600. Syngenta File No. SYN508272_10910
28-Day Oral (Dietary) Toxicity Study in the Han Wistar Rat. GLP, OECD 407  <i>Acceptable</i>	NOAEL = 500 ppm (~37/42.5 mg/kg bw/d in males/females) Reductions in body weight gain and food consumption.	██████████ (2015). Report No. ██████████ 35015. Syngenta File No. SYN508272

\* Exposure of the target tissue (bone marrow) to SYN508272 has been demonstrated in a blood analysis study (K-CA 5.8.1/14; ██████████ (2018)).

In conclusion, SYN508272 appears to be of higher toxicity than the parent substance, with moderate acute oral toxicity compared to the low acute toxicity of the parent ( $LD_{50} > 5000$  mg/kg bw). In the 28-day toxicity study in the rat, reductions in body weight gain and food consumption were observed at 143 mg/kg bw/d (males)/243.5 mg/kg bw/d (females). In comparison, the same effect (decrease BW gains) was observed at 10 fold higher dosage (i.e. 1322 mg/kg bw/d) in the equivalent 28-d study in rat performed with

pydiflumetofen. One explanation of these differences may be a higher oral absorption of the metabolite compared to the parent. Indeed, ADME studies demonstrated that oral absorption of pydiflumetofen is limited by the dose level: 19-24% at 300 mg/kg bw in males and 50-55% at 100 mg/kg bw in females.

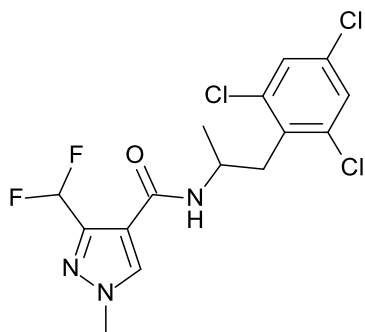
Therefore, from a toxicological point of view, SYN508272 needs to be considered in the residue definition for risk assessment (RD-RA).

SYN508272 is a major rat metabolite of pydiflumetofen as it was detected in plasma accounting for up to 14.8% of the total radioactivity AUC (TRA). On this basis, its toxicological profile can be considered covered by that of the parent and the parent dietary reference values could be used in the risk assessment.

However, HSE agrees with the EU, that given its higher toxicity potential compared to the parent, it would be more appropriate to set metabolite specific reference values on the basis of the available data. An ADI of 0.04 mg/kg bw/d was set at EU level from the NOAEL of the 28-day study with the application of an UF of 1000 (extra assessment factor of 10 to account for the limited database, as no long-term, multigeneration or developmental toxicity studies are available). The ARfD was set at the same level of the ADI. It should be noted that this metabolite-specific ADI is lower than the parent ADI (0.09 mg/kg bw/d), confirming the relative higher toxicity of the metabolite.

If from a residue perspective, a dietary risk assessment is required for this metabolite, SYN508272 glucuronide/sulphate could be included in the RD-RA together with parent by applying a Relative Potency Factor (RPF) of 2.25. Alternatively, a separate RD-RA could be set for this metabolite using its specific ADI and ARfD.

#### **B.6.8.1.3. Metabolite SYN545547 glucuronide/sulphate (CSCD550897 glucuronide/sulphate)**



3-(difluoromethyl)-1-methyl-N-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]pyrazole-4-carboxamide (SYN545547 aglycon)

Metabolite SYN545547 glucuronide/sulphate is a plant and livestock metabolite. In the human gastrointestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN545547. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

SYN545547 is a metabolite of PYDIFLUMETOFEN (SYN545974), which has been identified in animal commodities, primary and rotated crops. An *in vitro* battery of genotoxicity assay was conducted on SYN545547. In addition, a comparative genotoxicity QSAR analysis has been conducted on SYN545547 and the parent substance.

#### **Comparative genotoxicity QSAR analysis**

<b>Report:</b>	K-CA 5.8.1/69 Syngenta Ltd. (2016). SYN545547: A Multi-(Q)SAR Genotoxicity Assessment Of SYN545974 and SYN545547. Syngenta Ltd. Jealott's Hill International Research, Bracknell, Berks RG42 6EY. Laboratory Report No. TK0288608, 07 January 2016. Unpublished. Syngenta File No. SYN545974_10433.
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**Guidelines:** This was a review article with no applicable guidelines.

**GLP:** Not applicable as no experimental work conducted.

## EXECUTIVE SUMMARY

Three (Q)SAR programs were selected working on different basis of expert knowledge rules and statistical methods for the assessment of genotoxicity. These were; DEREK Nexus (genotoxicity endpoints), CAESAR (mutagenicity) and ToxTree (structural alerts for *in vivo* micronucleus formation). Additionally, the OECD QSAR Toolbox was used to assess DNA and protein binding, DNA alerts for Ames, MN and CA, *in vivo* MN alerts and for functional group profiling. Hence, overall, using these tools genotoxicity endpoints of *in vitro* and *in vivo* mutagenicity, chromosome damage and DNA/protein binding were considered.

DEREK Nexus: No alerts for SYN545974 (pydiflumetofen) and SYN545547.

CAESAR: No alerts.

ToxTree: Identical alerts for SYN545974 (pydiflumetofen) and SYN545547: SA-34: hacceptor-path-hacceptor).

OECD QSAR Toolbox: Identical alerts for SYN545974 and SYN545547 (*in vitro* mutagenicity – aliphatic hydrocarbons, unsaturated carbonyls, hydrazines; *in vivo* mutagenicity – aliphatic hydrocarbons, unsaturated carbonyls, H-acceptor-path3-H-acceptor, hydrazine; organic functional groups – alkyl halide, aryl, aryl halide, N-hydroxylamine derivatives, pyrazole). In addition, SYN545547 had alerts for protein binding (acylation ester, aminolysis amides). This alert is implicated in skin sensitisation. No protein binding activity was identified by the “protein binding by OECD” profiler or by the “protein binding alerts for chromosome aberration” profiler, hence the confidence of this alert is doubted.

SYN545974 has been tested in a comprehensive battery of *in vitro* and *in vivo* genotoxicity assays. From this robust data set SYN545974 has been demonstrated to be non-genotoxic. Therefore, the alerts identified by the (Q)SAR performed on SYN545974 can be concluded to be falsely overpredictive and dismissed. SYN545974 and SYN545547 are highly structurally similar and consequently it is predicted that SYN545547 will be non-genotoxic.

## CONCLUSION

Genotoxicity predictions were obtained from 4 different models : Derek, Caesar, ToxTree and OECD QSAR Toolbox. Derek and Caesar identified no alerts. ToxTree and the OECD QSAR Toolbox identified some alerts; however, these were identical between the metabolite and the parent substance for which a comprehensive data package showed absence of genotoxicity. Therefore, the alerts identified by the latter two models were overpredictive and could be dismissed. Overall, SYN545547 and pydiflumetofen are highly structurally similar and consequently it is predicted that SYN545547 will be non-genotoxic.

EFSA requested further details of the QSAR analysis; however, this was not required, as a battery of *in vitro* genotoxicity tests on the metabolite was subsequently made available.

(Syngenta Ltd., 2016)

<b>Report:</b>	K-CA 5.8.1/70 [REDACTED] (2017). SYN545547 - Bacterial Reverse Mutation Test. Envigo CRS Limited, Woolley Road, Alconbury, Huntingdon, Cambridgeshire, PE28 4HS, United Kingdom. Laboratory Report No. MK43KP; 15 February 2017. Unpublished. Syngenta File No. SYN545547_10005.
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**Guidelines:** OECD 471 (1997); OPPTS 870.5100 (1998); 2000/32/EEC B.13/B.14 (2000)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

In this *in vitro* assessment of the mutagenic potential of SYN545547, histidine-dependent auxotrophic mutants of *Salmonella typhimurium*, strains TA1535, TA1537, TA98 and TA100, and a tryptophan-dependent mutant of *Escherichia coli*, strain WP2 *uvrA* (pKM101), were exposed to SYN545547 diluted in dimethyl sulfoxide (DMSO). DMSO was also used as a vehicle control.

Two independent mutation experiments were performed in the presence and absence of liver preparations (S9 mix) from rats treated with phenobarbital and 5,6-benzoflavone. The first experiment was a standard plate incorporation assay; the second included a pre-incubation stage.

Concentrations of SYN545547 up to 5000 µg/plate were tested. This is the standard limit concentration recommended in the regulatory guideline that this assay was conducted to. The other concentrations used were a series of ca half-log<sub>10</sub> dilutions of the highest concentration.

Toxicity (observed as a reduction in revertant colony numbers) was seen in strain TA98 at 500 µg/plate and above in the absence of S9 mix in both experiments. Precipitate was observed on all plates containing SYN545547 at 500 µg/plate and above in both experiments.

No evidence of mutagenic activity was seen at any concentration of SYN545547 in either experiment.

The concurrent positive controls verified the sensitivity of the assay and the metabolizing activity of the liver preparations. The mean revertant colony counts for the vehicle controls were within or close to the current historical control range for the laboratory.

**It was concluded that SYN545547 showed no evidence of mutagenic activity in this bacterial system under the test conditions employed.**

## MATERIALS AND METHODS

### Materials:

**Test Material:** SYN545547  
**Description:** White solid  
**Lot/Batch number:** BPS 1510/2  
**Purity:** 99% (w/w)  
**Stability of test compound:** Not indicated by the sponsor  
**Recertification date:** 07 December 2016

**Control Materials:**

**Negative:** Concurrent untreated and solvent controls were performed  
**Solvent control (final concentration):** 100µl/plate  
**Positive control:** Nonactivation:  
     Sodium azide (2 µg/plate) with TA100 and TA1535  
     9-Aminoacridine (50 µg/plate) with TA1537  
     2-Nitrofluorene (2 µg/plate) with strain TA98  
     4-Nitroquinoline-1-oxide (2 µg/plate) with strain WP2 uvrA  
   Activation:  
     2-Aminoanthracene (5 µg/plate) with TA100 and TA1535  
     2-Aminoanthracene (10 µg/plate) with WP2 uvrA  
     Benzo[a]pyrene (5 µg/plate) with TA98 and TA1537

**Mammalian metabolic system: S9 derived**

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

S9 mix contains: S9 fraction (10% v/v), MgCl<sub>2</sub> (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter sterilized before use.

**Test organisms:**

*S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

*E. coli* strains

X	WP2 (pKM101)		WP2 uvrA (pKM101)						
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Properly maintained?

X
X

Yes


No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?

Yes

No

**Test compound concentrations used**

The test substance was tested at the following concentrations in both experiments:

Experiment I: 5; 15; 50; 150; 500; 1500; and 5000 µg/plate

Experiment II: 0.5; 1.5; 5; 15; 50; 150; 500; 1500; and 5000 µg/plate

**Study Design and Methods:****In-life dates:** Start: 13 December 2016 End: 22 December 2016**TEST PERFORMANCE****Preliminary Cytotoxicity Assay:** Not performed.**Type of Bacterial assay:**

- X standard plate test (pre-experiment/experiment I; –S9, +S9)
- X pre-incubation (60 minutes) (second experiment ; –S9, +S9)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Study Design**

**Experiment 1 (Plate incorporation assay):** Aliquots of 0.1 mL of the test item solutions (seven concentrations up to 5000 µg/plate), positive control or vehicle control were placed in glass tubes. The vehicle control was DMSO. S9 mix (0.5 mL) or 0.1 M pH 7.4 sodium phosphate buffer (0.5 mL) was added, followed by 0.1 mL of a 10-hour bacterial culture and 2 mL of agar containing histidine (0.05 mM), biotin (0.05 mM) and tryptophan (0.05 mM). Three Petri dishes were used for each treatment. Plates were also prepared without the addition of bacteria in order to assess the sterility of the test item, S9 mix and sodium phosphate buffer. All plates were incubated at approximately 37°C for between 48 to 72 hours.

Any toxic effects of the test item may be detected by a substantial reduction in mean revertant colony counts, by a sparse or absent background bacterial lawn, or both. In the absence of any toxic effects, the maximum concentration selected for use in the Pre-incubation assay is the same as that used in the Plate incorporation assay. If toxic effects are observed, a lower concentration might be chosen, ensuring that signs of bacterial inhibition are present at this maximum concentration. Ideally, a minimum of four non-toxic concentrations should be obtained. If precipitate is observed on the plates at the end of the incubation period, at least four non-precipitating concentrations should be included in the second test, unless otherwise justified by the Study Director.

**Experiment 2 (Pre-incubation test):** As a clear negative response was obtained in the first experiment, a variation to the test procedure was used for the second experiment. The variation used was the pre-incubation assay in which the tubes, which contained mixtures of bacteria, buffer or S9 mix and test dilution, were incubated at 37°C for 30 minutes with shaking before the addition of the agar overlay. The maximum concentration chosen was again 5000 µg/plate.

**Statistics**

The results satisfied the criteria for a negative response and no statistical analysis was conducted.

**Evaluation criteria:**

If exposure to a test item produces a reproducible increase in mean revertant colony numbers of at least twice that of the concurrent vehicle controls, with some evidence of a positive concentration-response relationship, it is considered to exhibit mutagenic activity in this test system.

If exposure to a test item does not produce a reproducible increase in mean revertant colony numbers, it is considered to show no evidence of mutagenic activity in this test system. No statistical analysis is performed since additionally this is not required by the guideline.

If the results obtained fail to satisfy the criteria for a clear "positive" or "negative" response, even after additional testing, the test data may be subjected to analysis to determine the statistical significance of any

increases in revertant colony numbers. The statistical procedures used are those described by Mahon et al (1989) and are usually Dunnett's test followed, if appropriate, by trend analysis. Biological importance will be considered along with statistical significance. In general, treatment-associated increases in mean revertant colony numbers below two or three times those of the vehicle controls (as described above) are not considered biologically important. It should be noted that it is acceptable to conclude an equivocal response if no clear results can be obtained.

Occasionally, these criteria may not be appropriate to the test data and, in such cases, the Study Director would use their scientific judgment.

## RESULTS

**Experiment 1 (Plate Incorporation Assay):** Toxicity, observed as a reduction in the number of revertant colonies, was obtained in strain TA98 at 500 µg/plate and above in the absence of S9 mix. Precipitate was observed on all plates containing SYN545547 at 500 µg/plate and above. A maximum exposure concentration of 5000 µg/plate was, therefore, selected for use in the second experiment.

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to SYN545547 at any concentration up to and including 5000 µg/plate in either the presence or absence of S9 mix.

**Experiment 2 (Pre-incubation Assay):** Toxicity, observed as a reduction in the number of revertant colonies, was obtained in strain TA98 at 500 µg/plate and above in the absence of S9 mix. Precipitate was observed on all plates containing SYN545547 at 500 µg/plate and above.

No substantial increases in revertant colony numbers over control counts were obtained with any of the tester strains following exposure to SYN545547 at any concentration up to and including 5000 µg/plate in either the presence or absence of S9 mix.

**Validity criteria:** For a test to be considered valid, the mean of the vehicle control revertant colony numbers for each strain should lie within or close to the current historical control range for the laboratory unless otherwise justified by the Study Director. The historical range is maintained as a rolling record over a maximum of two years or a minimum of twenty data sets.

The positive control compounds must induce an increase in mean revertant colony numbers of at least twice that of the concurrent vehicle controls.

Mean viable cell counts in the 10-hour bacterial cultures must be at least 109/mL.

A minimum of five analysable concentrations should be present with at least four showing no signs of toxic effects, evident as bacterial inhibition and/or a reduction in the number of revertants below the indication factor of 0.5.

**Table 6.8.1-45: Results Obtained in the Absence of Metabolic Activation:**

**Experiment 1** Without metabolic activation

Strain	Addition	Concentration per plate	Mean revertants per plate	Standard Deviation	Fold increase relative to vehicle	Individual revertant colony counts (Sorcerer)
TA98	DMSO		21.3	0.6		21, 21, 22
	SYN545547	5 µg	15.7	3.2	0.7	17, 12, 18
		15 µg	18.3	1.5	0.9	20, 18, 17
		50 µg	12.7	4.7	0.6	18, 11, 9
		150 µg	13.7	2.3	0.6	11, 15, 15
		500 µg	8.0	1.7	0.4	9 P, 9 P, 6 P
		1500 µg	8.0	1.0	0.4	9 P, 7 P, 8 P

		5000 µg	6.7	0.6	0.3	7 P, 6 P, 7 P
<b>TA100</b>	<b>DMSO</b> <b>SYN545547</b>		213.7	17.5		233, 209, 199
		5 µg	215.7	2.9	1.0	214, 214, 219
		15 µg	209.0	17.6	1.0	189, 216, 222
		50 µg	207.7	18.5	1.0	226, 208, 189
		150 µg	218.0	7.9	1.0	221, 209, 224
		500 µg	188.7	4.9	0.9	183 P, 191 P, 192 P
		1500 µg	163.3	7.1	0.8	157 P, 162 P, 171 P
		5000 µg	146.7	3.1	0.7	146 P, 150 P, 144 P
<b>TA1535</b>	<b>DMSO</b> <b>SYN545547</b>		14.0	1.7		15, 12, 15
		5 µg	10.3	1.2	0.7	11, 9, 11
		15 µg	13.7	2.1	1.0	13, 16, 12
		50 µg	10.3	1.2	0.7	11, 11, 9
		150 µg	8.3	4.2	0.6	5, 7, 13
		500 µg	11.7	2.9	0.8	10 P, 15 P, 10 P
		1500 µg	9.3	1.5	0.7	8 P, 11 P, 9 P
		5000 µg	8.3	3.5	0.6	8 P, 12 P, 5 P
<b>TA1537</b>	<b>DMSO</b> <b>SYN545547</b>		9.7	2.3		11, 7, 11
		5 µg	11.3	3.2	1.2	15, 9, 10
		15 µg	12.0	5.6	1.2	7, 18, 11
		50 µg	10.0	3.5	1.0	12, 6, 12
		150 µg	7.0	0.0	0.7	7, 7, 7
		500 µg	8.0	3.5	0.8	10 P, 10 P, 4 P
		1500 µg	5.7	1.5	0.6	7 P, 4 P, 6 P
		5000 µg	7.0	1.0	0.7	7 P, 8 P, 6 P
<b>WP2 uvrA</b> <b>(pKM101)</b>	<b>DMSO</b> <b>SYN545547</b>		91.3	4.9		88, 97, 89
		5 µg	100.7	8.0	1.1	93, 109, 100
		15 µg	111.3	11.9	1.2	115, 98, 121
		50 µg	77.3	12.1	0.8	90, 66, 76
		150 µg	72.3	8.4	0.8	68, 67, 82
		500 µg	77.0	8.7	0.8	67 P, 82 P, 82 P
		1500 µg	79.3	4.5	0.9	84 P, 75 P, 79 P
		5000 µg	56.7	7.4	0.6	54 P, 51 P, 65 P
<b>TA98</b>	<b>2NF</b>	2 µg	302.0	93.5	14.2	210, 299, 397
<b>TA100</b>	<b>NaN3</b>	2 µg	596.0	54.7	2.8	656, 583, 549
<b>TA1535</b>	<b>NaN3</b>	2 µg	670.3	87.5	47.9	570, 710, 731
<b>TA1537</b>	<b>AAC</b>	50 µg	143.7	16.2	14.9	154, 152, 125
<b>WP2 uvrA</b> <b>(pKM101)</b>	<b>NQO</b>	2 µg	1308.7	100.7	14.3	1424, 1238, 1264

## Key to Positive Controls

2NF	2-Nitrofluorene
NaN3	Sodium azide
AAC	9-Aminoacridine
NQO	4-Nitroquinoline-1-oxide

## Key to Plate Postfix Codes

P	Precipitate
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**Table 6.8.1-46: Results Obtained in the Presence of Metabolic Activation:****Experiment 1 With metabolic activation**

Strain	Addition	Concentration per plate	Mean revertants per plate	Standard Deviation	Fold increase relative to vehicle	Individual revertant colony counts (Sorcerer)
<b>TA98</b>	<b>DMSO</b> <b>SYN545547</b>		19.3	2.1		21, 17, 20
		5 µg	14.3	3.8	0.7	16, 10, 17
		15 µg	14.3	1.2	0.7	15, 15, 13
		50 µg	14.7	4.6	0.8	20, 12, 12
		150 µg	15.3	2.5	0.8	13, 18, 15
		500 µg	20.0	4.6	1.0	21 P, 24 P, 15 P
		1500 µg	13.3	1.5	0.7	15 P, 12 P, 13 P



		5000 µg	13.3	1.2	0.7	14 P, 14 P, 12 P
<b>TA100</b>	<b>DMSO</b> <b>SYN545547</b>		204.7	19.1		199, 226, 189
		5 µg	206.0	10.8	1.0	203, 197, 218
		15 µg	213.3	8.5	1.0	205, 213, 222
		50 µg	237.7	11.9	1.2	246, 243, 224
		150 µg	220.3	21.0	1.1	199, 221, 241
		500 µg	229.7	14.2	1.1	221 P, 222 P, 246 P
		1500 µg	184.7	7.8	0.9	187 P, 191 P, 176 P
		5000 µg	184.3	8.7	0.9	182 P, 194 P, 177 P
<b>TA1535</b>	<b>DMSO</b> <b>SYN545547</b>		11.3	0.6		11, 11, 12
		5 µg	7.3	3.2	0.6	5, 6, 11
		15 µg	7.7	2.1	0.7	7, 10, 6
		50 µg	10.3	2.9	0.9	7, 12, 12
		150 µg	8.3	3.2	0.7	7, 6, 12
		500 µg	11.3	3.2	1.0	15 P, 10 P, 9 P
		1500 µg	6.7	1.5	0.6	7 P, 8 P, 5 P
		5000 µg	7.0	1.0	0.6	6 P, 7 P, 8 P
<b>TA1537</b>	<b>DMSO</b> <b>SYN545547</b>		8.7	1.5		7, 9, 10
		5 µg	7.0	2.0	0.8	9, 7, 5
		15 µg	8.7	1.5	1.0	9, 10, 7
		50 µg	7.0	1.7	0.8	6, 9, 6
		150 µg	7.0	3.0	0.8	7, 4, 10
		500 µg	7.3	2.3	0.8	6 P, 6 P, 10 P
		1500 µg	5.3	0.6	0.6	5 P, 6 P, 5 P
		5000 µg	6.3	0.6	0.7	6 P, 7 P, 6 P
<b>WP2 uvrA</b> <b>(pKM101)</b>	<b>DMSO</b> <b>SYN545547</b>		115.7	15.6		101, 114, 132
		5 µg	93.0	21.3	0.8	70, 97, 112
		15 µg	90.7	9.1	0.8	84, 101, 87
		50 µg	98.3	11.5	0.9	87, 110, 98
		150 µg	110.7	4.5	1.0	115, 111, 106
		500 µg	110.7	4.2	1.0	106 P, 112 P, 114 P
		1500 µg	88.0	5.6	0.8	94 P, 83 P, 87 P
		5000 µg	79.0	2.6	0.7	76 P, 81 P, 80 P
<b>TA98</b>	<b>B[a]P</b>	5 µg	149.3	8.7	7.7	159, 142, 147
<b>TA100</b>	<b>AAN</b>	5 µg	2656.0	43.6	13.0	2667, 2693, 2608
<b>TA1535</b>	<b>AAN</b>	5 µg	249.7	50.5	22.0	308, 222, 219
<b>TA1537</b>	<b>B[a]P</b>	5 µg	69.3	3.8	8.0	72, 71, 65
<b>WP2 uvrA</b> <b>(pKM101)</b>	<b>AAN</b>	10 µg	1166.7	21.2	10.1	1144, 1186, 1170

## Key to Positive Controls

B[a]P Benzo[a]pyrene  
AAN 2-Aminoanthracene

## Key to Plate Postfix Codes

P Precipitate

**Table 6.8.1-47: Results Obtained in the Absence of Metabolic Activation: Experiment 2**  
**Without metabolic activation**

Strain	Addition	Concentration per plate	Mean revertants per plate	Standard Deviation	Fold increase relative to vehicle	Individual revertant colony counts (Sorcerer)
<b>TA98</b>	<b>DMSO</b> <b>SYN545547</b>		22.3	0.6		22, 22, 23
		0.5 µg	17.7	0.6	0.8	17, 18, 18
		1.5 µg	18.0	0.0	0.8	18, 18, 18
		5 µg	17.0	0.0	0.8	17, 17, 17
		15 µg	18.0	0.0	0.8	18, 18, 18

		50 µg	12.7	5.8	0.6	16, 6, 16
		150 µg	15.0	1.7	0.7	16, 13, 16
		500 µg	4.0	2.6	0.2	6 P, 1 P, 5 P
		1500 µg	5.7	1.5	0.3	7 P, 6 P, 4 P
		5000 µg	6.0	2.0	0.3	4 P, 6 P, 8 P
<b>TA100</b>	<b>DMSO SYN545547</b>		145.0	2.6		144, 143, 148
		0.5 µg	140.0	5.3	1.0	134, 144, 142
		1.5 µg	144.0	8.9	1.0	137, 154, 141
		5 µg	139.7	10.7	1.0	149, 128, 142
		15 µg	164.0	10.8	1.1	176, 161, 155
		50 µg	153.3	19.7	1.1	143, 176, 141
		150 µg	134.7	9.3	0.9	145, 132, 127
		500 µg	142.0	21.0	1.0	119 P, 147 P, 160 P
		1500 µg	134.0	7.0	0.9	127 P, 134 P, 141 P
		5000 µg	130.7	13.7	0.9	137 P, 140 P, 115 P
<b>TA1535</b>	<b>DMSO SYN545547</b>		15.3	2.9		17, 12, 17
		0.5 µg	9.7	0.6	0.6	10, 9, 10
		1.5 µg	10.3	0.6	0.7	10, 10, 11
		5 µg	10.3	3.8	0.7	13, 6, 12
		15 µg	14.7	6.8	1.0	7, 17, 20
		50 µg	12.3	6.4	0.8	5, 16, 16
		150 µg	16.3	0.6	1.1	16, 16, 17
		500 µg	14.3	3.1	0.9	15 P, 17 P, 11 P
		1500 µg	12.3	1.5	0.8	11 P, 14 P, 12 P
		5000 µg	10.3	1.5	0.7	9 P, 12 P, 10 P
<b>TA1537</b>	<b>DMSO SYN545547</b>		10.0	0.0		10, 10, 10
		0.5 µg	6.7	0.6	0.7	6, 7, 7
		1.5 µg	8.0	4.6	0.8	13, 4, 7
		5 µg	6.0	1.0	0.6	5, 6, 7
		15 µg	6.0	1.0	0.6	6, 5, 7
		50 µg	9.0	0.0	0.9	9, 9, 9
		150 µg	7.3	2.3	0.7	6, 10, 6
		500 µg	9.7	0.6	1.0	10 P, 10 P, 9 P
		1500 µg	6.7	0.6	0.7	7 P, 6 P, 7 P
		5000 µg	7.3	0.6	0.7	8 P, 7 P, 7 P
<b>WP2 uvrA (pKM101)</b>	<b>DMSO SYN545547</b>		90.3	5.7		84, 92, 95
		0.5 µg	82.3	5.1	0.9	81, 78, 88
		1.5 µg	91.0	9.2	1.0	93, 81, 99
		5 µg	101.3	8.1	1.1	100, 110, 94
		15 µg	95.3	11.6	1.1	82, 103, 101
		50 µg	79.0	8.7	0.9	89, 73, 75
		150 µg	70.7	6.4	0.8	66, 78, 68
		500 µg	79.0	9.5	0.9	70 P, 89 P, 78 P
		1500 µg	70.3	2.1	0.8	68 P, 72 P, 71 P
		5000 µg	61.0	4.6	0.7	60 P, 57 P, 66 P
<b>TA98</b>	<b>2NF</b>	2 µg	361.0	84.7	16.2	273, 368, 442
<b>TA100</b>	<b>NaN3</b>	2 µg	428.7	29.2	3.0	395, 444, 447
<b>TA1535</b>	<b>NaN3</b>	2 µg	454.3	37.9	29.6	422, 496, 445
<b>TA1537</b>	<b>AAC</b>	50 µg	224.7	24.6	22.5	227, 248, 199
<b>WP2 uvrA (pKM101)</b>	<b>NQO</b>	2 µg	1604.3	91.1	17.8	1693, 1511, 1609

## Key to Positive Controls

2NF	2-Nitrofluorene
NaN3	Sodium azide
AAC	9-Aminoacridine
NQO	4-Nitroquinoline-1-oxide

## Key to Plate Postfix Codes

P	Precipitate
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**Table 6.8.1-48: Results Obtained in the Presence of Metabolic Activation: Experiment 2  
With metabolic activation**

Strain	Addition	Concentration per plate	Mean revertants per plate	Standard Deviation	Fold increase relative to vehicle	Individual revertant colony counts (Sorcerer)
TA98	DMSO SYN545547		22.3	0.6		23, 22, 22
		0.5 µg	18.0	4.4	0.8	13, 21, 20
		1.5 µg	15.0	3.6	0.7	16, 18, 11
		5 µg	18.0	0.0	0.8	18, 18, 18
		15 µg	16.3	4.7	0.7	18, 20, 11
		50 µg	18.7	6.7	0.8	26, 13, 17
		150 µg	14.7	2.5	0.7	12, 17, 15
		500 µg	21.0	4.6	0.9	20 P, 26 P, 17 P
		1500 µg	17.3	1.2	0.8	16 P, 18 P, 18 P
		5000 µg	18.3	2.3	0.8	17 P, 21 P, 17 P
TA100	DMSO SYN545547		128.0	13.1		142, 116, 126
		0.5 µg	111.0	14.1	0.9	98, 109, 126
		1.5 µg	114.7	6.1	0.9	108, 116, 120
		5 µg	136.3	23.4	1.1	119, 163, 127
		15 µg	137.3	5.1	1.1	133, 143, 136
		50 µg	139.0	2.6	1.1	137, 142, 138
		150 µg	150.0	14.9	1.2	161, 133, 156
		500 µg	163.7	7.6	1.3	167 P, 155 P, 169 P
		1500 µg	145.0	5.6	1.1	140 P, 151 P, 144 P
		5000 µg	124.7	9.7	1.0	133 P, 114 P, 127 P
TA1535	DMSO SYN545547		7.7	1.2		7, 7, 9
		0.5 µg	10.3	4.2	1.3	15, 7, 9
		1.5 µg	7.0	2.6	0.9	6, 5, 10
		5 µg	9.7	2.3	1.3	7, 11, 11
		15 µg	9.7	2.5	1.3	10, 7, 12
		50 µg	5.0	1.0	0.7	4, 6, 5
		150 µg	11.7	5.0	1.5	11, 7, 17
		500 µg	13.7	3.2	1.8	16 P, 10 P, 15 P
		1500 µg	8.0	1.0	1.0	7 P, 8 P, 9 P
		5000 µg	8.3	3.2	1.1	12 P, 7 P, 6 P
TA1537	DMSO SYN545547		7.3	1.5		9, 6, 7
		0.5 µg	8.0	1.7	1.1	6, 9, 9
		1.5 µg	6.7	0.6	0.9	6, 7, 7
		5 µg	7.0	0.0	1.0	7, 7, 7
		15 µg	11.0	5.3	1.5	7, 17, 9
		50 µg	6.7	2.1	0.9	9, 6, 5
		150 µg	7.0	4.4	1.0	9, 10, 2
		500 µg	7.3	1.5	1.0	9 P, 6 P, 7 P
		1500 µg	5.7	1.5	0.8	7 P, 6 P, 4 P
		5000 µg	7.3	1.2	1.0	8 P, 6 P, 8 P
WP2 <i>uvrA</i> (pKM101)	DMSO SYN545547		109.0	10.5		119, 110, 98
		0.5 µg	102.3	8.6	0.9	93, 104, 110
		1.5 µg	90.3	10.0	0.8	79, 94, 98
		5 µg	109.3	19.7	1.0	97, 99, 132
		15 µg	109.3	3.8	1.0	105, 111, 112
		50 µg	102.0	13.1	0.9	111, 108, 87
		150 µg	92.7	7.2	0.9	88, 89, 101
		500 µg	105.0	17.0	1.0	105 P, 122 P, 88 P
		1500 µg	88.7	17.6	0.8	107 P, 72 P, 87 P
		5000 µg	82.3	4.9	0.8	80 P, 79 P, 88 P

TA98	B[a]P	5 µg	224.3	15.5	10.0	209, 224, 240
TA100	AAN	5 µg	1579.3	200.5	12.3	1380, 1781, 1577
TA1535	AAN	5 µg	249.3	32.1	32.5	216, 252, 280
TA1537	B[a]P	5 µg	79.0	6.1	10.8	82, 83, 72
WP2 uvrA (pKM101)	AAN	10 µg	1162.0	44.3	10.7	1184, 1191, 1111

Key to Positive Controls		Key to Plate Postfix Codes	
B[a]P	Benzo[a]pyrene	P	Precipitate
AAN	2-Aminoanthracene		

## CONCLUSION:

In a GLP and OECD compliant Ames test, the potential of SYN545547 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strain WP2 uvrA (pKM 101), was investigated up to the limit concentration of 5000 µg/plate (in DMSO) with and without metabolic activation.

Toxicity (observed as a reduction in revertant colony numbers) was seen in strain TA98 at 500 µg/plate and above in the absence of S9 mix in both experiments. Precipitate was observed on all plates containing SYN545547 at 500 µg/plate and above in both experiments. No evidence of mutagenic activity was seen at any concentration of SYN545547 in either experiment. The concurrent positive controls verified the sensitivity of the assay and the metabolizing activity of the liver preparations. The mean revertant colony counts for the vehicle controls were within or close to the current historical control range for the laboratory.

As a result, HSE agrees with the EU evaluation, that **SYN545547 is not mutagenic in bacteria when tested up to the concentrations causing cytotoxicity and/or precipitation.**

(██████████. 2017)

### In vitro micronucleus test

<b>Report:</b> K-CA 5.8.1/71 ██████████ (2017a). SYN545547 - In Vitro Micronucleus Test in Human Lymphocytes. Envigo CRS Limited, Woolley Road, Alconbury, Huntingdon, Cambridgeshire, PE28 4HS, United Kingdom. Laboratory Report No. NH83HP; 28 March 2017. Unpublished. Syngenta File No. SYN545547_10009.
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**Guidelines:** OECD 487 (2016)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

This study was designed to assess the potential of SYN545547 to cause an increase in the induction of micronuclei in cultured human peripheral blood lymphocytes *in vitro*.

The study consisted of a preliminary toxicity test, a main micronucleus test and an additional test for 3-hour in the presence of S9-mix. Human lymphocytes in whole blood culture were exposed to SYN545547 for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix) and for 20 hours

in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 696 µg/mL. This was considered to be the maximum achievable concentration based on the solubility of the test item. Vehicle (dimethyl sulphoxide [DMSO]) and positive control cultures were included in all appropriate test conditions.

Three SYN545547 concentrations were assessed for determination of induction of micronuclei. The highest concentration selected for all exposures was that which caused a reduction in CBPI equivalent to 55±5% cytotoxicity. Following a 3-hour treatment in the absence of S9 mix, reductions in CBPI equivalent to 60.0% cytotoxicity were obtained with SYN545547 at 135 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis were 10, 85 and 135 µg/mL. Following 3-hour treatment in the presence of S9 mix, reductions in CBPI equivalent to 50.2% cytotoxicity were obtained with SYN545547 at 140 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis were 5, 100 and 140 µg/mL. In the absence of S9 mix following 20-hour treatment, a reduction in CBPI equivalent to 56.0% cytotoxicity was obtained with SYN545547 at 29 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis were 1, 10 and 29 µg/mL.

In both the absence and presence of S9 mix, following 3-hour treatment, and in the absence of S9 mix, following 20 hour treatment, SYN545547 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls.

The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused statistically significant increases in the number of binucleate cells containing micronuclei under appropriate conditions, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

**It was concluded that SYN545547 did not show evidence of causing an increase in the induction of micronuclei in cultured human lymphocytes, in this *in vitro* test system under the experimental conditions described. Hence, SYN545547 is concluded to be negative in this assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545547
<b>Description:</b>	White solid
<b>Lot/Batch number:</b>	BPS 1510/2
<b>Molecular weight:</b>	396.7 g/mol
<b>Purity</b>	99% w/w
<b>Stability of test compound:</b>	Not indicated by the sponsor
<b>Control Materials:</b>	
<b>Negative:</b>	-
<b>Solvent control</b>	
<b>(final concentration):</b>	DMSO
<b>Positive control:</b>	Absence of S9 mix: Mitomycin C (0.3 and 0.1 µg/mL, 3 and 20-hour exposure respectively). Colchicine (0.07 and 0.01 µg/mL, 3 and 20-hour exposure respectively) Presence of S9 mix: Cyclophosphamide (10 µg/mL)

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

S9 mix contained: S9 fraction (10% v/v), MgCl<sub>2</sub> (8 mM), KCl (33 mM), sodium phosphate buffer pH 7.4 (100 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter sterilized before use.

**Test cells: mammalian cells in culture**

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the day of culture initiation from healthy, non-smoking donors aged between 18 and 35 years.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: RPMI 1640, supplemented with 10% fetal calf serum, 0.2 IU/mL sodium heparin, 20 IU/mL penicillin / 20 µg/mL streptomycin and 2.0 mM L-glutamine.				
Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

**Test compound concentrations used:**

Preliminary toxicity test 1.36, 2.72, 5.44, 10.88, 21.75, 43.5, 87, 174, 348 and 696 µg/mL

Main Experiment

Absence of S9 mix (3-hour) 10, 20, 50, 75, 85, 95, 105, 115, 125, 135, 155 and 175 µg/mL

Presence of S9 mix (3-hour) 5, 50, 100, 125, 130, 135, 140, 145, 150 and 155 µg/mL

Absence of S9 mix (20-hour) 1, 10, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47 and 50 µg/mL

**Study Design and Methods:**

**In-life dates:** Start: 12 December 2016, End: 07 February 2017

**Test performance:**

**Preliminary toxicity test:**

**3-Hour Treatment in the Absence and Presence of S9 Mix**

Lymphocyte cultures were incubated for approximately 48 hours following stimulation with PHA, before addition of the test item. The test item was prepared in the vehicle and dilutions made for both sets of cultures. Single cultures were prepared for each treatment level and duplicate cultures were prepared for vehicle controls. S9 homogenate was present in appropriate cultures at a final concentration of 2% v/v.

Before treatment all cultures were centrifuged and re-suspended in the required volume of fresh medium, taking into account the treatment volume and S9 mix volume, where required.

Test item preparations were added to cultures at 0.5% v/v. Cultures were incubated at 34 to 39°C for 3 hours.

The cells were centrifuged and the medium was replaced with fresh medium. Cytochalasin B, at a final concentration of 6 µg/mL, was then added to all cultures. The cultures were incubated for a further 17 hours until the scheduled harvest time.

**20-Hour Treatment in the Absence of S9 Mix**

Human lymphocyte cultures were set up as previously described. A 20-hour continuous treatment (1.5 to 2 normal cell cycles) at 34 to 39°C was used in the absence of S9 mix. Test item preparations were added to cultures at 0.5% v/v in the presence of Cytochalasin B (6 µg/mL).

**Harvesting and Fixation**

The cells were harvested by centrifugation at 500 g for 5 minutes. The supernatant was removed and the cell pellet re-suspended and treated with a 4 mL hypotonic solution (0.075M KCl) at 34 to 39°C, cultures were then incubated for 3 minutes at 34 to 39°C to cause swelling. Cultures were agitated, 4 mL of ice-cold fixative (3:1 v/v methanol: acetic acid) was added slowly onto the culture surface and the cultures were slowly inverted to mix.

The cultures were centrifuged at 500 g for five minutes. The supernatant was removed, and the cell pellet re-suspended. A further 4 mL of fresh fixative was then added and the cells stored at 2 to 8°C until slide preparation.

**Slide Preparation**

The cultures were centrifuged at 500 g for 5 minutes and the supernatant removed. A homogeneous cell suspension was prepared. Pre-cleaned microscope slides were prepared for each culture by aliquoting the re-suspended cells onto the slides, and allowing the slides to air-dry. One slide was prepared from each culture. Slides were stained using acridine orange. The remaining cell cultures were stored at 2 to 8°C until slide analysis was complete.

**Microscopic Examination**

The prepared slides were examined by fluorescence microscopy. The incidences of mononucleate, binucleate and polynucleate cells were assessed per culture. The presence of an unusual number of, for example, cells undergoing mitosis, polyploid cells, necrotic cells and debris, if any, was also noted.

From these results, SYN545547 concentrations were selected for evaluation in the main experiment. The highest concentration was intended to be that which caused a depression in the cytokinesis-block proliferative index (CBPI) equivalent to  $55 \pm 5\%$  cytotoxicity (approximately) when compared with the concurrent vehicle control or, where no cytotoxicity was observed, the limit of solubility.

**Main micronucleus test:**

The procedure for the main experiments was the same as that for the preliminary experiments, with the following exceptions: positive control cultures were included for all experiments; duplicate cultures were prepared for each treatment level and positive control cultures; quadruplicate cultures were prepared for vehicle controls; two slides were prepared from each culture.

**Microscopic Examination**

The prepared slides were examined by fluorescence microscopy. The incidences of mononucleate, binucleate and polynucleate cells were assessed per culture. The presence of an unusual number of, for example, cells undergoing mitosis, polyploid cells, necrotic cells and debris, if any, was also noted.

From these results, at least three concentrations were selected for micronucleus analysis. The highest concentration was intended to be that which caused a depression in the cytokinesis-block proliferative index (CBPI) equivalent to  $55 \pm 5\%$  cytotoxicity (approximately) when compared with the concurrent vehicle control or, where no cytotoxicity was observed, the limit of solubility.

Prior to micronucleus analysis, all slides were randomly coded. Interphase cells were examined by fluorescence microscopy and the incidence of micronucleated cells per 1000 binucleate cells per culture were scored where possible from at least 2000 binucleate cells per concentration (4000 for vehicle controls).

**Statistical Methods:**

The analysis assumed that the replicate was the experimental unit. An arcsine square-root transformation was used to transform the data. SYN545547 treated groups were then compared to control using Williams' tests. Positive controls were compared to control using *t*-tests. Trend tests have also been carried out using linear contrasts by group number. These were repeated, removing the top dose group, until there were only 3 groups.

Statistical significance was declared at the 5% level for all tests.

**Evaluation Criteria:**

Providing that all of the acceptance criteria have been met, the test item was considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test concentrations exhibits a statistically significant increase in the frequency of micronucleated cells compared with the concurrent negative control.
- The increase in the frequency of micronucleated cells is dose-related when evaluated with an appropriate trend test.
- Any of the results are outside the distribution of the historical negative control data.

If all of these criteria are met, the test item was considered able to induce chromosome breaks and/or gain or loss in the test system.

Providing that all of the acceptance criteria have been met, a clearly negative response will be claimed if, in all of the experimental conditions examined:

- None of the test concentrations exhibits a statistically significant increase in the frequency of micronucleated cells compared with the concurrent negative control.
- There is no concentration-related increase when evaluated with an appropriate trend test.
- All results are inside the distribution of the historical negative control data.

If all of these criteria are met, the test item was considered unable to induce chromosome breaks and/or gain or loss in the test system.

**RESULTS**

**Selection of Vehicle and Formulation of Test Item:** Prior to commencing testing, the solubility of the test item in DMSO was assessed as part of the concurrently performed Envigo study No.: XS02YK. SYN545547 was found to be soluble at 139.2 mg/mL which gave a maximum final concentration of 696 µg/mL when dosed at 0.5% (v/v).

The osmolality of the test item in medium was tested at 696 µg/mL; no fluctuations in osmolality of the medium of more than 50 mOsm/kg were observed compared with the vehicle control. No fluctuations in pH of the medium were observed at 696 µg/mL of more than 1.0 unit compared with the vehicle control. The maximum final concentration tested in the preliminary toxicity test was 696 µg/mL.

**Preliminary toxicity test:**

Precipitate was observed by eye at the end of treatment at 174 µg/mL and above and this was, therefore, the highest concentration assessed for cytostasis.

Following a 3-hour treatment in the absence of S9 mix, a reduction in CBPI compared with vehicle control values, equivalent to 34.2% cytostasis, was obtained with SYN545547 at 87 µg/mL. At higher analysed concentrations overt toxicity was observed.

Following a 3-hour treatment in the presence of S9 mix, a reduction in CBPI compared with vehicle control values, equivalent to 27.4% cytostasis, was obtained with SYN545547 at 87 µg/mL. At higher analysed concentrations overt toxicity was observed.

Following a 20-hour treatment in the absence of S9 mix, a reduction in CBPI compared to vehicle control values, equivalent to 77.4% cytostasis, was obtained with SYN545547 at 43.5 µg/mL. At higher analysed concentrations overt toxicity was observed.



Concentrations for the main micronucleus test were based upon these results.

### Main micronucleus test:

#### Main Test - 3-hour Treatment in the Absence of S9 Mix

Concentrations of SYN545547 used for the main micronucleus test were 10, 20, 50, 75, 85, 95, 105, 115, 125, 135, 155 and 175 µg/mL. Precipitate was observed by eye at the end of treatment at 155 µg/mL and above and this was, therefore, the highest concentration assessed for cytostasis. A reduction in CBPI compared with vehicle control values equivalent to 60.0% cytostasis, was obtained with SYN545547 at 135 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis were 10, 85 and 135 µg/mL.

SYN545547 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls. All measured values were within the control limits.

Mean micronucleus induction in the vehicle control was within the historical control range.

The positive control compounds (mitomycin C and colchicine) caused statistically significant increases in the number of binucleate cells containing micronuclei, demonstrating the sensitivity of the test system.

**Table 6.8.1-49: 3-Hour Treatment in the Absence of S9 Mix – Micronucleus Data**

Treatment/ Concentration (µg/mL)	CBPI	Mean CBPI	Mean Cytostasis (%)	Binucleated cells containing micronuclei			
				per 1000 cells	Mean	<i>p</i> -value <sup>b</sup>	Trend test <i>p</i> -value <sup>c</sup>
Vehicle <sup>a</sup>	1.75 1.79 1.78 1.76	1.77	0.0	8 10 12 9	9.8		
SYN545547 10	1.74 1.74	1.74	3.9	8 10	9.0	0.696	
SYN545547 85	1.51 1.53	1.52	32.1	9 8	8.5	0.613	0.513
SYN545547 135	1.29 1.33	1.31	60.0	11 6	8.5	0.578	0.434
MMC 0.3	1.61 1.58	1.60	22.3	32 35	33.5	<0.001 ***	
COL 0.07	1.49 1.42	1.45	41.1	25 19	22.0	<0.001 ***	

a. Vehicle control = DMSO (0.5% v/v)

b. *p*-values are for comparisons to control using Williams' test for SYN545547 and the *t*-test otherwise

c. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same compound

\*\*\* *p*<0.001

CBPI: Cytokinesis block proliferative index

MMC: Mitomycin C

COL: Colchicine

#### Main Test - 3-hour Treatment in the Presence of S9 Mix

Concentrations of SYN545547 used for the main micronucleus test were 5, 50, 100, 125, 130, 135, 140, 145, 150 and 155 µg/mL. A reduction in CBPI compared with vehicle control values, equivalent to 50.2% cytostasis, was obtained with SYN545547 at 140 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis were 5, 100 and 140 µg/mL.

SYN545547 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls. All measured values were within the control limits.

Mean micronucleus induction in the vehicle control was within the historical control range.

The positive control compound (cyclophosphamide) caused a statistically significant increase in the number of binucleate cells containing micronuclei, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

**Table 6.8.1-50: 3-Hour Treatment in the Presence of S9 Mix – Micronucleus Data**

Treatment/ Concentration (µg/mL)	CBPI	Mean CBPI	Mean Cytostasis (%)	Binucleated cells containing micronuclei			
				per 1000 cells	Mean	<i>p</i> -value <sup>b</sup>	Trend test <i>p</i> -value <sup>c</sup>
Vehicle <sup>a</sup>	1.81	1.83	0.0	5	8.3		
	1.82			12			
	1.78			8			
	1.89			8			
SYN545547 5	1.82	1.83	-0.4	6	8.0	0.925	
	1.84			10			
SYN545547 100	1.60	1.64	22.2	5	6.0	0.367	0.284
	1.68			7			
SYN545547 140	1.38	1.41	50.2	6	6.0	0.367	0.207
	1.44			6			
CPA 10	1.79	1.78	5.7	18	18.5	0.004**	
	1.77			19			

a. Vehicle control = DMSO (0.5% v/v)

b. *p*-values are for comparisons to control using Williams' test for SYN545547 and the *t*-test otherwisec. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same compound\*\* *p* < 0.01

CBPI: Cytokinesis block proliferative index

CPA: Cyclophosphamide

**Main Test - 20-hour Treatment in the Absence of S9 Mix**

Concentrations of SYN545547 used for the main micronucleus test were 1, 10, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47 and 50 µg/mL. A reduction in CBPI compared with vehicle control values, equivalent to 56.0% cytotoxicity, was obtained with SYN545547 at 29 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis were 1, 10 and 29 µg/mL.

In this experiment, no biologically relevant increase in the number of micronucleated cells was observed after treatment with the test substance. All the observed values were slightly outside the control limit; however, this was also including the control. SYN545547 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls and the trend test was negative.

The mean micronucleus induction in the vehicle control was acceptable because it was only slightly outside the control limit and still within the historical control range.

The positive control compounds (mitomycin C and colchicine) caused statistically significant increases in the number of binucleate cells containing micronuclei, demonstrating the sensitivity of the test system.

**Table 6.8.1-51: 20-Hour Treatment in the Absence of S9 Mix – Micronucleus Data**

Treatment/ Concentration (µg/mL)	CBPI	Mean CBPI	Mean Cytostasis (%)	Binucleated cells containing micronuclei			
				per 1000 cells	Mean	<i>p</i> -value <sup>b</sup>	Trend test <i>p</i> -value <sup>c</sup>
Vehicle <sup>a</sup>	1.83	1.84	0.0	10	9.3		
	1.90			10			
	1.72			6			
	1.90			11			
SYN545547 1	1.63	1.74	12.1	12	12.0	0.322	
	1.85			12			
SYN545547 10	1.60	1.63	25.0	13	11.0	0.322	0.303
	1.66			9			
SYN545547 29	1.35	1.37	56.0	11	10.5	0.322	0.566
	1.39			10			
MMC 0.1	1.67	1.66	21.6	30	31.0	<0.001***	
	1.64			32			
COL 0.01	1.64	1.61	27.7	22	21.0	<0.001***	
	1.58			20			

a. Vehicle control = DMSO (0.5% v/v)

b. *p*-values are for comparisons to control using Williams' test for SYN545547 and the *t*-test otherwise

c. Trend test *p*-values are for the linear contrast including the control group and lower concentrations of the same compound

\*\*\* *p* < 0.001

CBPI: Cytokinesis block proliferative index

MMC: Mitomycin C

COL: Colchicine

## CONCLUSION:

In a GLP and OECD compliant in vitro micronucleus assay, the potential of SYN545547 to cause an increase in the induction of micronuclei in cultured human peripheral blood lymphocytes was investigated.

The study consisted of a preliminary toxicity test, a main micronucleus test and an additional test involving treatment for 3-hour in the presence of S9-mix. Human lymphocytes in whole blood culture were exposed to SYN545547 for 3 hours in both the absence and presence of exogenous metabolic activation (S9 mix) and for 20 hours in the absence of S9 mix. The maximum final concentration to which the cells were exposed was 696 µg/mL. This was considered to be the maximum achievable concentration based on the solubility of the test item. Vehicle (DMSO) and positive control cultures were included in all appropriate test conditions.

Three SYN545547 concentrations were assessed for the determination of the induction of micronuclei. The highest concentration selected for all exposures was that which caused a reduction in CBPI equivalent to 55±5% cytotoxicity. Following a 3-hour treatment in the absence of S9 mix, reductions in CBPI equivalent to 60.0% cytotoxicity were obtained with SYN545547 at 135 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis without S9 were therefore 10, 85 and 135 µg/mL. Following 3-hour treatment in the presence of S9 mix, reductions in CBPI equivalent to 50.2% cytotoxicity were obtained with SYN545547 at 140 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis with S9 were therefore 5, 100 and 140 µg/mL. In the absence of S9 following 20-hour treatment, a reduction in CBPI equivalent to 56.0% cytotoxicity was obtained with SYN545547 at 29 µg/mL. Concentrations of SYN545547 selected for micronucleus analysis without S9 for 20 hours were therefore 1, 10 and 29 µg/mL.

In both the absence and presence of S9, following 3-hour treatment, and in the absence of S9 mix, following 20 hour treatment, SYN545547 did not cause any statistically significant increases in the number of binucleate cells containing micronuclei when compared with the vehicle controls.

The positive control compounds (mitomycin C, colchicine and cyclophosphamide) caused statistically significant increases in the number of binucleate cells containing micronuclei under appropriate conditions, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

In conclusion, in this guideline study, SYN545547 did not induce the formation of micronuclei in human lymphocytes in vitro with or without metabolic activation up to a concentration causing cytotoxicity.

(██████████. 2017a)

### In vitro mouse lymphoma test

<b>Report:</b>	K-CA 5.8.1/72 ██████████ (2017b). SYN545547 - <i>In Vitro</i> Mutation Test using Mouse Lymphoma L5178Y Cells. Envigo CRS Limited, Woolley Road, Alconbury, Huntingdon, Cambridgeshire, PE28 4HS, United Kingdom. Laboratory Report No. XS02YK; 01 March 2017. Unpublished. Syngenta File No. SYN545547_10007.
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**Guidelines:** OECD 490 (2016)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

SYN545547 was tested for mutagenic potential in an *in vitro* mammalian cell mutation assay. This test system is based on detection and quantitation of forward mutation in the subline 3.7.2c of mouse lymphoma L5178Y cells, from the heterozygous condition at the thymidine kinase locus (TK+/-) to the thymidine kinase deficient genotype (TK-/-).

The study consisted of a preliminary toxicity test and two independent mutagenicity assays. The cells were exposed for 3 hours in the absence or presence of exogenous metabolic activation (S9 mix).

SYN545547 was found to be soluble at 139.2 mg/mL in dimethyl sulphoxide (DMSO). A final concentration of 1392 µg/mL, dosed at 1% v/v, was used as the maximum concentration in the preliminary toxicity test, in order to test up to the limit of solubility.

Precipitate was observed by eye at the end of treatment at concentrations of 87 µg/mL and above in the preliminary toxicity test and this was, therefore, the highest concentration assessed for cytotoxicity. Following a 3-hour exposure to SYN545547 at concentrations from 2.72 to 87 µg/mL, relative suspension growth (RSG) was reduced from 109 to 56% and from 106 to 54% in the absence and presence of S9 mix respectively. The concentrations assessed for determination of mutant frequency in the main test were based upon these data, the objective being to test up to the lowest concentration at which precipitate was observed by eye at the end of treatment.

Following 3-hour treatment in the absence and presence of S9 mix, there were no increases in the mean mutant frequencies of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor (GEF), within acceptable levels of toxicity. The maximum concentration assessed for mutant frequency in the absence and presence of S9 mix was 90 µg/mL (the lowest precipitating concentration). There were no significant reductions in relative total growth (RTG).

In all tests the concurrent vehicle and positive control were within acceptable ranges.

**It was concluded that SYN545547 did not demonstrate mutagenic potential in this *in vitro* cell mutation assay, and afforded a clearly negative result under the experimental conditions described.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545547
<b>Description:</b>	White solid
<b>Lot/Batch number:</b>	BPS 1510/2
<b>Molecular weight:</b>	Not applicable
<b>Purity</b>	99% w/w
<b>Stability of test compound:</b>	Not indicated by the sponsor
<b>Control Materials:</b>	
<b>Negative:</b>	-
<b>Solvent control (final concentration):</b>	DMSO
<b>Positive control:</b>	Absence of S9 mix: Methyl methanesulphonate (10 µg/mL)

Presence of S9 mix: Benzo[a]pyrene (1.5 µg/mL)

**Mammalian metabolic system: S9 derived**

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

S9 mix contained: S9 fraction (5% v/v), glucose-6-phosphate (6.9 mM), NADP (1.4 mM) in R0. The co factors were prepared, neutralized with 1N NaOH and filter sterilized before adding to S9 fraction and R0.

**Test cells: mammalian cells in culture**

X	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media: RPMI 1640</b>			
Properly maintained?		X	Yes
Periodically checked for Mycoplasma contamination?		X	Yes
Periodically checked for karyotype stability?		X	Yes
Periodically “cleansed” against high spontaneous background?		X	Yes

X indicates those that apply

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na <sup>+</sup> /K <sup>+</sup> ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	Trifluorothymidine (TFT)				

X indicates those that apply

**Test compound concentrations used:**

Preliminary toxicity test 2.72, 5.44, 10.88, 21.75, 43.5, 87, 174, 348, 696 and 1392 µg/mL

Main Experiment

Absence of S9 mix 15, 30, 50, 70, 90 and 110 µg/mL

Presence of S9 mix 15, 30, 50, 70, 90 and 110 µg/mL

**Study Design and Methods:**

**In-life dates:** Start: 14 December 2016, End: 16 January 2017

**Test performance:**

**Preliminary toxicity test:**

A cell suspension at 1x 10<sup>6</sup> cells/mL was prepared in R10p. Aliquots of the cell suspension, R0 (without metabolic activation) or S9 mix (with metabolic activation) and the test substance solution at each concentration or DMSO were combined and incubated at 34 to 39°C for three hours. Each culture contained 6 x 10<sup>6</sup> cells in 10 mL.

At the end of the 3-hour exposure period, the cells were washed once, re-suspended in R10p to nominally  $2 \times 10^5$  cells/mL (assuming no cell loss), incubated (at 34 to 39°C, 5% (v/v) CO<sub>2</sub>) and sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to  $2 \times 10^5$  cells/mL with R10p where necessary.

**Main mutation test:** A cell suspension at  $1 \times 10^6$  cells/mL was prepared in R10p. Aliquots of the cell suspension, R0 (without metabolic activation) or S9 mix (with metabolic activation) and the test substance solution at each concentration or DMSO were combined and incubated at 37°C for three hours. Each culture contained  $1.2 \times 10^7$  cells in 20 mL.

Each treatment group included vehicle and positive control cultures. The vehicle control cultures were treated with DMSO at a final concentration of 1% (v/v). The positive control cultures were treated with methyl methanesulphonate (in the absence of S9 mix) or benzo[a]pyrene (in the presence of S9 mix).

At the end of the 3-hour exposure period, the cells were washed once, re-suspended in R10p to nominally  $2 \times 10^5$  cells/mL (assuming no cell loss) and incubated for a further 48 hours to allow for expression of mutant phenotype. The cultures were sampled after 24 and 48 hours to assess growth in suspension. After sampling at 24 hours the cell density was readjusted to  $2 \times 10^5$  cells/mL with R10p where necessary. After 48 hours cultures with a density of more than  $1 \times 10^5$  cells/mL were assessed for cloning efficiency (viability) and mutant potential by plating in 96-well plates. Cloning efficiency was assessed by plating 1.6 cells/well, two plates being prepared per culture. Mutant potential was assessed by plating  $2 \times 10^3$  cells/well in selective medium, two plates being prepared per culture. The plates were placed in a humidified incubator at 34 to 39°C in an atmosphere of 5% CO<sub>2</sub> in air.

After the plates had been incubated for 10 to 12 days, the number of empty wells was assessed for each 96 well plate (P0). P0 was used to calculate the cloning efficiency (CE) and mutant frequency (MF). The colony size distribution in the vehicle and positive controls was examined to ensure that there was an adequate recovery of small colony mutants. The maximum concentration assessed for mutant frequency in the main test was 90 µg/mL in both the absence and presence of S9 mix.

The criteria for sizing colonies was based on morphology and generally was less than 25% of the well's diameter for small colonies and greater than 25% of the well's diameter for large colonies.

**Statistical Methods:** The data were analysed using Fluctuation application SAFESat (SAS statistical applications for end users) version 1.1, which follows the methods described by Robinson et al. (1989). Statistics were only reported if the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor was exceeded, and this was accompanied by a significant positive linear trend ( $p < 0.05$ ).

**Evaluation Criteria:** The following criteria were applied for assessment of individual assay results using data for MF where the RTG normally exceeded 10%:

Definitions: GEF = Global Evaluation Factor. For microwell assays this is  $126 \times 10^{-6}$ .

- Providing that all acceptability criteria were fulfilled, the test item was considered to be clearly positive if, in any of the experimental conditions examined the increase in MF above the concurrent background exceeded the GEF and the increase was concentration related (i.e., there is a significant positive linear trend). The test item is then considered able to induce mutation in this test system.
- Providing that all acceptability criteria are fulfilled, the test item was considered to be clearly negative if, in all experimental conditions examined there is no concentration related response or, if there is an increase in MF, it does not exceed the GEF. The test item is then considered unable to induce mutations in this test system.

If the maximum concentration was based on cytotoxicity, the highest concentration aimed to achieve between 20 and 10% RTG. The consensus is that care should be taken when interpreting positive results only found between 20 and 10% RTG and a result would not be considered positive if the increase in MF occurred only at or below 10% RTG (if evaluated).

## RESULTS

**Selection of Vehicle and Formulation of Test Item:** Prior to commencing testing, the solubility of the test item in DMSO was assessed. SYN545547 was found to be soluble at 139.2 mg/mL which gave a maximum final concentration of 1392 µg/mL when dosed at 1% (v/v).

The osmolality of the test item in medium was tested at 1392 µg/mL; no fluctuations in osmolality of the medium of more than 50 mOsm/kg were observed compared with the vehicle control. No fluctuations in pH of the medium were observed at 1392 µg/mL of more than 1.0 unit compared with the vehicle control. The maximum final concentration tested in the preliminary toxicity test was 1392 µg/mL.

### Preliminary toxicity test:

Precipitate (observed by eye at the end of treatment) was observed at concentrations of 87 µg/mL and above in the absence and presence of S9 mix, therefore, this was the highest concentration assessed for cytotoxicity. Exposure to SYN545547 at concentrations from 2.72 to 87 µg/mL in the absence and presence of S9 mix resulted in relative suspension growth (RSG) values from 109 to 56% and from 106 to 54% respectively.

Concentrations used in the main test were based upon these data.

### Main mutation assay:

#### Main Mutation Test - 3-hour Treatment in the Absence of S9 Mix

Cultures were exposed to SYN545547 at concentrations from 15 to 110 µg/mL. Precipitate (assessed by eye at the end of treatment) was observed at concentrations of 90 µg/mL and above and this was, therefore, the highest concentration carried forward following treatment. Cultures exposed to SYN545547 at concentrations from 15 to 90 µg/mL were assessed for determination of mutation frequency. Relative total growth (RTG) values from 108 to 65% were obtained relative to the vehicle control. There were no increases in the mean mutant frequencies of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor (GEF), within acceptable levels of toxicity.

The positive control, methyl methanesulphonate, induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants.

**Table 6.8.1-52: 3-Hour Treatment in the Absence of S9 Mix**

Treatment / Concentration		Cell Concentration (x10 <sup>5</sup> /mL)		Mean Suspension Growth	Viability Plate Count <sup>a</sup>		Mutant Plate Count <sup>a</sup>		Mean Cloning Efficiency (%)	RTG (%)	Mean RTG (%)	Mean MF (x10 <sup>-6</sup> )
(µg/mL)	Replicate ID	24 h	48 h		Day 2		Day 2					
Vehicle Control <sup>b</sup>	A	7.32	8.92	16.4	40	(192)	168	(192)	91	100	100	98
	B	6.67	10.07		47	(192)	159	(192)				
	C	6.15	10.00		45	(192)	163	(192)				
	D	7.07	9.69		47	(192)	152	(192)				
SYN545547 15	A	6.13	10.86	16.5	38	(192)	155	(192)	97	113	108	108
	B	5.86	11.16		43	(192)	156	(192)				
SYN545547 30	A	4.62	10.55	12.0	40	(192)	146	(192)	95	80	77	130
	B	4.24	11.18		44	(192)	154	(192)				
SYN545547 50	A	4.70	12.36	13.1	48	(192)	169	(192)	87	84	77	94
	B	4.35	10.81		47	(192)	157	(192)				
SYN545547 70	A	3.60	12.03	10.8	41	(192)	158	(192)	90	70	65	114
	B	3.58	11.92		50	(192)	155	(192)				
SYN545547	A	4.84	10.54	12.1	40	(192)	168	(192)	97	84	78	75

Treatment / Concentration		Cell Concentration (x10 <sup>5</sup> /mL)		Mean Suspension Growth	Viability Plate Count <sup>a</sup>		Mutant Plate Count <sup>a</sup>		Mean Cloning Efficiency	RTG	Mean RTG	Mean MF
(µg/mL)	Replicate ID	24 h	48 h		Day 2		Day 2		(%)	(%)	(%)	(x10 <sup>-6</sup> )
90 <sup>c</sup>	B	4.22	10.91		42	(192)	164	(192)		73		
SYN545547 100 <sup>d</sup>	A											
	B											
MMS 10	A	6.10	11.10	16.1	74	(192)	46	(192)	69	68	75	1090
	B	6.39	9.61		53	(192)	39	(192)		83		

a. Number of non-colony bearing wells (total number of wells)

b. Vehicle control = DMSO (1% v/v)

c. Precipitate observed by eye at the end of treatment

d. Precipitate observed by eye at the end of treatment, therefore cultures discarded

MMS - Methyl methanesulphonate

RTG - Relative Total Growth

MF - Mutant Frequency

### Main Mutation Test - 3-hour Treatment in the Presence of S9 Mix

Cultures were exposed to SYN545547 at concentrations from 15 to 110 µg/mL. Precipitate (assessed by eye at the end of treatment) was observed at concentrations of 90 µg/mL and above and this was, therefore, the highest concentration carried forward following treatment. Cultures exposed to SYN545547 at concentrations from 15 to 90 µg/mL were assessed for determination of mutation frequency. RTG values from 107 to 62% were obtained relative to the vehicle control. There were no increases in the mean mutant frequencies of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control mutant frequency and the GEF, within acceptable levels of toxicity.

The positive control, benzo[a]pyrene, induced an acceptable increase in mutation frequency and an acceptable increase in the number of small colony mutants.

**Table 6.8.1-53: 3-Hour Treatment in the Presence of S9 Mix**

Treatment / Concentration		Cell Concentration (x10 <sup>5</sup> /mL)		Mean Suspension Growth	Viability Plate Count <sup>a</sup>		Mutant Plate Count <sup>a</sup>		Mean Cloning Efficiency	RTG	Mean RTG	Mean MF
(µg/mL)	Replicate ID	24 h	48 h		Day 2		Day 2					
Vehicle Control <sup>b</sup>	A	8.97	9.36	21.2	53	(192)	161	(192)	85	100	100	102
	B	9.13	9.35		50	(192)	161	(192)				
	C	10.35	8.00		52	(192)	164	(192)				
	D	9.65	8.99		41	(192)	159	(192)				
SYN545547 15	A	8.38	10.34	21.2	52	(192)	161	(192)	91	98	107	97
	B	8.02	10.37		38	(192)	161	(192)		116		
SYN545547 30	A	7.57	10.40	19.2	72	(192)	168	(192)	65	67	69	116
	B	7.93	9.46		63	(192)	162	(192)		72		
SYN545547 50	A	6.68	10.98	18.2	51	(192)	166	(192)	72	84	73	105
	B	7.38	9.75		70	(192)	164	(192)		63		
SYN545547 70	A	6.27	10.70	16.8	46	(192)	158	(192)	89	83	82	110
	B	6.73	10.02		47	(192)	158	(192)		82		
SYN545547 90 <sup>c</sup>	A	4.88	10.26	12.6	43	(192)	158	(192)	89	65	62	117
	B	5.05	10.01		50	(192)	154	(192)		59		
SYN545547 100 <sup>d</sup>	A											
	B											
MMS 10	A	5.90	7.06	10.2	82	(192)	43	(192)	57	31	32	1394
	B	5.88	6.77		73	(192)	36	(192)		33		

a. Number of non-colony bearing wells (total number of wells)

b. Vehicle control = DMSO (1% v/v)

c. Precipitate observed by eye at the end of treatment

d. Precipitate observed by eye at the end of treatment, therefore cultures discarded

BaP - Benzo[a]pyrene

RTG - Relative Total Growth

MF - Mutant Frequency

### CONCLUSION:



In a GLP and OECD compliant mammalian cell gene mutation assay, the potential of SYN545547 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y was investigated. The study consisted of a preliminary toxicity test and two independent mutagenicity assays. The cells were exposed for 3 hours in the absence or presence of exogenous metabolic activation (S9 mix).

SYN545547 was found to be soluble at 139.2 mg/mL in DMSO. A final concentration of 1392 µg/mL, dosed at 1% v/v, was used as the maximum concentration in the preliminary toxicity test, in order to test up to the limit of solubility.

Precipitate was observed by eye at the end of treatment at concentrations of 87 µg/mL and above in the preliminary toxicity test and this was, therefore, the highest concentration assessed for cytotoxicity. Following a 3-hour exposure to SYN545547 at concentrations from 2.72 to 87 µg/mL, relative suspension growth (RSG) was reduced from 109 to 56% and from 106 to 54% in the absence and presence of S9 mix respectively. The concentrations assessed for determination of mutant frequency in the main test were based upon these data, the objective being to test up to the lowest concentration at which precipitate was observed by eye at the end of treatment.

Following 3-hour treatment in the absence and presence of S9 mix, there were no increases in the mean mutant frequencies of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control mutant frequency and the Global Evaluation Factor (GEF), within acceptable levels of cytotoxicity. The maximum concentration assessed for mutant frequency in the absence and presence of S9 mix was 90 µg/mL (the lowest precipitating concentration). There were no significant reductions in relative total growth (RTG).

In all tests the concurrent vehicle and positive control were within acceptable ranges.

As a result, HSE agrees with the EU evaluation, that **SYN545547 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation when tested up to concentrations causing precipitation.**

██████████. (2017b)

### **Summary of toxicity data on metabolite SYN545547**

#### **Summary of toxicity studies and derivation of dietary reference values for metabolite SYN545547 glucuronide/sulphate**

Metabolite SYN545547 glucuronide/sulphate is a plant and livestock metabolite. In the human gastrointestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN545547. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

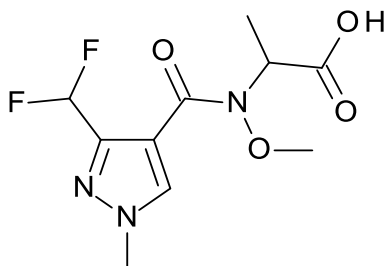
Overall, GLP and OECD compliant in vitro genotoxicity assays (supported by a comparative genotoxicity QSAR analysis) are available on metabolite SYN545547. The metabolite was negative in the standard battery of 3 in vitro tests and therefore it is considered to be non-genotoxic.

The table below summarises the available studies on SYN545547:

Study & Acceptability	Result	Reference
Ames test in bacteria GLP, OECD 471  <i>Acceptable</i>	Negative up to cytotoxicity/precipitation	██████ (2017). Report No. MK43KP. Syngenta File No. SYN545547_10005
Micronucleus Test in Human Lymphocytes <i>In Vitro</i> . GLP, OECD 487  <i>Acceptable</i>	Negative up to cytotoxicity	██████ (2017a). Report No. NH83HP Syngenta File No. SYN545547_10009
Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells. GLP, OECD 490  <i>Acceptable</i>	Negative up to precipitation	██████ (2017b). Report No. Report No. XS02YK; Syngenta File No. SYN545547_10007.
Genotoxicity QSAR analysis of SYN545547 and pydiflumetofen  <i>Acceptable</i>	Non-genotoxic	Syngenta Ltd., (2016). Report No. TK0288608. Syngenta File No. SYN545974_10433

It is noted that SYN545547 is only a minor rat metabolite; therefore it is not covered by the parent dataset. **However, based on the available data, if a dietary risk assessment were to be required, the TTC Cramer Class III values (chronic value = 1.5 µg/kg bw/d and acute value = 5 µg/kg bw) could be used. This is in contrast to the advice given by the EU peer-review process.**

**B.6.8.1.4. Metabolite SYN548263 glucuronide/sulphate (CSCZ159698 glucuronide/sulphate)**



2-[[3-(difluoromethyl)-1-methyl-pyrazole-4-carbonyl]-methoxy-amino]propanoic acid (SYN548263 aglycon)

Metabolite SYN548263 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN548263. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

SYN548263 is a metabolite of pydiflumetofen (SYN545974), which has been identified in livestock (hen, goat). After an oral administration of pydiflumetofen in rat, SYN548263 was detected in plasma accounting for up to 7% of total radioactivity AUC (TRA) and for up to 8.9% in urine. SYN548263 is also the precursor of SYN508272, the major pyrazole specific metabolite (found for up to 14.8% TRA in plasma of rats).

**EFSA Request for additional information (February 2018), Question 35:** Applicant to provide further assessment of the toxicological profile of the metabolite SYN548263.

A standard battery of three genotoxicity tests have been conducted with the metabolite SYN548263 by the applicant and are presented below.

Ames test

**Report:** K-CA 5.8.1/73 [REDACTED] (2018) SYN548263 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1880400, issue date: 16 March 2018. Unpublished Syngenta File No. SYN548263\_10002.

**Guidelines:** OECD 471 (1997): OPPTS 870.5100 (1998): 2000/32/EEC B.13/B.14 (2000)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

This study was performed to investigate the potential of SYN548263 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* (*S. typhimurium*) strains TA1535, TA1537, TA98, and TA100, and the *Escherichia coli* (*E. coli*) strains WP2 *uvrA* pKM101 and WP2 pKM101.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in all strains with and without metabolic activation.

No relevant increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548263 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations and all mutation rates were within the range of normal biological variability.

Appropriate reference mutagens were used as positive controls, which showed a distinct increase of induced revertant colonies.

**In conclusion, it can be stated that during the described mutagenicity tests and under the experimental conditions reported, SYN548263 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore SYN548263 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN548263
<b>Description:</b>	White, solid
<b>Lot/Batch number:</b>	MES 346/2
<b>Content of SYN547407:</b>	99% (w/w)
<b>Stability of test compound:</b>	Not indicated by the Sponsor
<b>Recertification date:</b>	30 November 2019

**Control Materials:**

**Negative:** Concurrent untreated and solvent controls were performed

**Solvent control (final concentration):** 100µl/plate

**Positive control:** Nonactivation:  
 Sodium azide 10 µg/plate TA100, TA1535  
 4-nitro-o-phenylene-diamine,  
 50 µg/plate TA 1537, 10 µg/plate TA98  
 methyl methane sulfonate 2 µL/plate WP2 (pKM101),  
 WP2 *uvrA* (pKM101)

Activation:  
 2-Aminoanthracene  
 2.5 µg/plate TA 1535, TA 1537, TA100, TA98  
 10 µg/plate WP2 (pKM101), WP2 *uvrA* (pKM101)

**Mammalian metabolic system: S9 derived**

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM MgCl<sub>2</sub>  
 33 mM KCl  
 5 mM Glucose-6-phosphate  
 4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.

During the experiment the S9 mix was stored in an ice bath.

**Test organisms:**

*S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

*E. coli* strains

X	WP2 (pKM101)	X	WP2 <i>uvrA</i> (pKM101)						
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Properly maintained?

X
X

Yes


No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?

X
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Yes

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No

**Test compound concentrations used**

Pre-experiment / Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

**Study Design and Methods:**

**In-life dates:** Start: 05 December 2017

End: 18 December 2017

**TEST PERFORMANCE**

**Preliminary Cytotoxicity Assay:** Not performed.

**Type of Bacterial assay:**

- X standard plate test (pre-experiment/experiment I; –S9, +S9)
- X pre-incubation (60 minutes) (second experiment ; –S9, +S9)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Protocol:**

For each strain and dose level including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL Test solution at each dose level, solvent (negative control) or reference mutagen solution (positive control),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL Bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution, 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspensions were mixed in a test tube and shaken at 37° C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45° C) was added to each tube. The mixture was poured on selective agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37° C in the dark .

\* Substitution buffer: 7 parts of the 100 mM sodium-ortho-phosphate-buffer pH 7.4 with 3 parts of KCl solution 0.15 M

**Statistical analysis:** None – see Evaluation Criteria below.

**Evaluation criteria:** A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control is observed (1).

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (6).

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

**RESULTS**

**Preliminary cytotoxicity assay:** Not performed.

**Mutagenicity assay:**

The test substance, SYN548263, was assessed for its potential to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and the *E. coli* strains WP2 uvrA pKM101 and WP2 pKM101.

In the pre-experiment the concentration range of the test substance tested was 3 - 5000 µg/plate. The pre-experiment is reported as Experiment I since the acceptability criteria of the assay were met. Since no cytotoxic effects were observed 5000 µg/plate was chosen as the maximal concentration in Experiment II.

The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The concentration range included two logarithmic decades. The test substance was tested at the following concentrations:

Pre-experiment / Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate  
 Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

No precipitation of the test item occurred up to the highest investigated dose.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in all strains with and without metabolic activation.

No biologically relevant ( $\geq 2$  fold) increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548263 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations and all mutation rates were within the range of normal biological variability.

Appropriate reference mutagens were used as positive controls, which showed a distinct increase of induced revertant colonies.

In Experiment II the number of colonies did not quite reach the lower limit of our historical control data in the untreated control of strain WP2 uvrA pKM101 without metabolic activation (S9 mix). Since this deviation is small and the vehicle control was within the historical control data range, this effect is judged to be based on biological fluctuations and has no detrimental impact on the outcome of the study.

In Experiment II the historical range of positive controls was exceeded in strain TA 1537 without metabolic activation. This effect indicates the sensitivity of the strains rather than compromising the assay.

**Table 6.8.1-54: Results Obtained in the Absence and Presence of Metabolic Activation:**

**Experiment 1**

<u>Metabolic Activation</u>	<u>Test Group</u>	<u>Concentration (per plate)</u>	<u>Revertant Colony Counts (Mean ± SD)</u>					
			<u>TA 1535</u>	<u>TA 1537</u>	<u>TA 98</u>	<u>TA 100</u>	<u>WP2 pKM101</u>	<u>WP2 uvrA pKM101</u>
Without Activation	DMSO Untreated SYN548263	3 µg	10 ± 4	8 ± 1	26 ± 4	200 ± 3	205 ± 27	331 ± 23
			10 ± 1	7 ± 2	34 ± 3	205 ± 6	235 ± 18	374 ± 4
			8 ± 2	9 ± 2	27 ± 8	205 ± 20	204 ± 18	341 ± 21
		10 µg	11 ± 1	9 ± 2	30 ± 7	217 ± 13	197 ± 22	332 ± 17
		33 µg	9 ± 3	8 ± 3	26 ± 5	211 ± 14	215 ± 16	299 ± 16
		100 µg	9 ± 2	10 ± 3	25 ± 2	216 ± 13	204 ± 10	309 ± 13
		333 µg	8 ± 3	11 ± 2	29 ± 8	206 ± 15	210 ± 7	340 ± 17
		1000 µg	10 ± 1	8 ± 3	28 ± 6	224 ± 12	208 ± 12	334 ± 17

		2500 µg	11 ± 3	8 ± 3	26 ± 7	212 ± 26	203 ± 28	322 ± 17
		5000 µg	11 ± 1	8 ± 2	29 ± 6	194 ± 16	196 ± 11	317 ± 19
	NaN3	10 µg	1093 ± 62			1828 ± 105		
	4-NOPD	10 µg			292 ± 14			
	4-NOPD MMS	50 µg 2.0 µL		138 ± 6			3739 ± 27	3678 ± 443
With Activation	DMSO		10 ± 3	13 ± 3	34 ± 7	190 ± 3	220 ± 17	374 ± 20
	Untreated		12 ± 3	14 ± 3	47 ± 6	189 ± 15	256 ± 22	417 ± 30
	SYN548263	3 µg	13 ± 3	11 ± 1	34 ± 4	204 ± 10	270 ± 16	427 ± 16
		10 µg	11 ± 4	10 ± 3	39 ± 15	197 ± 21	216 ± 4	395 ± 10
		33 µg	10 ± 1	14 ± 3	41 ± 7	190 ± 18	198 ± 19	368 ± 25
		100 µg	13 ± 3	12 ± 1	31 ± 10	204 ± 7	188 ± 15	380 ± 23
		333 µg	10 ± 3	12 ± 2	43 ± 13	199 ± 18	224 ± 19	393 ± 12
		1000 µg	11 ± 2	12 ± 4	33 ± 7	170 ± 7	206 ± 6	385 ± 10
		2500 µg	13 ± 2	9 ± 2	30 ± 4	210 ± 3	215 ± 22	372 ± 29
		5000 µg	11 ± 3	12 ± 3	39 ± 8	195 ± 6	197 ± 35	355 ± 5
	2-AA	2.5 µg	459 ± 33	107 ± 10	4010 ± 216	3765 ± 523		
	2-AA	10.0 µg					1135 ± 47	2266 ± 150

## Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Table 6.8.1-55: Results Obtained in the Absence and Presence of Metabolic Activation:

## Experiment 2

Metabolic Activation	Test Group	Concentration (per plate)	Revertant Colony Counts (Mean ± SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		9 ± 0	10 ± 4	28 ± 10	171 ± 5	243 ± 17	243 ± 9
	Untreated		11 ± 4	10 ± 4	31 ± 4	214 ± 11	259 ± 17	279 ± 38
	SYN548263	33 µg	10 ± 3	9 ± 4	23 ± 4	173 ± 16	249 ± 2	230 ± 15
		100 µg	9 ± 3	15 ± 1	25 ± 4	171 ± 16	231 ± 13	190 ± 24
		333 µg	10 ± 1	13 ± 2	34 ± 8	157 ± 14	199 ± 11	211 ± 16
		1000 µg	7 ± 2	13 ± 3	23 ± 5	152 ± 11	233 ± 22	242 ± 20
		2500 µg	9 ± 1	15 ± 2	25 ± 6	171 ± 20	225 ± 18	197 ± 32
		5000 µg	9 ± 1	8 ± 1	22 ± 6	169 ± 3	223 ± 31	192 ± 25
	NaN3	10 µg	1095 ± 42			1608 ± 81		
	4-NOPD	10 µg			336 ± 14			
	4-NOPD	50 µg		182 ± 15				
	MMS	2.0 µL					2827 ± 172	3358 ± 341
With Activation	DMSO		8 ± 2	18 ± 1	42 ± 10	184 ± 12	268 ± 7	294 ± 25
	Untreated		15 ± 5	14 ± 2	46 ± 9	192 ± 12	274 ± 40	307 ± 20
	SYN548263	33 µg	10 ± 1	21 ± 7	44 ± 7	157 ± 12	237 ± 18	337 ± 75
		100 µg	11 ± 4	24 ± 4	38 ± 7	160 ± 15	229 ± 44	256 ± 10
		333 µg	10 ± 4	17 ± 3	35 ± 6	157 ± 12	224 ± 24	229 ± 14
		1000 µg	15 ± 4	20 ± 1	40 ± 6	155 ± 9	250 ± 8	328 ± 33
		2500 µg	12 ± 2	20 ± 3	53 ± 1	166 ± 8	240 ± 24	266 ± 24
		5000 µg	14 ± 5	13 ± 2	39 ± 2	164 ± 16	192 ± 18	240 ± 11

2-AA	2.5 µg	488 ± 41	95 ± 13	3587 ± 192	2581 ± 438		
2-AA	10.0 µg					1065 ± 28	1921 ± 101

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Key to Positive Controls

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NaN <sub>3</sub>	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

**CONCLUSION:**

In a GLP and OECD compliant Ames test, the potential of SYN548263 to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100, and the *Escherichia coli* strains WP2 *uvrA* pKM101 and WP2 pKM101, was investigated up to the limit concentration of 5000 µg/plate with and without metabolic activation.

No precipitation of the test item occurred up to the highest investigated concentration.

No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in all strains with and without metabolic activation.

No biologically relevant ( $\geq 2$  fold) increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548263 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations and all mutation rates were within the range of normal biological variability. The concurrent positive controls verified the sensitivity of the assay and the metabolizing activity of the liver preparations. The mean revertant colony counts for the vehicle controls were within the current historical control range for the laboratory.

As a result, HSE agrees with the EU evaluation, that **SYN548263 is not mutagenic in bacteria when tested up to the limit concentrations for this test.**

(██████████. 2018)

In vitro micronucleus test

<b>Report:</b>	K-CA 5.8.1/74 ██████████. (2018) SYN548263 - Micronucleus Test in Human Lymphocytes In vitro. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1880200, issue date: 09 April 2018. Unpublished. Syngenta File No. SYN548263_10003.
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**Guidelines:** Micronucleus Test in Human Lymphocytes *In vitro*. OECD 487 (2016); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable



## EXECUTIVE SUMMARY

The test substance SYN548263, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments.

In each experimental group two parallel cultures were analysed. Per culture at least 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (2000 µg/mL of the test substance) was chosen with respect to the current OECD Guideline 487.

Concentration selection of the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance. The mean percentage of the micronuclei in all treated conditions was within the 95% historical control limits and none of the values were statistically significant increased, when compared with the vehicle control. The outcome of the study is a clear negative.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

**In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.**

**Therefore, SYN548263 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN548263
<b>Description:</b>	White solid
<b>Lot/Batch number:</b>	MES 346/2
<b>Purity:</b>	99% w/w
<b>CAS#:</b>	-
<b>Stability of test compound:</b>	Not indicated by the sponsor

### Control Materials:

<b>Negative control:</b>	-
<b>Solvent control</b>	
<b>(final concentration):</b>	DMSO (0.5 %)
<b>Positive control:</b>	Absence of S9 mix: MMC, 0.8 µg/mL (Experiment I), Absence of S9 mix: Demecolcin, 125 ng/ml (Experiment II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other

		X	Other β-naphthoflavone		Other		
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X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained MgCl<sub>2</sub> (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

#### Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

#### Test compound concentrations used:

Absence of S9 mix	Experiment 1 Experiment 2	684, 1026, 1538, 2000 µg/mL 373, 653, 1143, 2000 µg/mL
Presence of S9 mix	Experiment 1	684, 1026, 1538, 2000 µg/mL

#### Study Design and Methods:

In-life dates: Start: 06 December 2017, End: 05 February 2018

#### TEST PERFORMANCE

**Preliminary Cytotoxicity Assay:** A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. The pre-test was performed with 11 concentrations of the test item separated by no more than a factor of  $\sqrt{10}$  and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

#### Cytogenetic Assay:

Cell exposure time:		Test Material	Solvent Control	Positive Control
- S9 mix + S9 mix - S9 mix	Experiment 1	4h	4h	4h
		4h	4h	4h
	Experiment 2	20h	20h	20h

Cytokinesis block:	Cytochalasin B (4 µg/mL)
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Cell harvest time after termination of treatment:	Test Material	Solvent Control	Positive Control
- S9 mix (4 hour treatment)	36h	36h	36h
+ S9 mix (4 hour treatment)	36h	36h	36h
- S9 mix (20 hour treatment)	0h	0h	0h

**Details of slide preparation****Pulse exposure**

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm<sup>2</sup> cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H<sub>2</sub>O, 192 mg/L Na<sub>2</sub>HPO<sub>4</sub> • 2 H<sub>2</sub>O and 150 mg/L KH<sub>2</sub>PO<sub>4</sub>). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

**Continuous exposure (without S9 mix)**

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm<sup>2</sup> cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

**Preparation and analysis of cells:** The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were washed and fixated. The slides were added to a microscope slide and stained with Giemsa. Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criteria. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

**Evaluation criteria:** The percentages of micronuclei in binucleate cells were calculated for each treatment scored. The data have been interpreted as follows:

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

- None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- There is no concentration-related increase
- The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

- At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
- The increase is concentration-related in at least one experimental condition
- The results are outside the range of the laboratory historical solvent control data

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

**Statistical analysis:** Statistical significance was confirmed by the Chi-squared test ( $\alpha < 0.05$ ) using a validated R Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

A linear regression was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to assess a possible dose dependent increase of mutant frequencies. The number of micronucleated cells obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

However, both, biological and statistical significance was considered together.

## RESULTS

**Preliminary cytotoxicity assay:** A pre-experiment to evaluate the cytotoxicity of the test item was performed. The pre-experiment is reported as the main Experiment I since the criteria mentioned under Acceptability of the assay were met.

**Cytogenetic assay:** The test substance SYN548263, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analysed. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration. To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in Experiment I, 2000 µg/mL was chosen with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

No precipitation was observed at the end of treatment.

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Exp. I	Solvent control	-	395	7.5
	SYN548263	2000	390	7.1

No relevant influence on the osmolarity and pH was observed.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In the absence (4 h and 20 h exposures) and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance. The mean percentage of the micronuclei in all treated conditions was within the 95% control limit of the historical solvent control data and none of the values were statistically significant increased, when compared with the vehicle control. The outcome of the study is a clear negative.

Demecolcine (125 ng/mL), MMC (0.8 µg/mL) or CPA (15.0 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei demonstrating the sensitivity of the system and the efficacy of the S9 mix.

**Table 6.8.1-56: Summary of results of the Micronucleus Assay with SYN548263**

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in % *	Micronucleated cells in %**
Exposure period 4 hrs without S9 mix					
I	40 h	Solvent control <sup>1</sup>	2.16		0.50
		Positive control <sup>2</sup>	1.66	43.2	17.65 <sup>S</sup>
		684	2.10	5.3	0.60
		1026	2.12	3.0	0.40
		1538	2.12	3.1	0.50
		2000	2.11	3.8	0.55
Exposure period 20 hrs without S9 mix					
II	40 h	Solvent control <sup>1#</sup>	1.89		1.00
		Positive control <sup>3</sup>	1.77	13.7	4.60 <sup>S</sup>
		373 <sup>#</sup>	1.87	2.5	0.95
		653 <sup>#</sup>	1.91	n.c.	0.83
		1143 <sup>#</sup>	1.86	3.8	0.95
		2000 <sup>#</sup>	1.86	3.8	0.93
Exposure period 4 hrs with S9 mix					
I	40 h	Solvent control <sup>1</sup>	2.06		0.50
		Positive control <sup>4</sup>	1.65	38.7	4.75 <sup>S</sup>
		684	2.15	n.c.	0.15
		1026	2.12	n.c.	0.45
		1538	2.18	n.c.	0.30
		2000	2.12	n.c.	0.25

\* For the positive control groups and the test item treatment groups the values are related to the solvent controls

\*\* The number of micronucleated cells was determined in a sample of 2000 binucleated cells

# The number of micronucleated cells was determined in a sample of 4000 binucleated cells

<sup>S</sup> The number of micronucleated cells is statistically significantly higher than corresponding control values

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

<sup>1</sup> DMSO 0.5 % (v/v)

<sup>2</sup> MMC 0.8 µg/mL

<sup>3</sup> Demecolcine 125 ng/mL

<sup>4</sup> CPA 15.0 µg/mL

## CONCLUSION:

In a GLP and OECD compliant in vitro micronucleus assay, the potential of SYN548263 (in DMSO) to cause an increase in the induction of micronuclei in cultured human peripheral blood lymphocytes was investigated in two independent experiments. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix.

In each experimental group two parallel cultures were analysed. Per culture at least 1000 binucleated cells were evaluated for cytogenetic damage. The highest applied concentration in this study (2000 µg/mL) was equivalent to 10 mM, the limit concentration for this assay. To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture.

No precipitation was observed at the end of treatment. In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In the absence and presence of S9 mix, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance. The mean percentage of the micronuclei in all treated conditions was within the 95% historical control limits and none of the values were statistically significant increased, when compared with the vehicle control. Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei, demonstrating the efficacy of the S9 mix and the sensitivity of the test system.

In conclusion, in this guideline study, **SYN548263 did not induce the formation of micronuclei in human lymphocytes in vitro with or without metabolic activation up to the limit concentration for this test.**

(██████████. 2018)

#### In vitro mouse lymphoma test

<b>Report:</b>	K-CA 5.8.1/75 ██████████ (2018), SYN548263 - Cell Mutation Assay at the Thymidine Kinase Locus (TK +/-) in Mouse Lymphoma L5178Y Cells. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1880500 issue date: 06 April 2018. Unpublished. Syngenta File No. SYN548263_10001.
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**Guidelines:** *In Vitro* Mammalian Cell Gene Mutation Test OECD 490 (2016)

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

**HSE comment:** the study was considered acceptable

## EXECUTIVE SUMMARY

### Study Design

The study was performed to investigate the potential of SYN548263 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y.

The experiment was performed using the microwell method with and without liver microsomal activation using two parallel cultures with four hour treatment.

### Results

#### Pre-Experiment

Test item concentrations between 15.6 µg/mL and 2000 µg/mL were used in the pre-experiment. The highest concentration was chosen with respect to the OECD guideline 490 (2016).

No relevant cytotoxic effects were observed up to the maximum concentration in the presence and absence of metabolic activation

The test medium was checked for precipitation or phase separation at the end of the treatment period (4 hours) before the test item was removed. No precipitation or phase separation was noted up to the highest concentration with and without metabolic activation.

There was no relevant shift of osmolarity and pH of the medium even at the maximum concentration of the test item measured in the main experiment (solvent control: 376 mOsm, pH 7.48 versus 360 mOsm and pH 7.10 at 2000.0 µg/mL).

### Main Experiment

Based on the results of the pre-experiment the following concentrations were applied in Experiment I with and without metabolic activation:

62.5; 125.0; 250.0; 500.0; 1000.0; and 2000.0 µg/mL

Neither precipitation nor phase separation was observed.

The cultures at 62.5 and 125.0 µg/mL with and without metabolic activation were not evaluated for mutagenicity as a minimum of only four concentrations are required by the guideline.

No relevant cytotoxic effect indicated by a relative total growth of less than 50% was noted in the presence and absence of metabolic activation for concentrations between 250 and 2000 µg/mL.

No substantial dose dependent increase in the mutation frequency exceeding the threshold of 126 above the corresponding solvent control was observed with and without metabolic activation for the evaluated concentrations.

The statistical analysis was performed based on the mean values of culture I and II. No statistically significant concentration dependent trend was observed in the absence and presence of metabolic activation.

The reported solvent control fulfilled the acceptability criteria as stated in the OECD 490.

**In conclusion it can be stated that during the mutagenicity test described and under the experimental conditions reported the test item SYN548263 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation. Therefore, SYN548263 is considered to be non-mutagenic in this mouse lymphoma assay.**

### MATERIALS AND METHODS

#### Materials:

<b>Test Material:</b>	SYN548263
<b>Description:</b>	White, solid
<b>Lot/Batch number:</b>	MES 346/2
<b>Molecular weight:</b>	277.2 g/mol
<b>Purity</b>	99% w/w
	concentration calculation not adjusted to purity
<b>Stability of test compound:</b>	Not indicated by the sponsor
<b>Control Materials:</b>	
<b>Negative:</b>	-
<b>Solvent control</b>	
<b>(final concentration):</b>	DMSO (0.5%)
<b>Positive control:</b>	Absence of S9 mix: Methylmethanesulphonate, 19.5 µg/mL
	Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

#### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM MgCl<sub>2</sub>  
 33 mM KCl  
 5 mM glucose-6-phosphate  
 4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

**Test cells: mammalian cells in culture**

X	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media:</b> RPMI 1640			
Properly maintained?		X	Yes
Periodically checked for Mycoplasma contamination?		X	Yes
Periodically checked for karyotype stability?		X	Yes
Periodically “cleansed” against high spontaneous background?			Yes

X indicates those that apply

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na <sup>+</sup> /K <sup>+</sup> ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	Trifluorothymidine (TFT)				

X indicates those that apply

**Test compound concentrations used:**

Absence of S9 mix 62.5; 125.0; **250.0; 500.0; 1000.0; 2000.0** µg/mL

Presence of S9 mix 62.5; 125.0; **250.0; 500.0; 1000.0; 2000.0** µg/mL

Numbers in bold indicate concentrations analysed for mutagenicity

**Study Design and Methods:**

**In-life dates:** Start: 04 December 2017, End: 13 February 2018

**Test performance:**

**Cell treatment:** Cells (1x10<sup>7</sup> cells /flask) were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

After washing, cells were cultured for 2 days (expression period) before cell selection.

After the expression period the cultures were selected. Cells from each experimental group were seeded into 2 microtiter plates so that each well contained approximately 4x10<sup>3</sup> cells in selective medium with TFT (SERVA, 69042 Heidelberg, Germany). The viability (cloning efficiency) was determined by seeding about 2 cells per well into microtiter plates (same medium without TFT). The plates were incubated at 37°±1.5°C in 4.5% COB<sub>2B</sub>/95.5% water saturated air for 10 - 15 days.

Cell growth in individual microwell plates was assessed after 10-15 days using a dissecting microscope. The survival plates and viability plates were scored for the number of wells containing no cell growth (negative wells). The mutation plates were scored so that each well contained either a small colony



(considered to be associated with clastogenic effects), a large colony (considered to be associated with gene mutation effects), or no colony.

**Statistical Methods:**

The statistical analysis was performed in the mean values of culture I and II.

A linear regression (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using the validated R Script LM.Rnw statistics software. The number of mutant colonies obtained for the groups treated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological relevance and statistical significance were considered together.

**Size distribution of the colonies:** Each well of the mutation plates (those containing TFT) was scored as containing either, a small colony, a large colony or no colony according to the following criteria:

Colonies were counted manually. In accordance with their size the colonies were classified into two groups. The colony size distribution was determined in the controls and at all concentrations of the test item. Criteria to determine colony size were the absolute size of the colony (more than 25% of a well for large colonies) and the optical density of the colonies (the optical density of the small colonies is generally higher than the large colonies).

**Acceptability of the Assay**

A mutation assay is considered acceptable if, for at least four concentrations, the mean values of both parallel cultures meet the following criteria:

Reasonable reproducibility between cultures should be given.

All plates, from either the cloning efficiency or the TFT resistance-testing portion of the experiment are analysable.

The absolute cloning efficiency at the time of mutant selection (CE) of the solvent controls is 65 – 120 %.

The total suspension growth of the solvent control calculated by the day 1 fold-increase in cell number multiplied by the day 2-fold-increase in cell number is 8 – 32.

The range of the solvent control mutant frequency is in the range of 50 – 170 x 10<sup>-6</sup> cells.

The positive controls (MMS and CPA) should yield an absolute increase in total MF (Mutation Frequency, number of mutant colonies per 10<sup>6</sup> cells), that is an increase above spontaneous background MF (an induced MF [IMF]) of at least 300 x 10<sup>-6</sup> cells. At least 40 % of the induced mutation frequency (IMF) should be reflected in the small colony MF. Alternatively, the positive controls should induce at least 150 small colonies.

The upper limit of cytotoxicity observed in the positive control culture should be the same as for the experimental cultures (i.e. the relative total growth – RTG – should be greater than 10 % of the concurrent selective control group).

The highest concentration of the test substance should be 10 mM or 2 µL/mL or 2 mg/mL, unless limited by toxicity, solubility or molecular weight of the test substance. If toxicity occurred, the highest concentration should lower the relative total growth to approximately 10 to 20 % of survival. If precipitation or phase separation is noted, the highest analysed concentration should be the lowest concentration where insolubility is observed by the naked eye.

**Evaluation of Results**

A test item is classified as clearly mutagenic if the induced mutation frequency reproducibly exceeds a threshold of 126 colonies per 10<sup>6</sup> cells above the mean concurrent background.

This threshold is called the Global Evaluation Factor (GEF).

A relevant increase of the mutation frequency should be dose-dependent when evaluated by a trend test.

A mutagenic response is considered to be reproducible if it occurs in both parallel cultures.

A test item is considered equivocal in this assay if the threshold is reproducibly exceeded but the increase of the mutation frequency is not dose dependent.

However, in the evaluation of the test results the historical variability of the mutation rates in the solvent controls of this study are taken into consideration.

Results of test groups are generally rejected if the relative total growth is less than 10 % of the vehicle control.

Whenever a test item is considered mutagenic according to the above mentioned criteria, the ratio of small versus large colonies is used to differentiate point mutations from clastogenic effects. If the increase of the mutation frequency is accompanied by a reproducible and dose dependent shift in the ratio of small versus large colonies clastogenic effects are indicated.

A test item is classified as non-mutagenic if the induced mutation frequency does not reproducibly exceed a threshold of 126 colonies per  $10^6$  cells above the corresponding solvent control.

## RESULTS

The study was performed to investigate the potential of SYN548263 to induce mutations at the mouse lymphoma thymidine kinase locus of the cell line L5178Y.

The assay was performed using the microwell method with and without liver microsomal activation using two parallel cultures with four hours treatment.

### **Preliminary toxicity assay:**

Test item concentrations between 15.6 µg/mL and 2000 µg/mL were used in the pre-experiment. The highest concentration was chosen with respect to the OECD guideline 490 (2016).

No relevant cytotoxic effects were observed up to the maximum concentration in the presence or absence of metabolic activation.

The test medium was checked for precipitation or phase separation at the end of the treatment period (4 hours) before the test item was removed. No precipitation or phase separation was noted up to the highest concentration with or without metabolic activation.

There was no relevant shift of osmolarity and pH of the medium even at the maximum concentration of the test item measured in the main experiment (solvent control: 376 mOsm, pH 7.48 versus 360 mOsm and pH 7.1 at 2000.0 µg/mL).

### **Mutation experiment:**

#### **Experiment I in the absence of S9 mix**

Based on the results of the pre-experiment the following concentrations were applied:

62.5; 125.0; 250.0; 500.0; 1000.0; and 2000.0 µg/mL

No precipitation or phase separation was noted up to the highest concentration.

The cultures at 62.5 µg/mL and 125.0 µg/mL were not evaluated for mutagenicity as a minimum of only four concentrations are required by the guideline.

No relevant cytotoxic effects indicated by a relative total growth of less than 50% occurred up to the highest concentration.

No substantial or reproducible dose dependent increase in the mutation frequency exceeding the threshold of 126 above the corresponding solvent control was observed. The statistical analysis was performed based on the mean values of culture I and II. No statistically significant concentration dependent trend was observed. Therefore, this experimental condition is concluded to be clearly negative.

MMS was used as a positive control and showed a distinct increase in both small and large mutant colonies thus demonstrating the sensitivity of the test system.

Since the solvent control in the experimental part with metabolic activation did not fulfil the acceptability criteria (mutant frequency > 170), this experiment was judged as invalid (data not reported) and was repeated under identical conditions as experiment IA.

#### Experiment IA in the presence of S9 mix (not reported)

Due to the high variability in relative total growth between culture I and II, Experiment IA was terminated prior to the generation of mutagenicity data and repeated as Experiment IB. The data of Experiment IA are not reported.

#### Experiment IB in the presence of S9 mix (reported as part of experiment I)

The following concentrations were used in Experiment IB:

62.5; 125.0; 250.0; 500.0; 1000.0; and 2000.0 µg/mL

No precipitation or phase separation was noted up to the highest concentration.

The cultures in Experiment IB at 62.5 and 125.0 µg/mL were not evaluated for mutagenicity as a minimum of only four concentrations are required by the guideline.

No relevant cytotoxic effects indicated by a relative total growth of less than 50% occurred up to the highest concentration.

No substantial or dose dependent increase in the mutation frequency exceeding the threshold of 126 above the corresponding solvent control was observed. The statistical analysis was performed based on the mean values of culture I and II. No statistically significant concentration dependent trend was observed. Therefore, this experiment is concluded to be clearly negative.

The data in this experiment is reported under Experiment I.

CPA was used as a positive control and showed a distinct increase in both small and large mutant colonies. Thus demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

Since the test substance gave a negative result in both the presence and absence of metabolic activation, and in accordance with the OECD test guideline for this assay, no further experiments were required.

**Table 6.8.1-57: Summary of results of the Mouse Lymphoma Assay with SYN548263**

conc. µg/mL	S9 mix	relative total growth	mutant colonies/ 10 <sup>6</sup> cells	threshold	linear regression analysis*
Experiment I / 4 h treatment		mean of culture I and II			
Solv. control with DMSO	-	100.0	120	246	0.254
Pos. control with MMS	-	12.2	602	246	
Test item	-	#	#	#	
Test item	-	86.1	#	#	
Test item	-	89.4	168	246	
Test item	-	108.4	167	246	
Test item	-	96.5	159	246	
Test item	-	82.0	176	246	

Experiment I / 4 h treatment		mean of culture I and II			
Solv. control with DMSO		+	100.0	127	253
Pos. control with CPA	3.0	+	81.4	243	253
Pos. control with CPA	4.5	+	34.5	465	253
Test item	62.5	+	#	#	#
Test item	125.0	+	121.2	#	#
Test item	250.0	+	105.3	180	253
Test item	500.0	+	99.2	212	253
Test item	1000.0	+	102.2	188	253
Test item	2000.0	+	105.6	185	253

threshold = number of mutant colonies per  $10^6$  cells of each solvent control plus 126

\* p-value (significant trend =  $p < 0.05$ )

# culture was not continued as a minimum of only four analysable concentrations is required

## CONCLUSION:

In a GLP and OECD compliant mammalian cell gene mutation assay, the potential of SYN548263 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y was investigated. The study consisted of a preliminary toxicity test and two independent mutagenicity assays (experiment I and IB). The cells were exposed for 4 hours in the absence or presence of exogenous metabolic activation (S9 mix).

In the pre-experiment, test item concentrations between 15.6 µg/mL and 2000 µg/mL were used. The highest concentration was equivalent to 10 mM, the limit concentration for this test. No relevant cytotoxic effects were observed up to the maximum concentration in the presence and absence of metabolic activation. No precipitation or phase separation was noted up to the highest concentration with and without metabolic activation.

In the main experiment, the following concentrations were applied in experiment I and IB with and without metabolic activation:

62.5; 125.0; 250.0; 500.0; 1000.0; and 2000.0 µg/mL

Neither precipitation nor phase separation was observed. No relevant cytotoxic effect indicated by a relative total growth of less than 50% was noted in the presence and absence of metabolic activation up to the top concentration. No substantial dose dependent increase in the mutation frequency exceeding the threshold of 126 (GEF) above the corresponding solvent control was observed with and without metabolic activation for the evaluated concentrations. No statistically significant concentration dependent trend was observed in the absence and presence of metabolic activation.

CPA was used as a positive control and showed a distinct increase in both small and large mutant colonies, thus demonstrating the sensitivity of the test system and the efficacy of the S9 mix. The reported solvent control fulfilled the acceptability criteria of the guideline.

As a result, HSE agrees with the EU evaluation, that **SYN548263 did not induce mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation when tested up to the limit concentration of this assay.**

(██████████, 2018)

## Summary of toxicity data on metabolite SYN548263

### Summary of toxicity studies and derivation of dietary reference values for metabolite SYN548263 glucuronide/sulphate

Metabolite SYN548263 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN548263. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

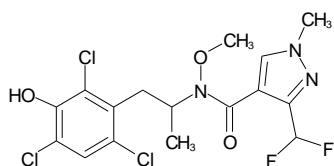
Overall, GLP and OECD compliant in vitro genotoxicity assays are available on metabolite SYN548263. The metabolite was negative in the standard battery of 3 in vitro tests and therefore it is considered to be non-genotoxic.

The table below summarises the available studies on SYN548263:

Study & Acceptability	Result	Reference
Ames test in bacteria GLP, OECD 471  <i>Acceptable</i>	Negative up to limit concentration	██████ (2018). Report No. 1880400. Syngenta File No. SYN548263_10002
Micronucleus Test in Human Lymphocytes <i>In Vitro</i> . GLP, OECD 487  <i>Acceptable</i>	Negative up to limit concentration	██████ (2018). Report No. 1880200. Syngenta File No. SYN548263_10003
Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells. GLP, OECD 490  <i>Acceptable</i>	Negative up to limit concentration	██████ (2018). Report No. 1880500. Syngenta File No. SYN548263_10001

It is noted that SYN548263 is only a minor rat metabolite (< 10% AD in urine and plasma); however, it is a direct precursor of SYN508272, which is a major rat metabolite (14.8% TRA in blood). On this basis, it can be assumed that at some point, SYN548263 must have also been present at similar levels in plasma; thus it can be considered a major rat metabolite, covered by the parent dataset. **Therefore, if a dietary risk assessment were to be required, the dietary reference values of the parent could be used. This is in contrast to the advice given by the EU peer-review process.**

#### **B.6.8.1.5. Metabolite SYN547897**



3-(difluoromethyl)-N-methoxy-1-methyl-N-[1-methyl-2-(2,4,6-trichloro-3-hydroxyphenyl)ethyl]pyrazole-4-carboxamide

SYN547897 is a metabolite of SYN545974, which has been identified in animal commodities in goat liver and goat kidney. After an oral administration of SYN545974 in rat, SYN547897 was detected in plasma accounting for up to 4.3% of total radioactivity AUC (TRA) and for up to 0.9% in urine. Based on the structural similarity to parent (hydroxylated parent on the benzene ring) and its presence in rat metabolism, comparative genotoxicity QSAR analysis of the metabolite and parent are considered appropriate to assess SYN547897. Therefore, the Threshold of Toxicological Concern (TTC) for non-genotoxic compounds ('Cramer Class III') are considered appropriate for assessing dietary exposure to SYN547897 if necessary.

**EFSA Request for additional information (February 2018), Question 41:** Applicant to provide a detailed assessment of the QSAR analysis as well read across analysis (including genotoxicity and other toxicological endpoints) in order to characterize the toxicological profile of the metabolite SYN547897.

A detailed assessment of the QSAR analysis as well as read across analysis for genotoxicity of metabolite SYN547897 has been conducted by the applicant and summarized below. SYN547897 is considered not to be of genotoxic concern based on either similarity of the (Q)SAR alerts or *via* chemical read-across to SYN545974 with a known negative genotoxicity assessment (K-CA 5.8.1/76).

The applicant did not provide QSAR and read across analysis on toxicological end-points other than genotoxicity that would have permit to characterize the complete toxicological profile of SYN545974. The toxicological profile of SYN547897 has been discussed in expert meeting PPR 182 (September 2018). The experts agreed that the metabolite can be considered non genotoxic. However, the database for general toxicity was considered insufficient to set an ADI .

Comparative genotoxicity QSAR analysis of metabolite and parent, structural similarity, read-across and conclusion

<b>Report:</b>	K-CA 5.8.1/76 (2018) SYN548263 - SYN547897 - Chemical Read-Across for Genotoxicity Potential Using a Multi-(Q)SAR Approach. Syngenta - Jealott's Hill, Bracknell, United Kingdom. Unpublished. Syngenta File No. SYN545974_10625.
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**Guidelines:** Not applicable.

**GLP:** This report contains an assessment of data from additional scientific information and is not a study per se, a Good Laboratory Practice (GLP) statement as defined by OECD is not appropriate.

## EXECUTIVE SUMMARY

The purpose of this document is to provide chemical read-across evidence for genotoxicity potential for SYN547897 using a multi-(Q)SAR approach, in order to in order to characterize the molecules genotoxicity profile. SYN547897 is a rat metabolite at levels <10% (mainly fecal excreta) of the administered dose, with close structural relation to parent as its simple hydroxylate. All of the available genotoxicity data on the following similar chemical structures of SYN545974 (parent) were used.

## RESULTS AND DISCUSSION

### Multi – (Q)SAR assessment

The genotoxicity of SYN547897 was assessed using multi-(Q)SAR and a chemical read-across approach.

Three (Q)SAR programs were selected working on different basis of expert knowledge rules and statistical methods for the assessment of genotoxicity. These were; DEREK Nexus (genotoxicity alerts), CAESAR (mutagenicity model) and OECD QSAR Toolbox to assess DNA and protein binding and for organic functional group profiling. Hence, overall, using these tools the genotoxicity endpoints related to molecular initiating events relevant to genotoxicity were evaluated, i.e. chromosome damage and DNA/protein binding. The models used in each respective program are included in the references.

The structures assessed using this (Q)SAR methodology SYN547897 (target molecule) and the the parent molecule SYN545974 which are presented in Table 89.

SYN547897 (target molecule) exists as a a rat metabolite at levels <10% (mainly fecal excreta) of the administered dose as demonstrated by the biotransformation study for SYN545974 ( and , 2015). The genotoxicity potential of SYN547897 is evaluated using the above mentioned predictive tools.

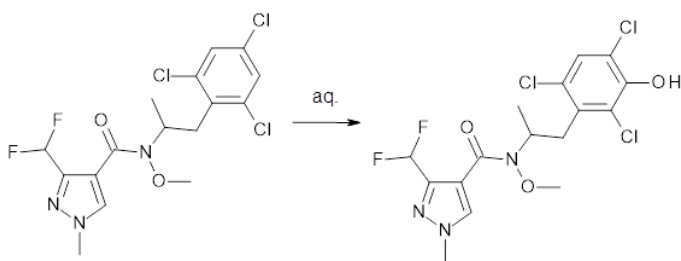
SYN545974 is the parent molecule, and it has a full *in vitro* and *in vivo* genotoxicity battery available, which demonstrates that SYN545974 is not of genotoxic concern.

### Chemical read-across

In addition to the (Q)SAR assessment, chemical read-across was conducted using knowledge of chemical reactivity and structural feature similarities. The principles of chemical read-across rely on using chemical reactivity to bridge between a structure of interest (target molecule) for which there can be limited data to a chemically similar molecule (source) where data is available.

In the current context SYN547897 are designated as the target structure and SYN545974 is designated as the source structure.

SYN545974 forms SYN547897 through hydroxylation



**Figure 6.8.1-2: Hydrolysis of SYN545974 to SYN547897**

A full negative genotoxicity package (*in vitro* and *in vivo*) is available for SYN545974. The *in vitro* genotoxicity studies are conducted in aqueous conditions; under these conditions the hydroxyl group is added to SYN547897. Therefore, it is reasonable to assume that SYN547897 was assessed for genotoxicity as part of the studies on the parent compound, SYN545974.

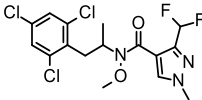
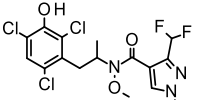
### DISCUSSION

SYN547897 returned no alerts for genotoxicity using DEREK Nexus and was considered to be “out of domain” by CEASAR. A number of alerts relevant to initiating events for genotoxicity were identified by two profilers in the OECD QSAR Toolbox (Table 6.8.1-58). An additional profiler for protein binding was also triggered.

With the exception of the alert for protein binding, direct read-across of SYN545974 to SYN547897 is possible due to same structural activity alerts in the (Q)SAR outputs. The protein binding alert for SYN547897 is based on structural alerts for protein interactions which may be a secondary mechanism in the formation of chromosomal aberrations. It should be noted however that the other profilers for protein binding in the OECD QSAR Toolbox were not triggered by SYN547897, nor was any chromosomal aberration activity identified by the expert knowledge based system DEREK Nexus. Hence, given the lack of congruence between the different QSAR tools (DEREK Nexus and OECD QSAR Toolbox) the relevance of this protein binding alert is highly questionable.

Overall therefore, SYN547897 can be considered to be of no genotoxic concern based on (Q)SAR and read-across analysis, supported by the comprehensive *in vitro* and *in vivo* genotoxicity data available for SYN545974.

Table 6.8.1-58: Summary of (Q)SAR Alerts and Existing Genotoxicity Data

Compound identifier	Existing genotoxicity data	DEREK Nexus alerts	CEASAR alerts (Ames mutagenicity)	OECD QSAR Toolbox (Genotoxicity relevant profiling)	Read-across use
SYN545974 	Ames -ve  in vitro mammalian mutagenicity –ve  in vitro chromosomal aberration +ve  In vivo mammalian erythrocyte micronucleus mouse -ve  mouse -ve  rat -ve	No genotoxicity alerts	Non-mutagenic (outside the Applicability Domain)	(in vivo mutagenicity (Micronucleus) alerts by ISS) H-acceptor-path3-H-acceptor  (DNA binding by OASIS v.1.4) AN2 >> Schiff base formation by aldehyde formed after metabolic activation >> Geminal Polyhaloalkane Derivatives  Radical >> Radical mechanism via ROS formation (indirect) >> Geminal Polyhaloalkane Derivatives  SN2 >> Acylation involving a leaving group after metabolic activation >> Geminal Polyhaloalkane Derivatives  SN2 >> Nucleophilic substitution at sp3 carbon atom after thiol (glutathione) conjugation >> Geminal Polyhaloalkane Derivatives	Source
SYN547897 	None	No genotoxicity alerts	Non-mutagenic (outside the Applicability Domain)	(in vivo mutagenicity (Micronucleus) alerts by ISS)H-acceptor-path3-H-acceptor  (Protein binding alerts for Chromosomal aberration by OASIS v.1.2) AN2 >> Michael addition to the quinoid type structures >> Substituted Phenols - <a href="#">questionable</a>  (DNA binding by OASIS v.1.4) AN2 >> Schiff base formation by aldehyde formed after metabolic activation >> Geminal Polyhaloalkane Derivatives  Radical >> Radical mechanism via ROS formation (indirect) >> Geminal Polyhaloalkane Derivatives  SN2 >> Acylation involving a leaving group after metabolic activation >> Geminal Polyhaloalkane Derivatives  SN2 >> Nucleophilic substitution at sp3 carbon atom after thiol (glutathione) conjugation >> Geminal Polyhaloalkane Derivatives	Target



**CONCLUSION:** SYN547897 is considered not to be of genotoxic concern based on either similarity of the (Q)SAR alerts or *via* chemical read-across to SYN545974 with a known negative genotoxicity assessment.

#### REFERENCES:

██████████ (2013). SYN545974 - Chromosome Aberration Test in Human Lymphocytes In Vitro. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1498902, issue date: 21 January 2013. Unpublished. Syngenta File No. SYN545974\_10048.

██████████ (2014). SYN545974 - Micronucleus Assay in Bone Marrow Cells of the Mouse. ██████████. Laboratory Report No. 1648702, issue date: 12 December 2014, Unpublished. Syngenta File No. SYN545974\_10141.

██████████ and ██████████ (2015). SYN545974 - Biotransformation of [14C]-SYN545974 in Rat, ██████████. Report No. 34216, issue date 31 July 2015. Unpublished. Syngenta File No. SYN545974\_10259.

██████████ (2017). Rat Bone Marrow Chromosome Aberration Assay. ██████████. Laboratory Report No. 8359217, issue date: 11 October 2017, Unpublished. Syngenta File No. SYN545974\_10513.

██████████ (2012). SYN545974 – Micronucleus Assay in Bone Marrow Cells of the Mouse. ██████████. Laboratory Report No. 1498904, issue date: 20 December, 2012, Unpublished. Syngenta File No. SYN545974\_10045.

██████████ (2012). SYN545974: Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan, Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1498901, 22 October 2012. Unpublished. Syngenta File No. SYN545974\_10018.

██████████ (2014). SYN545974 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1648701, issue date: 03 December 2014. Unpublished. Syngenta File No. SYN545974\_10127.

██████████ (2013). SYN545974 - Cell Mutation Assay at the Thymidine Kinase Locus (TK <sup>+/+</sup>) in Mouse Lymphoma L5178Y Cells. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1498903, issue date: 24 January 2013. Unpublished. Syngenta File No. SYN545974\_10049.

(I). Derek Nexus v.5.0.1; LHASA Limited, Leeds <http://www.lhasalimited.org/>

Knowledgebase Derek KB 2015 1.0  
All alerts used.

(II). Vega V 1.1.4; (build date 13/02/17 [www.vega-qsar.eu](http://www.vega-qsar.eu)) Mario Negri.

Mutagenicity (Ames test) model (CEASAR) – v. 2.1.13

(III). OECD QSAR Toolbox Version 4.2; 2018.

Mechanistic, endpoint specific and empiric (functional group) profilers were used.

Mechanistic: DNA binding by OASIS v1.5  
DNA binding by OECD v2.3  
Protein binding by OASIS v1.5  
Protein binding by OECD v2.3

Endpoint specific: DNA alerts for AMES by OASIS v1.5  
DNA alerts for CA and MNT by OASIS v1.2  
In vitro mutagenicity (AMES test) by ISS v2.3

*In vivo mutagenicity (MN) by ISS v2.3*

*Protein binding alerts for Chromosomal aberration by OASIS v.1.2*

*Empiric: Organic functional groups*

(██████████, 2018)

An updated comparative genotoxicity QSAR analysis of metabolite SYN547897 and pydiflumetofen was performed by the applicant in Feb 2021 (██████████ & ██████████, 2021). This provided further details requested by EFSA and confirmed the findings of ██████████ (2018).

**Comparative genotoxicity QSAR analysis of metabolite SYN547897 and pydiflumetofen, structural similarity and read-across (██████████, 2018; ██████████ & ██████████, 2021)**

Genotoxicity predictions were obtained from three different models: Derek Nexus (V6.1.0), Caesar (v2.1.13) and the OECD QSAR Toolbox (v4.4). Derek Nexus identified no alerts for both substances. Caesar also identified no alerts for both compounds, but since they were out-of-domain, these predictions are not fully reliable. The OECD QSAR Toolbox identified some alerts; however, with one exception (protein binding), these were identical between the metabolite and the parent substance for which a comprehensive data package showed absence of genotoxicity. Therefore, the alerts identified by the OECD QSAR Toolbox were overpredictive and could be dismissed. The protein binding alert for SYN547897 was based on structural alerts for protein interactions which may be a secondary mechanism in the formation of chromosomal aberrations. It should be noted however that the other profilers for protein binding in the OECD QSAR Toolbox were not triggered by SYN547897, nor was any chromosomal aberration activity identified by the expert knowledge based system Derek Nexus. Hence, given the lack of congruence between the different QSAR tools (Derek Nexus and OECD QSAR Toolbox) the relevance of this protein binding alert is highly questionable.

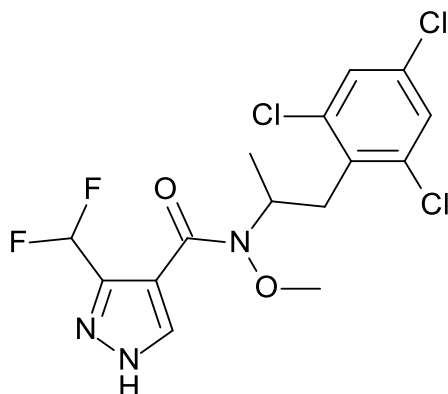
Overall, SYN547897 was predicted not be genotoxic on the basis of a comparative genotoxicity QSAR analysis (involving three different models) with the parent substance.

In addition, SYN547897 is structurally very similar to pydiflumetofen, being the hydroxylated parent on the benzene ring. Therefore, read-across of the genotoxicity data of pydiflumetofen to SYN547897 is appropriate. In conclusion, SYN547897 is not genotoxic.

EFSA requested a comparative QSAR and read across analysis for toxicological end-points other than genotoxicity to permit the characterization of the complete toxicological profile of SYN545974. This was performed by the applicant using Derek Nexus (V6.1.0). The same alerts (carcinogenicity alert 116 – plausible; nephrotoxicity alert 035 – equivocal; and skin sensitisation alert 892 – plausible) were triggered by the parent substance and the metabolite. However, in addition the metabolite presented mitochondrial disfunction alert 097 – equivocal; and a supplementary skin sensitisation alert 439 – plausible, which were triggered by the phenol substructure, which is specific to SYN547897. The applicant did not discuss the significance of these additional alerts. Therefore, no clear conclusions can be drawn on the general toxicity profile of metabolite SYN547897.

**Overall conclusion**

HSE notes that SYN547897 is not a major rat metabolite; therefore it cannot be considered covered by the parent dataset. However, given the lack of genotoxicity based on a comparative QSAR analysis with the parent, HSE concludes that, if required, the **TTC Cramer Class values (chronic value = 1.5 µg/kg bw/day and acute value = 5 µg/kg bw) can be used in the dietary risk assessment**. This is in contrast to the EU decision not to set a toxicological reference values for SYN547897.

***B.6.8.1.6. Metabolite SYN547891 glucuronide/sulphate***

3-(difluoromethyl)-N-methoxy-N-[1-methyl-2-(2,4,6-trichlorophenyl)ethyl]-1H-pyrazole-4-carboxamide (SYN547891 aglycon)

Metabolite SYN547891 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN547891. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

Following the EU peer-review process, EFSA identified a data gap for metabolite SYN547891 related to its genotoxic potential. A comparative genotoxicity QSAR analysis of metabolite SYN547891 and pydiflumetofen was performed by the applicant in Feb 2021 (██████████ & ██████████, 2021). In addition, structural similarity with the parent substance and potential read-across of its genotoxicity package was considered.

Genotoxicity predictions were obtained from three different models: Derek Nexus (V6.1.0), Caesar (v2.1.13) and the OECD QSAR Toolbox (v4.4). Derek Nexus identified no alerts for both substances. Caesar also identified no alerts for both compounds, but since they were out-of-domain, these predictions are not fully reliable. The OECD QSAR Toolbox identified some alerts; however, these were identical between the metabolite and the parent substance for which a comprehensive data package showed absence of genotoxicity. Therefore, the alerts identified by the OECD QSAR Toolbox were overpredictive and could be dismissed. Overall, SYN547891 was predicted not be genotoxic on the basis of a comparative genotoxicity QSAR analysis (involving three different models) with the parent substance (see Table B.6.8.1-59 below).

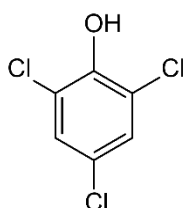
In addition, SYN547891 is structurally very similar to pydiflumetofen, being the desmethylated parent on the pyrazole ring. Therefore, read-across of the genotoxicity data of pydiflumetofen to SYN547891 is appropriate. In conclusion, SYN547891 is not genotoxic.

**Overall conclusion**

HSE notes that SYN547891 is not a major rat metabolite; therefore it cannot be considered covered by the parent dataset. However, given the lack of genotoxicity based on a comparative QSAR analysis with the parent, HSE concludes that, if required, the **TTC Cramer Class values (chronic value = 1.5 µg/kg bw/day and acute value = 5 µg/kg bw) can be used in the dietary risk assessment.**

Table 6.8.1-59: Comparative genotoxicity (Q)SAR analysis of SYN547891 and read-across from parent

Compound identifier	Existing genotoxicity data	DEREK Nexus alerts	CEASAR alerts  (Ames mutagenicity)	OECD QSAR Toolbox  (Genotoxicity relevant profiling)	QSAR/read-across conclusion for genotoxicity
Pydiflumetofen	Ames -ve  in vitro mammalian mutagenicity –ve  in vitro chromosomal aberration +ve  In vivo mammalian erythrocyte micronucleus mouse -ve  Mouse MN -ve  Rat MN -ve	No genotoxicity alerts	Non-mutagenic (outside the Applicability Domain)	(in vivo mutagenicity (Micronucleus) alerts by ISS) H-acceptor-path3-H-acceptor  (DNA binding by OASIS v.1.4) AN2 >> Schiff base formation by aldehyde formed after metabolic activation >> Geminal Polyhaloalkane Derivatives  Radical >> Radical mechanism via ROS formation (indirect) >> Geminal Polyhaloalkane Derivatives  SN2 >> Acylation involving a leaving group after metabolic activation >> Geminal Polyhaloalkane Derivatives  SN2 >> Nucleophilic substitution at sp3 carbon atom after thiol (glutathione) conjugation >> Geminal Polyhaloalkane Derivatives	<b>Full genotoxic package available. No genotoxic concern</b>
<b>SYN547891</b>	None	No genotoxicity alerts	Non-mutagenic (outside the Applicability Domain)	Same alerts as parent	<b>SYN547891 is of no genotoxic concern based on read-across from parent. Same QSAR alerts and chemically similar with respect to genotoxicity</b>

**B.6.8.1.7. Metabolite 2,4,6-Trichlorophenol sulphate (2,4,6-TCP sulphate)**

## 2,4,6-TCP

Metabolite 2,4,6-TCP sulphate is a livestock metabolite. In the human gastro-intestinal tract, the sulphate will be easily cleaved, leading to systemic exposure to the aglycon, 2,4,6-TCP. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugate. In addition, it is noted that 2,4,6-TCP sulphate is a major rat metabolite covered by the parent.

No toxicity studies have been conducted with the mammalian (rat and mouse) and livestock metabolite 2,4,6- TCP by Syngenta. However, in accordance with Article 8(5) of Regulation (EC) No 1107/2009 a search of the published literature data on relevant metabolites is required, therefore summaries of relevant literature data identified are included in this section.

Following the EU peer-review process, it became evident that the genotoxic potential of 2,4,6-TCP required further investigation and that more robust repeated dose toxicity studies were needed to characterise its general toxicity profile. On this basis, Syngenta has recently submitted a new genotoxicity package (Ames, in vitro micronucleus assay, mammalian cell gene mutation test and an in vivo TGR study), a 14-day range-finder study and a 28-day oral study in the rat on 2,4,6-TCP.

These new studies have been fully evaluated by HSE.

Although historic toxicity data from the open literature are available for TCP, it is considered that the mammalian toxicity data package on SYN545974 sufficiently assesses the toxicity of SYN545974 and its mammalian metabolites including TCP and its conjugates. In both rats and mice, TCP and TCP-conjugates were the major circulating metabolites after administration of SYN545974 and the plasma concentration of TCP-species exceeds that of parent SYN545974; therefore, the mammalian toxicity data package on SYN545974 adequately assesses the toxicity of TCP and its conjugates. Consumer risk assessment endpoints for SYN545974 are proposed using NOAELs from studies conducted in the rat or mouse, where it is known that TCP was a major circulating metabolite; therefore are considered appropriate for risk assessment (OECD, 2009)<sup>9</sup>.

2,4,6-TCP was identified as a metabolite in animal commodities and 2,4,6-TCP is included in the definition of residue for animal commodities only. However, in the rat and mouse, 2,4,6-TCP was the major circulating metabolite of SYN545974 in plasma (██████ and ██████, 2015 and ██████ et al., 2015 see Section 5.1.1/05 and 5.1.1/08 respectively). Therefore, the mammalian toxicity database on SYN545974 also assesses the toxicity of 2,4,6-TCP and risk assessment endpoints for SYN545974 are considered appropriate also for 2,4,6-TCP. Although the dose levels of SYN545974 were limited in the long term repeat dose studies, based on non-proportional kinetics, because this was due to dose limited absorption, systemic levels of metabolites would also not increase if the dose of SYN545974 was higher. In addition, it is known that systemic 2,4,6-TCP in rats is rapidly excreted and does not accumulate (██████ et al., 1981; ██████ et al., 1986). The literature studies on 2,4,6-TCP were designed to understand hazard and not to establish NOAEL; however, it is clear that toxicity observations were only observed at very high dose levels, with the lowest LOAEL cited at 240 mg/kg/day and a NOAEL of 80 mg/kg/day (██████ et al., 1990). For further information on the toxicokinetics of SYN545974 see section B.6.1.

In the rat following administration of SYN545974, the nature and identity of metabolites present in samples of urine, plasma, feces, bile and cage wash obtained from male and females following single oral administration of <sup>14</sup>C-SYN545974 were investigated (see K-CA 5.1.1/05 ██████ and ██████, 2015). Male and female rats received a single oral administration at either 5 mg/kg or 300 mg/kg (male rats) and 100 mg/kg (female rats). 2,4,6-TCP and its related metabolites (hydroxyl TCP sulphate and

<sup>9</sup> OECD (2009). Guidance Document on the Definition of Residue (as revised in 2009). ENV/JM/MONO(2009)30.

TCP sulphate) were identified in urine and plasma, but not faeces, confirming that 2,4,6-TCP was systemically circulating in the rat with 2,4,6-TCP sulphate accounting for >10% of the administered dose in urine. The maximum total mg/kg 2,4,6-TCP related material rats were exposed to over 72 h following oral administration was 17.1 mg/kg for males at 300 mg SYN545974/kg and 10.9 mg/kg for females at 100 mg SYN545974/kg (Table 6.8.1-23). Parent molecule recovered from urine was negligible.

#### **Is 2,4,6-TCP, as a major rat and mouse metabolite, covered by the parent dataset?**

HSE is of the view that where specific and extensive data exist on a metabolite, these should take priority in characterising the toxicity profile of the metabolite over and above the predictive approach of using the parent's data because the substance is a major rat metabolite of the parent. Therefore, a toxicological characterisation of 2,4,6-TCP based on specific data is considered more appropriate.

During the commenting period, the applicant was invited to provide further rationale demonstrating that the dose levels selected in the long term studies with pydiflumetofen were also sufficient to detect the expression of the carcinogenic potential of 2,4,6-TCP (EFSA request, question 15). This additional information provided by the applicant has been added above. The applicant based his argumentation and calculation from the urine excretion data available for 2,4,6-TCP. HSE choose to base his prediction on the profile of 2,4,6-TCP in plasma by using total plasma AUC data (argumentation presented below in paragraph: "HSE consideration regarding the carcinogenic potential of 2,4,6 TCP and the dose level selection for SYN545974 toxicity studies"). It should be noted that this method of calculation (from 2,4,6 TCP profile in plasma) might overestimate the systemic dose of 2,4,6 TCP and should be considered as a worst case assumption. Anyway, even using this worst case approach, the conclusion of HSE is the same than those of the application namely that it can be reasonably considered that the mammalian toxicity data package on SYN545974 has sufficiently assessed the toxicity of 2,4,6 TCP (and in particular its carcinogenicity potential).

#### **EFSA Request for additional information (February 2018), Question 15:**

Applicant to provide further rationale demonstrating that the dose levels selected in the long term toxicity studies with pydiflumetofen were also sufficient to detect the expression of the carcinogenic potential of 2,4,6-TCP.

#### **Applicant Response:**

- The high dose level of SYN545974 used in the carcinogenicity studies (2 year study in rats and 80 week study in mice) complies with the OECD TG 453 and OECD Guidance (2012); it achieved a maximum tolerated dose and the use of dose limited absorption (toxicokinetics) was also appropriate to assess HAZARD to SYN545974 and its mammalian metabolites including 2,4,6-TCP and its conjugates.
- It is appropriate to use the risk assessment endpoints derived from the mammalian toxicology database for SYN545974 to assess RISK to exposure to 2,4,6-TCP and its conjugates, because following administration of SYN545974 to rats and mice 2,4,6-TCP and its conjugates are major circulating metabolites.

2,4,6-TCP is a mammalian metabolite of SYN545974 (parent); the formation of 2,4,6-TCP is dependent on the availability of SYN545974. Therefore, if the concentration of SYN545974 does not increase then the concentration of 2,4,6-TCP cannot increase.

The percentage of SYN545974 converted to TCP and related conjugates (HTCP sulphate, TCPM glucuronide, 2,4,6-TCP sulphate) following single oral dosing is 55% of the total area under the time

concentration curve in plasma (■■■■■ and ■■■■■ 2015). It is noted that this calculation (from 2,4,6-TCP profile in plasma) provides only an estimation and might overestimate the systemic dose of 2,4,6-TCP. Indeed, based on urine excretion data, the maximum total mg/kg 2,4,6-TCP related material, rat were exposed to over 72 h following oral administration was 17.1 mg/kg for males at 300 mg SYN545974/kg and 10.9 mg/kg for females at 100 mg SYN545974/kg.

The formation of 2,4,6-TCP occurs via hydroxylation of SYN545974 on the carbon adjacent to the trichlorophenyl ring to give SYN547948. This then cleaved following oxygen insertion adjacent to the carbonyl to yield the 2,4,6 trichlorophenol (TCP) and SYN548263. These oxidations are likely mediated via cytochrome P450's in the liver. Once formed, TCP is rapidly conjugated and excreted, and does not accumulate upon repeat administration. 90 % of a 25 mg/kg ip dose of TCP was excreted in 4-6 h predominantly as conjugated material. At 10 h only trace amounts of TCP related material was found in the blood and tissues. Thirty minutes after the TCP dose, 70% of the administered trichlorophenol in blood was identified as conjugates, with the conjugated fraction increasing with time, facilitating excretion. The half-life of trichlorophenol related material was very similar in all the tissues studied, blood, brain, fat, kidney, liver and muscle, at 1.4-1.8 h (■■■■■ et al, 1981; ■■■■■ et al 1986).

The toxicokinetic evaluations of SYN545974 following gavage and dietary dosing in rats and mice (see Section 6.1) demonstrates that the absorption SYN545974 is limited by non-linear kinetics. Therefore, increasing the dose does not relate to an increase in exposure in the non-linear range. In the rat study the non-linear range is triggered at doses above 100 mg/kg in females and 300 mg/kg in males, and in mice at concentrations above 300 mg/kg (males and females). Since the concentration of SYN545974 is concomitant to metabolite concentration, the exposure to metabolites must be the similar/same irrespective of dose. Therefore, the highest dose of SYN545974 tested in the carcinogenicity studies represented a maximal exposure to parent and the highest concentration of 2,4,6-TCP that could be formed has also been tested. Therefore, the mammalian toxicity database on SYN545974 also assesses the toxicity of 2,4,6-TCP and risk assessment endpoints for SYN545974 are considered appropriate also for 2,4,6-TCP.

**Table 6.8.1-60: Maximum total mg/kg TCP related material in rats over 72 h following a single oral dose of SYN545974.**

Compound	% administered dose recovered in urine (equivalent mg/kg)			
	Male (0-72 h)		Female (0-72 h)	
	5 mg/kg	300 mg/kg	5 mg/kg	100 mg/kg
Hydroxy (2,4,6-TCP) sulphate	-	0.1 (0.3)	-	0.1 (0.1)
2,4,6-TCP sulphate	14.9 (0.75)	2.5 (7.5)	7.8 (0.39)	4.6 (4.6)
2,4,6-TCP	4 (0.2)	3.1 (9.3)	6.6 (0.33)	6.2 (6.2)
Total	18.9 (0.95)	5.7 (17.1)	14.4 (0.72)	10.9 (10.9)

In plasma, the largest circulating 2,4,6-TCP related material was the sulphate conjugate at up to 44% of the total radioactivity AUC, equivalent to 140 µg equiv.h/g in male. In female, the sulphate conjugate accounted for up to 32% of the total radioactivity AUC, equivalent to 36 µg equiv.h/g (6.8.1-61). If these are converted into average concentrations over the 96 h period this equates to 1.46 and 0.375 µg equivalents/g. As 2,4,6-TCP sulphate this equates to, (1.46/MW SYN545974 \* MW metabolite), 0.95 and 0.24 µg/g or 3.4 µM and 0.87 µM. The parent molecule accounted for up to 5% of the total radioactivity AUC, equivalent to 6 µg equiv.h/g in female (Table 6.8.1-61).

**Table 6.8.1-61: Mean plasma circulating levels of TCP related material over 96 hour period, following single oral dose SYN545974 to rats.**

Compound	Concentration expressed as:
----------	-----------------------------

	µg equiv.h/g (% Total radioactivity AUC))			
	Male		Female	
	5 mg/kg	300 mg/kg	5 mg/kg	100 mg/kg
	0-96 h	0-96 h	0-96 h	0-96 h
SYN545974	0.16 (1.9)	4 (1.3)	0.22 (2.8)	6 (5)
Hydroxy (2,4,6 TCP) sulphate	0.53 (6.1)	15 (4.8)	0.73 (9.3)	10 (9.2)
2,4,6-TCP sulphate	3.60 (41)	140 (44)	3.23 (41)	36 (32)
2,4,6-TCP	0.37 (4.3)	7 (2.4)	0.41 (5.2)	6 (5.3)

In mouse, a <sup>14</sup>C-study was conducted to investigate the excretion and metabolite profile following a single oral dose (see K-CA 5.1.1/08 [REDACTED] et al., 2015). A single oral dose of 10 or 300 mg SYN545974/kg was administered to male and female mice and excretion samples collected over a 7 day period and analysed for the nature and identity of the metabolites in excreta and carcass. [<sup>14</sup>C]-SYN545974 was extensively metabolised giving rise to numerous metabolites. The major metabolite in urine was 2,4,6-TCP sulphate, 3-hydroxy-2,4,6-TCP sulphate and 2,4,6-TCP-glucuronide, free 2,4,6-TCP was not observed. 2,4,6-TCP and its conjugates were not detected in feces. 2,4,6-TCP and its conjugates accounted for up to 9.87 mg/kg for males and 18.9 mg/kg for females at 300 mg/kg oral dose of SYN545974 (Table 6.8.1-62).

**Table 6.8.1-62: Maximum total mg/kg TCP related material in mice exposed to over a 7 day period following a single oral dose of SYN545974.**

Compound	% administered dose recovered in urine (equivalent mg/kg)			
	Male (0-48 h)	Male (0-72 h)	Female (0-48 h)	Female (0-72 h)
	10 mg/kg	300 mg/kg	10 mg/kg	300 mg/kg
Hydroxy 2,4,6 TCP sulphate	0.08 (0.008)	0.21 (0.63)	0.33 (0.033)	2.99 (8.97)
2,4,6 TCP Glucuronide	0.2 (0.02)	0.98 (2.94)		
2,4,6-TCP sulphate	1.02 (0.102)	2.1 (6.3)	0.52 (0.052)	3.31 (9.93)
Total	1.3 (0.13)	3.29 (9.87)	0.85 (0.085)	10.9 (18.9)

In both rats and mice, 2,4,6-TCP and 2,4,6-TCP-conjugates were the major circulating metabolites after administration of SYN545974. The plasma concentration of 2,4,6-TCP-species exceeds that of parent SYN545974; therefore, the mammalian toxicity data package on SYN545974 adequately assesses the toxicity of 2,4,6-TCP and its conjugates. The toxicity profile of SYN545974 and its metabolites in mammalian species (including 2,4,6-TCP has been determined in a full regulatory data package). In both long-term studies performed with SYN545974 (rats and mice), 2,4,6-TCP was the major circulating metabolite and the plasma concentration of 2,4,6-TCP exceeded that of parent (SYN545974); therefore, the mammalian toxicity data package on SYN545974 adequately assess the toxicity of 2,4,6-TCP. Consumer risk assessment endpoints for SYN545974 are proposed using NOAELs from studies conducted in the rat or mouse, (where it is known that 2,4,6-TCP was a major circulating metabolite) and therefore are considered sufficient to conduct human risk assessments.

#### **Consideration regarding the carcinogenic potential of 2,4,6 TCP and the dose level selection for SYN545974 toxicity studies (July 2017):**

As toxicological data regarding the metabolite 2,4,6 TCP are available in the published literature as well as in reviews from regulatory agencies, HSE questioned the derivation of specific reference values for this compound to conduct risk assessment. As presented above, the applicant considered that the mammalian toxicity data package on SYN545974 sufficiently assesses the toxicity of 2,4,6 TCP and its conjugates. However, as previously mentioned, HSE had some reservations regarding the dose level



selection which has been proposed by the applicant on the basis of pharmacokinetic data (see section B.6.1). HSE questioned on the possibility that the doses of SYN545974 selected by the applicant for the long-term studies might be not sufficiently high to cover the carcinogenic potential of TCP, especially in rat. Indeed, the highest tested dose of SYN545974 in the carcinogenicity study in rat was 300 mg/kg bw/day (males) whereas leukemias were observed from the near dose of 250 mg/kg bw/day in male rat in a long-term toxicity study from the NTP (NCI, 1979).

In order to verify whether higher doses of SYN545974 (>300 and up to 1000mg/kg bw/day) would have covered actually the carcinogenic potential of TCP, an extrapolation of systemic exposure to 2,4,6 TCP (and related compound) have been proposed for these doses levels (Table 6.8.1-34). This estimation has taken into account the oral absorption of SYN545974 (which decreases with increasing dose levels) and the proportion of 2,4,6 TCP measured in plasma (% of AUC) after an oral administration of radiolabeled SYN545974. The study of [REDACTED] and [REDACTED] (2015) showed that the metabolite profile in plasma of rats following a single administration of [Phenyl U-<sup>14</sup>C] or [pyrazole U-<sup>14</sup>C]SYN545974 was similar, irrespective of the dose or gender. Table 6.8.1-33 shows that the metabolic pathway leading to compounds related to 2,4,6 TCP and its conjugated metabolites (HTCP sulphate, TCPM glucuronide, 2,4,6 TCP sulphate) represents about 55% of the total AUC of [phenyl U-<sup>14</sup>C]-SYN545974.

It is noted that this calculation (from 2,4,6 TCP profile in plasma) provides only a estimation and might overestimate the systemic dose of 2,4,6 TCP. Indeed, based on urine excretion data, the maximum total mg/kg 2,4,6-TCP related material rat were exposed to over 72 h following oral administration was 17.1 mg/kg for males (Table 6.8.1-60 vs 39.6 mg/kg in Table 6.8.1-64) at 300 mg SYN545974/kg and 10.9 mg/kg (Table 6.8.1-60 vs 28 mg/kg in Table 6.8.1-64) for females at 100 mg SYN545974/kg.

**Table 6.8.1-63: Metabolite profile in plasma of rats following a single oral dose of [Phenyl U-<sup>14</sup>C]-SYN545974 (expressed as percentage of total AUC)\***

Compound	% AUC			
	Male		Female	
	300 mg/kg	5 mg/kg	100 mg/kg	5 mg/kg
	0-96 h	0-96 h	0-96 h	0-96 h
SYN545974 (parent)	1.3	1.9	5.0	2.8
<b>2,4,6 TCP and related compounds (2,4,6 TCP sulphate, TCPM glucuronide, HTCP sulphate)</b>	<b>54.7</b>	<b>54.9</b>	<b>49.1</b>	<b>56.1</b>

\* Data extracted from the study of [REDACTED] and [REDACTED] (2015) presented in section 6.1.1/05

**Table 6.8.1-64: Extrapolation of systemic exposure of 2,4,6 TCP and related metabolites after oral administration of non-experimentally tested high doses of SYN545974 in rats**

		Doses tested in long-term studies		Extrapolated doses		Doses tested in long-term studies		Extrapolated doses		
External exposure doses (mg/kg bw/d)		5	300	500	1000	5	100	300	500	1000
		Male				Female				
Oral absorption*		80%	22.4%	22.4%		87.3%	48%	48%		
Estimated systemic doses (internal doses) (mg/kg bw/d)**	Total	4	67.2	112	224	4.4	48	144	240	480
	2,4,6 TCP ***	2.4	39.6	66	132	2.6	28	85	142	283

\* measured after single administration of [phenyl U-<sup>14</sup>C]-SYN545974

\*\* Internal doses were calculated taking into account the oral absorption

\*\*\* Calculated taking into account the proportion of 2,4,6 TCP and related compounds measured in blood (55% regardless of the dose or gender). As no information is available for high doses of SYN545974, the oral absorption value estimated for the highest tested dose in each sex have been used. This is a worst case approach as it is expected that oral absorption decrease with the increasing doses.

According to Table 6.8.1-64, the systemic exposure of 2,4,6 TCP (and related compounds) after an oral administration of SYN545974 at the dose level of 1000 mg/kg bw/day in male rat, was estimated to be 132 mg/kg bw/day. For comparative purposes, a T25 value for 2,4,6 TCP was calculated and corresponds to the estimated chronic dose rate eliciting 25% increase of monocytic leukemia incidences in males rat in the 2-year toxicity study (NCI 1979). 2,4,6 TCP being highly absorbed (oral absorption of 90%), the systemic dose of 2,4,6 TCP inducing leukemia in 25% of the male rats was estimated at 114 mg/kg bw/d (Table 6.8.1-65)

**Table 6.8.1-65: Estimated doses of systemic exposure after oral administration of 2,4,6 TCP at doses eliciting leukemia in males rats in the long-term study**

	2-year rat study with 2,4,6 TCP (NCI 1979)	
	LOAEL for leukemia	T25 for leukemia
External exposure doses (mg/kg bw/d)	250	127
Oral absorption*	90%	
Estimated systemic doses (internal doses (mg/kg bw/d)	225	114

\* Based on ████████ et al. (1981) study, see section 6.8.1

The maximum estimated internal dose of 2,4,6 TCP (and related compounds) tested in the long-term toxicity study with pydiflumetofen (corresponding to 300 mg/kg bw/d of SYN545974) is thus lower than the T25 (internal dose) calculated from the 2-year rat study (39.6 vs 114 mg/kg bw/d; see tables 6.8.1-64 and 6.8.1-65). This confirms that the higher tested dose of 300 mg/kg bw/d of pydiflumetofen in long term rat study was actually not sufficiently high to elicit the carcinogenic potential of its major circulating metabolite 2,4,6 TCP. On the other hand, if the dose of 1000 mg/kg bw/d (corresponding to an estimated systemic dose of 132 mg/kg bw/d of 2,4,6 TCP; see table 6.8.1-64) had been tested in male rat, the carcinogenic potential of 2,4,6 TCP would have been covered as this value is closed to the calculated T25. On the assumption that the dose of 1000 mg/kg bw/d of SYN545974 if it has been tested for 2 years in male rat, would also provoke leukemia in 25% of the animals, this dose could be considered as the T25 value for pydiflumetofen. Considering this T25 as the estimated LOAEL where a carcinogenic potential might be observed after 2-year exposure in rat, a margin of safety of 100 (1000/9.9) between the LOAEL and the NOAEL (9.9 mg/kg bw/day) of the study can be derived. This Margin of safety is considered sufficient as no indication of a genotoxic mode of action for SYN545974 or 2,4,6 TCP have been highlighted (especially in the genotoxic datapackage studies performed on SYN545974).

In conclusion, as the uncertainty regarding the dose selection for the long-term toxicity studies has been raised, it can be considered that the mammalian toxicity data package on SYN545974 has sufficiently assessed the toxicity of 2,4,6 TCP and its conjugates. The toxicological studies available on 2,4,6 TCP are less complete and reliable than the toxicological dataset performed on SYN545974: old and no OECD guideline compliant studies, absence of NOAEL determined for carcinogenicity and chronic toxicity with (only 2 high tested doses).

It should be noted that an evaluation of the carcinogenic potential of pydiflumetofen and also its residue of concern, 2,4,6-TCP, found in livestock commodities, was posted on the US EPA website on April 2018. This assessment was made by the Cancer Assessment Review Committee (CARC) of the Health Effects Division (HED) of the Office of Pesticide Programs (OPP) on the basis of the same toxicity data package than those submitted for the European approbation process.

After review of the NCI study (1979), which is presented below in this DAR, and studies from the open literature (the same than those presented below in the DAR), the CARC arrived at the following conclusion:

- The MCL findings in F344 rats are of questionable relevance to human health risk assessment given the strain specificity of the neoplastic lesion, the high variability in the historical control incidence, and the lack of a direct human correlate.

- The liver tumors observed in male and female mice could not be directly attributed to 2,4,6-TCP because the study did not account for known carcinogenic contaminants of commercial 2,4,6-TCP solutions that may have contributed to the induction of the liver tumors.
- The chronic NOAEL selected for pydiflumetofen (9.2 mg/kg/day) is 66 and 165x lower than the 2,4,6-TCP dose (on a molar basis) that induced leukemias in rats and liver tumors in mice, respectively.
- While a limited number of *in vitro* studies indicate 2,4,6-trichlorophenol is weakly aneugenic and clastogenic, the database of *in vivo* results is not sufficient to support the genotoxic or mutagenic potential of 2,4,6-trichlorophenol
- The ADME information available for 2,4,6-TCP is consistent with the ADME profile for pydiflumetofen: near complete absorption and extensive metabolism followed by rapid excretion without appreciable tissue accumulation.

Taking into account these elements and using a weight of evidence approach, the CARC concluded that a separate dietary cancer assessment for 2,4,6-TCP is not required. Thus, using the reference dose approach with the chronic NOAEL selected for the pydiflumetofen dietary assessment would be adequate for assessing direct dietary exposure to 2,4,6-TCP from the proposed pydiflumetofen uses.

US EPA (2017): Pydiflumetofen: Report of the Cancer Assessment Review Committee.

<https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0775-0019>

#### **Are the dose levels used in the pydiflumetofen carcinogenicity studies sufficient to investigate the carcinogenic potential of its major metabolite 2,4,6-TCP?**

2,4,6-TCP shows equivocal evidence of carcinogenicity in F344 rats (leukemia) and has harmonised classification with Carc Cat 2 under the CLP Regulation. HSE is of the view that the question above is irrelevant for two reasons:

- 1) Dose levels should be appropriate to maximise the potential of detecting a carcinogenic response of the tested substance (pydiflumetofen in this case). The MTD was reached in both studies and higher levels did not need to be used to try and investigate the carcinogenic potential of 2,4,6-TCP.

As specific and extensive toxicological data exist on 2,4,6-TCP, these should take priority in characterising its toxicity profile over and above the predictive approach of using the parent's data because the substance is a major rat metabolite of the parent.

Summaries of relevant studies identified in the published literature are included below. All the toxicity study summaries presented below were from a literature review performed by the applicant on the 2,4,6 TCP metabolite, according to the EFSA Guidance document as published in EFSA Journal 2011; 9(2):2092. These studies have been considered as supplementary by HSE. An assessment for reliability were performed according to the criteria described by Klimisch *et al* (1997)<sup>10</sup> using the ToxRTool ([http://ihcp.jrc.ec.europa.eu/our\\_labs/eurl-ecvam/archivepublications/toxrtool](http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/archivepublications/toxrtool))

#### **ADME STUDIES**

<b>Report:</b>	K-CA 5.8.1/16 Bahig M. <i>et al.</i> , (1981). Excretion And Metabolism of 2,4,6-Trichlorophenol- <sup>14</sup> C In Rats. Gesellschaft für Strahlen- und Umweltforschung, Institut für Ökologische Chemie, D – 8042, Neuherberg. Published: Bahig M, Kraus A and Klein W (1981). Excretion And Metabolism of 2,4,6-Trichlorophenol- <sup>14</sup> C In Rats. Chemosphere 10:323-327. Syngenta File No. NA_13756.
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<sup>10</sup> Klimisch H-J, Andreae M and Tillmann U (1997) A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data. Reg Tox Pharmacol 25, 1-5

**STUDY TYPE:** Excretion and Metabolism of Radiolabelled Material

**TEST MATERIAL (PURITY):** 2,4,6-TCP-<sup>14</sup>C (99%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**KLIMISCH SCORE: 1** Note: There is an inconsistency in the reporting of the results between the text and the data tables. The text cites that 92.5% of the daily dose of 2,4,6-TCP was excreted in faeces, however, the data tables cite this value for urine excretion. Based on the physical chemical properties of 2,4,6-TCP, the main route of elimination of this moiety following administration of SYN545974 (see Section 5.1.1/05) and data reported by Balikova (1988) (see Section 5.8.1/16), the summary has been amended to reflect the main route of excretion is via urine.

**HSE comment:** supportive study

#### **EXECUTIVE SUMMARY**

The study aimed to assess the rate of excretion of 2,4,6-trichlorophenol (2,4,6-TCP)-<sup>14</sup>C in urine and faeces, and in the characterization of metabolites formed by rats.

Rats were dosed by oral gavage with 0.5 ml of an Acetone/Keltrol solution (corresponding to a dose of 25 µg 2,4,6-TCP-<sup>14</sup>C) for 15 days.

The storage of radioactivity by rats after daily administration of 2,4,6-TCP-<sup>14</sup>C reached a plateau after three days. At this level the excretion was 92.5 % ± 0.7 in urine of the daily applied dose.

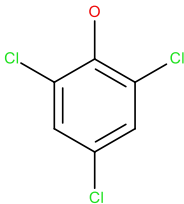
No radioactivity could be detected in the tissues of the 3 rats examined on day after stopping the TCP-<sup>14</sup>C application. The excretion of radioactivity in the other 3 rats decreased sharply after the application stopped and reached in 3 days about 4.3 % in urine and 1.9 % in faeces of a daily dose

The chloroform extract of urine contained about 63 % of the applied dose. GLC/MS analysis revealed 4 trichlorophenol isomers. One of these isomers could be identified as the parent 2,4,6-TCP-<sup>14</sup>C, and others are the 2,3,6- and 2,4,5-isomers. After chloroform extraction the urine contained only very polar compounds (28 % of the total activity). Upon acid or enzymatic hydrolysis at least 2 isomers of TCP could be characterized by GLC/MS. The nature of the polar metabolites was TCP-conjugates with glucuronic acid, since 80 % of the conjugates were hydrolysed by β-glucuronidase. In faeces, only free TCP was detected.

**Under the conditions of the study reported, 2,4,6-TCP-<sup>14</sup>C was not stored in rats. 2,4,6-TCP-<sup>14</sup>C is not significantly degraded by rats, it is rather conjugated and to a certain extent isomerized.**

## MATERIALS AND METHODS

### Materials:

<b>Radiolabelled Test Material:</b>	2,4,6-TCP- <sup>14</sup> C
<b>Specific activity:</b>	5 mCi/mM
<b>Radiochemical purity:</b>	99%
<b>Source:</b>	Synthesised
<b>Lot/Batch number:</b>	Not indicated
<b>Structure:</b>	 <p style="text-align: right;">position of [<sup>14</sup>C]-label not indicated</p>

### Preparation of dosing solutions:

A concentrated acetone solution was diluted with aqueous 0.1% Keltrol D (xanthan gum, a high molecular weight polysaccharide) to a concentration of 50 µg/ml. Further details of acetone and Keltrol are not provided.

<b>Test Animals:</b>	
<b>Species:</b>	Male rats
<b>Strain:</b>	Wistar, Sprague Dawley
<b>Age/weight at dosing:</b>	225-250 g
<b>Source:</b>	Not indicated
<b>Housing:</b>	Individually in metabolism cages.
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	Powdered standard diet <i>ad libitum</i>
<b>Water:</b>	Not indicated
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Photoperiod: Not indicated

### Study Design and Methods:

**In-life dates:** Not indicated

### Dosing and sample collection:

Six rats were dosed daily by a stomach tube with 0.5 ml of the Keltrol solution (25 µg 2,4,6-TCP-<sup>14</sup>C, which was reported to correspond to 1 ppm in the diet) for 15 days. Faeces and urine were collected separately on a daily basis and frozen directly after radioactivity measurements.

On the 16<sup>th</sup> day 3 rats were sacrificed, samples of liver, kidney, mesenteric and subcutaneous fat were analyzed for radioactivity by automatic combustion. The other 3 rats were kept for further 3 days for measuring the excretion of radioactivity.

### Radioactivity measurements:

The radioactivity of urine was measured directly by liquid scintillation counting (Packard, Tricarb 3385 and 3375 with external standards, scintillator based on dioxane). Faeces were ground in a mixer, and an

aliquot (200 mg) was subjected to automatic combustion and liquid scintillation counting (Oximat, Intertechnique) and scintillator based on toluene containing phenethylamine for trapping  $^{14}\text{CO}_2$ . The radioactivity in tissue samples was measured using the same technique. Thin-layer chromatography (TLC) plates were scanned for radioactive substances on a scanner (Berthold-Friesseke, Karlsruhe).

#### Isolation and identification of metabolites:

The combined urine was extracted 5 times with chloroform. The combined chloroform extracts were concentrated using a rotary evaporator (condenser cooled with methanol,  $-15^\circ\text{C}$ ). The radioactive fractions were separated and purified repeatedly by TLC on precoated plates  $20 \times 20$  cm, silicagel F 254, using dichloromethane as solvent.

For controlling the purification steps a TLC spectrodensitometer was used (SD 3000, Schoeffel, U.S.A.). The isolated metabolites were examined by GLC/MS. After chloroform extraction the urine was slightly acidified and extracted 5 times with ether, and the combined ether extracts were evaporated and separated by TLC in dichloromethane as solvent. Only a very polar radioactive zone could be obtained, which was hydrolysed with 8N  $\text{H}_2\text{SO}_4$  for 8 hours at  $40^\circ\text{C}$ , and extracted 5 times with ether. After evaporation of the solvent, it was purified several times by TLC and subjected to GLC/MS. The combined homogenized faeces were mixed with anhydrous sodium sulphate and extracted 5 times with hot methanol. The combined methanol extracts were evaporated and identified only by TLC since the concentration of TCP (-derivatives) was insufficient for proper clean-up. For identification a combined gas-liquid chromatography/mass-spectrometry system (GLC/MS) was used (LKB 9000 A, LKB-productor, Bromma, Sweden, GLC-column: 4 mm, length 2.0 m, chromosorb W-AW-DMCS 80-100 mesh 1% OV<sub>1</sub>, temp.  $150\text{--}250^\circ\text{C}$ , carrier gas 50 ml He/min). Mass spectra were compared with those of authentic samples.

## RESULTS

#### Storage and excretion:

The storage of radioactivity by rats after daily administration of 2,4,6-TCP- $^{14}\text{C}$  reached a plateau after three days. At this level the excretion was  $92.5 \pm 0.7$  % in urine of the daily applied dose. No radioactivity could be detected in the tissues of the 3 rats examined on day after stopping the TCP application, since excretion of radioactivity was nearly quantitative.

**Table 6.8.1-66: Balance of radioactivity in excreta of rats after administration of 2,4,6-TCP- $^{14}\text{C}$  (in % of applied radioactivity)**

Urine			Faeces	Total excreted
Water layer (conjugates)	Chloroform layer (TCP isomers)	Total		
28	63	$92.5 \pm 2.7$	$6.4 \pm 0.7$	$98.9 \pm 2.8$

The excretion of radioactivity in the other 3 rats decreased sharply after the application stopped and reached in 3 days about 4.3 % in urine and 1.9 % in faeces of a daily dose.

#### Metabolism:

The chloroform extract of urine contained about 63 % of the applied dose (Table 6.8.1-66). Using TLC, only one radioactive zone ( $R_f$  0.66 in dichloromethane as solvent) could be found. GLC/MS analysis of this zone showed 4 TCP isomers. One of these isomers could be identified as the parent 2,4,6-TCP, and others are the 2,3,6- and 2,4,5-isomers upon comparison of the mass spectra with the authentic isomers. These isolated isomers must be conversion products of the applied 2,4,6-TCP- $^{14}\text{C}$  in the rat, since the chemical used had been analysed by GLC/MS and proved to be pure. After chloroform extraction the urine contained only very polar compounds (28 % of the total activity, Table 6.8.1-66). Upon acid or enzymatic hydrolysis at least 2 isomers of TCP could be characterized by GLC/MS. The nature of the polar metabolites was TCP-conjugates with glucuronic acid, since 80 % of the conjugates were hydrolysed by  $\beta$ -glucuronidase. In faeces, only free TCP was identified.

**CONCLUSION:**

The study aimed to assess the rate of excretion of radio-labelled 2,4,6-trichlorophenol (2,4,6-TCP) in urine and faeces, and to characterize the metabolites formed by rats following repeated dosing.

Rats were dosed by oral gavage with a dose of 25 µg 2,4,6-TCP-<sup>14</sup>C for 15 days. Faeces and urine were collected separately on a daily basis. On the 16<sup>th</sup> day, 3 rats were sacrificed, and samples of liver, kidney, mesenteric and subcutaneous fat were analyzed for radioactivity. The other 3 rats were kept for further 3 days for measuring the excretion of radioactivity.

Radioactivity in urine reached a plateau after three days. At this timepoint, the level of excretion was 92.5 % of the daily applied dose in urine and 6.4% of the daily applied dose in faeces. No radioactivity could be detected in the tissues of the 3 rats examined on day 16. The excretion of radioactivity in the other 3 rats decreased sharply after the application stopped and reached about 4.3 % in urine and 1.9 % in faeces of a daily dose 3 days after the end of the treatment.

The chloroform extract of urine contained about 63 % of the applied dose. GLC/MS analysis revealed 4 trichlorophenol isomers. One of these isomers could be identified as the parent 2,4,6-TCP, and others were the 2,3,6- and 2,4,5-isomers. The urine contained only very polar compounds (28 % of the total activity). These polar metabolites were TCP-conjugates with glucuronic acid. In faeces, only free TCP was detected.

Overall, this publication showed that 2,4,6-TCP is not significantly degraded by rats, but it is rather conjugated and to a certain extent isomerized.

(Bahig M *et al.*, 1981)

<b>Report:</b>	K-CA 5.8.1/17 Pekari K. <i>et al.</i> , (1986). Kinetics of 2,4,6-Trichlorophenol in Different Organs of the Rat. Institute of Occupational Health, Laboratory of Biochemistry, Arinatie 3, SF-00370 Helsinki 37, Finland. Published: Pekari K, Boudène C, Aitio A (1986). Kinetics of 2,4,6-trichlorophenol in different organs of the rat. Archives of Toxicology 59:41-44. Syngenta File No. NA_13760.
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**STUDY TYPE:** Kinetic studies in different organs of the rat.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (~99%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

The concentrations of 2,4,6-trichlorophenol (2,4,6-TCP) were measured in the blood and various other tissues of male Wistar rats after a single IP administration of the compound at 25 mg/kg body weight.

The extent of conjugation of 2,4,6-TCP was also investigated by measuring total and free TCP in the blood.

The highest concentration,  $329 \pm 117 \text{ nmol g}^{-1}$ , was found in the kidney. Half-times were between 1.4 and 1.8 hours in the blood, brain, fat, kidney, liver and muscle.

Approximately 80% of 2,4,6-TCP was found to be conjugated.

**After IP administration of 2,4,6-TCP to male Wistar rats, the highest concentrations of test substance were found in the kidney, blood and liver. 2,4,6 TCP was rapidly and extensively conjugated (with glucuronic acid) and excreted with half times (free and conjugated) similar in all tissues (1.4 to 1.8 hours).**

## MATERIALS AND METHODS

### Materials:

<b>Non-Radiolabelled Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Source:</b>	Fluka AG Buchs, Switzerland
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	~99%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

**Internal Standard:** 2,3,4,6-tetrachlorophenol was used as an internal standard (source Fluka AG Buchs, Switzerland).

**Vehicle:** Propylene glycol (no further details are provided)

**Other reagents:** Other reagents were purchased from Merk (Darmstadt, F.R.G), J.T. Baker Chemicals (Deventer, Netherlands) or Sigma Chemical Company (St. Louis, Missouri, USA). No further details were provided.

**Preparation of dosing solutions:** Unlabelled 2,4,6-TCP in propylene glycol was administered by intraperitoneal (IP) injection. Details of dose concentration or dose volume are not provided.

<b>Test Animals:</b>	
<b>Species:</b>	Rat (males)
<b>Strain:</b>	Wistar
<b>Age/weight at dosing:</b>	Mean body weight $182 \pm 6 \text{ g}$
<b>Source:</b>	Not indicated
<b>Housing:</b>	Not indicated
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	Not indicated
<b>Water:</b>	Not indicated
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Photoperiod: Not indicated

### Study Design and Methods:



**In-life dates:** Not indicated

**Dosing and sample collection:** 2,4,6-TCP, 25 mg/kg body weight, in propylene glycol was administered IP to male Wistar rats. The number of rats dosed was not provided. At 0.5, 1, 2, 4, 6, 8, and 10 hours after the administration, blood samples were taken into heparin tubes by cardiac puncture from rats anaesthetized with ether. Upon blood sampling, tissue samples were excised from the liver, kidney, muscle, fat and brain and weighed. The tissues were frozen in liquid nitrogen and stored at -20°C. The blood samples were stored in a refrigerator and analysed within 4 days.

**Analytical procedure:** The amount of blood used in the analysis was 100 or 200 µl diluted with 1000 µl distilled water for the further procedure. Weighed specimens of liver, kidney, muscle, fat and brain were homogenized in distilled water with an Ultra-Turrax cutting blade homogenizer. The exact amount of the water was measured by weighing. The homogenate was stored frozen until analysed. To ensure complete hydrolysis of all conjugates (even other than glucuronides) acid hydrolysis was used in the measurement of total chlorophenol content. Hydrochloric acid, 6 mol<sup>-1</sup>, at a volume half that of the homogenate, was added and the mixture boiled in stoppered tubes in a water bath for 15 min. Depending on the concentration of TCP present, 3-6 ml hexane-isopropanol (5+1) containing 0.2 µmol<sup>-1</sup> of an internal standard (2,3,5,6-TCP) was added. After 15 min in a mechanical shaker, the phases were separated by centrifugation (2000 rpm). The aqueous phase was discarded and the chlorophenols were back-extracted into 3 ml sodium borate (pH 9.5). The organic phase was removed and 10 ml hexane containing 100 µl acetylation reagent (acetic acid anhydride + pyridine, 2+5) was added. The acetylated chlorophenols were extracted into the hexane phase (mixing 5 min, centrifugation). A 1 µl aliquot of the hexane layer was then injected into the gas chromatograph. For the isolation of the free, distilled water instead of the hydrochloric acid was added to the tissue homogenates. The pH of the mixture was adjusted to pH 2 with 1.5 N HCl. The hydrolysis at 100 °C was omitted; otherwise the extraction as well as the rest of the sample preparation were similar to those for the analysis of total 2,4,6-TCP. For the standard curve, 2,4,6-TCP in methanol (10 mg<sup>-1</sup>) was added to the blood and homogenized tissue samples of control animals at amounts corresponding to 1-4 nmol g<sup>-1</sup>. The slope of the standard curve was similar for all the tissues.

**Quantification.** The peak height ratio of 2,4,6-TCP and the internal standard was used in the calculations. The concentrations were obtained from five-point standard curves in the linear range of the detector response. The amount of conjugated TCP in the blood was calculated as the difference between the total and the free chlorophenol concentrations.

The limit of detection of the analysis was about 0.05 nmol g<sup>-1</sup>, calculated on the basis of the determinations made from brain tissue. The coefficient of variation was 5.3% (n=4) and 2.1% (n=5) at concentration levels of 1.0 and 4.0 nmol g<sup>-1</sup>, respectively. Recovery, calculated from spiked brain specimens, was 99.5 ± 3.8% (mean ± SD, n = 11), the added amount being 4.1 nmol g<sup>-1</sup>. The recovery was also tested for blood, liver and kidney and found to be similar. No interfering peaks were seen in chromatograms from tissues of unexposed animals.

**Statistics:** Not applicable.

## RESULTS

After a single IP administration of 2,4,6-TCP in the rat, the peak concentrations in all tissues were seen 30 minutes after the dose, the highest concentration, 329 ± 117 nmol g<sup>-1</sup> (mean ± SD, n=3) was found in the kidney, this was 2, 7, 10, 13, and 26 times the concentration found in the blood, liver, fat, muscle and brain, respectively. Ninety percent of TCP found was excreted in 4-6 hours. At 10 hours only minute amounts of 2,4,6-TCP were found in the blood and tissues.

Thirty minutes after the dose, 70% of the administered TCP in the blood was in the conjugated form with the conjugated fraction increasing with time. The overall average extent of conjugation of TCP in the blood was  $83 \pm 11\%$  (mean  $\pm$  SD).

The half-time of 2,4,6-TCP was very similar in all the tissues studied: 1.4-1.8 hours. The half-time of the conjugated 2,4,6-TCP in the blood was similarly 1.4 hours.

#### CONCLUSION:

In another publication, the concentrations of 2,4,6-trichlorophenol (2,4,6-TCP) were measured in the blood and various other tissues of male Wistar rats after a single IP administration of the compound at 25 mg/kg bw. The extent of conjugation of 2,4,6-TCP was also investigated by measuring total and free TCP in blood.

The peak concentrations in all tissues were seen 30 minutes after dose administration. The highest concentration,  $329 \pm 117 \text{ nmol g}^{-1}$ , was found in the kidney; this was 2, 7, 10, 13, and 26 times the concentration found in the blood, liver, fat, muscle and brain, respectively. Half-times were between 1.4 and 1.8 hours in the blood, brain, fat, kidney, liver and muscle. Ninety percent of recovered 2,4,6-TCP was excreted in 4-6 hours. At 10 hours post-dosing, only minute amounts of 2,4,6-TCP were found in the blood and tissues.

Approximately 80% of 2,4,6-TCP was found to be conjugated. The half-time of the conjugated 2,4,6-TCP in blood was 1.4 hours.

(Pekari K *et al.*, 1986)

<b>Report:</b>	K-CA 5.8.1/18 Balikova M. <i>et al.</i> , (1988). Process of Excretion and Toxicity of Lindane and its Metabolite 2,4,6-Trichlorophenol in Rats After Acute Oral Dosage. Institute of Toxicology and Forensic Chemistry, Faculty of General Medicine, Charles University, Prague. Published. Balikova M, Stipek S, Crkovska J (1988). Process of excretion and toxicity of lindane and its metabolite 2,4,6-Trichlorophenol in rats after acute oral dosage. Biochemia Clinica Bohemoslovaca, 17: 243-248. Syngenta File No. NA_13791.
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**STUDY TYPE:** Urinary excretion and nephrotoxicity of lindane and 2,4,6-TCP after acute oral dosage

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**The original publication was in Czechoslovakian and a translation to English obtained.**

**Only data and discussion relevant to the testing of 2,4,6-TCP are discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The rate of elimination of 2,4,6-Trichlorophenol (2,4,6-TCP) in urine was measured in male Wistar rats following administration by oral gavage of either 5 or 46 mg/kg. Analysis was performed using gas chromatography. The potential nephrotoxicity of 2,4,6-TCP was also investigated by measuring the levels of  $\gamma$ -glutamyltranspeptidase (GGT) and N-acetyl- $\beta$ -D-glucoaminidase (NAG) in the urine.

The elimination of 2,4,6-TCP was greatest between 0-24 hours following exposure. The rate of elimination decreased between 48-96 hours after exposure.

2,4,6-TCP (either 46 mg/kg or 5 mg/kg) had no effect on the excretion rate of GGT or NAG in the urine of rats in the first week after dosing. The authors concluded that at the doses employed in the study, 2,4,6-TCP was not found to be nephrotoxic to Wistar rats under the conditions of this study.

**2,4,6-TCP was found to be rapidly eliminated in urine from male Wistar rats following administration via oral gavage. 2,4,6-TCP was not found to impact on the levels of GGT and NAG in the urine and therefore under the conditions of this study not believed to be nephrotoxic.**

## MATERIALS AND METHODS

### Materials:

<b>Non-Radiolabelled Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>Contaminants:</b>	Not indicated
<b>CAS#:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated

**Vehicle:** Olive oil

**Preparation of dosing solutions:** 2,4,6-TCP was administered via oral gavage in olive oil. No details on dose volume or concentration were provided.

<b>Test Animals:</b>	
<b>Species:</b>	Rat (males)
<b>Strain:</b>	Wistar
<b>Age/weight at dosing:</b>	Not indicated
<b>Source:</b>	Not indicated
<b>Housing:</b>	Metabolic cages
<b>Acclimatisation period:</b>	Four days
<b>Diet:</b>	Not indicated
<b>Water:</b>	Not indicated
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Photoperiod: Not indicated

The number of animals dosed was not provided.

### Study Design and Methods:

**In-life dates:** Not indicated

**Dosing and sample collection:** 2,4,6-TCP was administered to male Wistar rats at either 46 mg/kg or 5 mg/kg in olive oil via oral gavage. Urine samples were taken from each rat daily. Pairs of rats were sacrificed at specific intervals, taking samples of blood and serum. The urine and serum were stored at -20°C until evaluation. The urine for the evaluation of  $\gamma$ -glutamyltranspeptidase (GGT) activity was dialysed before freezing against a volume of water.

#### Evaluation of $\gamma$ -glutamyltranspeptidase and N-acetyl- $\beta$ -D-glucoaminidase in Urine

The urine designated for evaluation of GGT activity was dialysed before freezing against a volume of water. GGT activity was evaluated using the Lachema Brno company test method, NAG activity was evaluated by a photometric technique using p-nitrophenyl-N-acetyl- $\beta$ -D-glucoaminidase.

#### Analytical procedure:

0.5 ml of serum or urine was acidified with 0.5 ml of 1M HCl, and extracted with 3 ml of n-hexane. Part of the hexane layer (2.5 ml) was washed with 1 ml of 0.5 M NaOH. The alkaline water layer containing phenolates was acidulated with 1 ml of 1M HCL, and extracted with 1 ml of hexane. Part of the hexane extract (0.5 ml) containing phenols was derivatised using trifluoroacethanhydride [8], adding to this part of the extract (0.5 ml) a catalyst made up of 200  $\mu$ l of 0.1M triethylamine, and 25  $\mu$ l of trifluoroacethanhydride. After 15 minutes of reaction at laboratory temperature, the reaction mixture was washed with 0.5 ml of 1M phosphate buffer, pH 6.

2,4,6-TCP in the form of trifluoroacetyl derivative were determined using gas chromatography and electron capture detection to record peak levels.

Conditions of GLC: a CHROM 42 gas chromatograph fitted with a Soviet-made  $^{63}\text{Ni}$  electron capture detector, a 1.25 m/3 mm glass column, column with filling of 3% SP 2250/Supelcoport 80/100 mesh. Total nitrogen was 80 ml/min, and the temperatures of the coating and detector were 250°C, and 300°C, respectively. The column temperature for the analysis of 2,4,6-TCP was 110°C. TZ 4221 recorder, range: 10 mV.

The minimum detectable quantity (MDQ) of 2,4,6-TCP was 10 pg (this corresponds to 30 ng/ml of urine). The detector linearity complied with the regulations within the range of the concentration levels of both substances.

**Statistics:** Not applicable.

## RESULTS

#### Elimination Studies:

Data of analysis of blood and serum for 2,4,6-TCP are not provided.

The elimination of 2,4,6-TCP in free form was greatest 0-24 hours after administration (Table 6.8.1-67).

**Table 6.8.1-67: Rates of elimination in urine of 2,4,6-TCP in free form (% of a single oral dose)**

Time after intoxication (hours)	2,4,6-TCP dose	
	46 mg/kg	5mg/kg
	%	%
	5	6
0 – 24	20.8	27.4
24 – 48	4.3	14.7
48 – 72	1.5	2.7

72 – 96	0.6	1.1
96 – 120	0.4	0.5
120 – 144	0.4	0.4
144 – 160	0.2	0.3
$\Sigma$ 0 – 100	28.2	57.1

[5, 6 – rat numbers]

When 2,4,6-TCP was administered orally, the initial rate of elimination and the quantity of the free-form substance eliminated in the monitoring period depended on the size of dose. A limit to the quantity that the organism was able to eliminate in a given period of time, which is why the elimination of smaller doses is more complete during the week monitored. Both the distribution and the elimination of 2,4,6-TCP in its free form were relatively fast processes.

### Nephrotoxicity

2,4,6-TCP dosed either at 46 mg/kg or 5 mg/kg) had no effect on the excretion rate of GGT or NAG in the urine of rats in the first week after administration. The authors concluded that at the doses employed in the study, 2,4,6-TCP was found not to be nephrotoxic to Wistar rats under the conditions of this study.

### CONCLUSION:

The rate of elimination of 2,4,6-Trichlorophenol (2,4,6-TCP) in urine was measured in groups of 6 male Wistar rats following administration by oral gavage of either 5 or 46 mg/kg bw of the test substance. Analysis was performed using gas chromatography. The potential nephrotoxicity of 2,4,6-TCP was also investigated by measuring the levels of  $\gamma$ -glutamyltranspeptidase (GGT) and N-acetyl- $\beta$ -D-glucosaminidase (NAG) in urine.

The elimination of 2,4,6-TCP was greatest between 0-24 hours following exposure. The rate of elimination decreased between 48-96 hours after exposure.

2,4,6-TCP had no effect on the excretion rate of GGT or NAG in the urine of rats in the first week after dosing. The authors concluded that at the doses employed in the study, 2,4,6-TCP was not found to be nephrotoxic to Wistar rats following a single oral administration.

Overall, in this publication, 2,4,6-TCP was found to be rapidly eliminated in urine from male Wistar rats following a single administration via oral gavage.

(Balikova M *et al*, 1988)

### SHORT-TERM TOXICITY

<b>Report:</b>	K-CA 5.8.1/19 Bercz J. <i>et al.</i> , (1990). Subchronic Toxicity Studies of 2,4,6-Trichlorophenol in Sprague-Dawley Rats. Environmental Toxicology Division. Health Effects Research Laboratory. U.S Environmental Protection Agency. Published: Bercz JP, Robinson M, Jones L, Page NP, Parnell MJ, Wolfe GW (1990). Subchronic Toxicity Studies of 2,4,6-Trichlorophenol in Sprague-Dawley Rats. Journal of the American College of Toxicology, 9(5): 497-506. Syngenta File No. NA_13762.
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**STUDY TYPE:** Subchronic toxicity studies in Sprague-Dawley rats, for 90 days by oral gavage dosing.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (99.8%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

Male and female Sprague-Dawley rats (10/sex/dose) were gavaged with 2,4,6-Trichlorophenol (2,4,6-TCP) administered in corn oil (2 ml/kg body weight) for 90 consecutive days at dose levels of 0, 80, 240, and 720 mg/kg per day.

Treatment-related effects were observed at the highest dose (720 mg/kg/day) and consisted of salivation, urine stains on the fur, increase in absolute and relative weights of the kidneys, liver, adrenal glands, and testes. At this dose, increases were seen in serum protein, albumin, and alanine aminotransferase (ALT), with a decrease in urinary pH. Some effects observed at 240 mg/kg per day were an increase in the absolute and relative weights of the liver and adrenal glands in females, relative liver weights in males, and an increase in serum albumin in males. No treatment-related effects were observed at 80 mg/kg per day.

No mortality or significant effects were observed at any dose level for body weight, food consumption, ophthalmic lesions, haematology, gross pathology, or histopathology.

**No mortality was observed on the study. Based on clinical chemistry and organ weight changes, it appears that the liver, kidney, and adrenal glands were target organs for systemic toxicity to 2,4,6-TCP in this study, although this was not correlated with histopathology lesions. It was concluded that 240 mg/kg/day represents a lowest observed adverse effect level (LOAEL), although the toxic effects were minimal. The no observed adverse effect level (NOAEL) for subchronic exposure to 2,4,6-TCP by the oral route was 80 mg/kg per day.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	99.8%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

**Vehicle and/or positive control:** The test substance was administered via oral gavage in corn oil (2 ml/kg body weight). No positive control was included on this study.

<b>Test Animals:</b>	
<b>Species</b>	Rat
<b>Strain</b>	Sprague-Dawley
<b>Age/weight at dosing</b>	7 weeks
<b>Source</b>	
<b>Housing</b>	Individually
<b>Acclimatisation period</b>	Not indicated
<b>Diet</b>	Purina Certified Chow 5002 (Ralston-Purina Co., St. Louis, MO) ad libitum
<b>Water</b>	Tap water ad libitum
<b>Environmental conditions</b>	Temperature: 72 ± 6°F. Humidity: 50 ± 20%. Air changes: Not indicated Photoperiod: 12h light/dark cycle

**Study Design and Methods:****In-life dates:** Not indicated**Animal assignment:**

Rats were assigned to eight groups according to a computer-generated set of random numbers.

**Study design**

Test group	Dose to animal (mg/kg)	# male	# female
Control	0	10	10
Low	80	10	10
Mid	240	10	10
High	720	10	10

**Observations:** Clinical observations were conducted daily for physiological and behavioural responses and other overt signs of toxicity. Mortality and morbidity checks were made twice daily.**Bodyweight:** The bodyweight of each rat was recorded prior to randomization, at initiation of dosing, at the end of the first week and weekly thereafter. A final body weight was measured at necropsy.**Ophthalmoscopic examination:** Ophthalmoscopic examination of dilated pupils was performed twice in the study, once prior to treatment and during the last week of the study**Haematology and clinical chemistry:** Blood was collected at terminal sacrifice via the orbital sinus. Haematology samples were evaluated using a Coulter Counter; the following data points were gathered.

Erythrocyte count

Corrected leukocyte count

Hematocrit.

Leukocyte count

Hemoglobin.

Platelet count

A differential analysis of leukocytes was performed for segmented neutrophils, lymphocytes, monocytes, eosinophils and other types of WBCs.

**Clinical chemistry:** The following clinical chemistry determinations were performed:

Sodium,

Total protein

Potassium,

Albumin.

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Calcium.	Total bilirubin
Creatinine,	Aspartate aminotransferase
Serum alanine aminotransferase	Alkaline phosphatase
Lactate dehydrogenase	Blood urea nitrogen

**Urinalysis:** Urine was collected from fasted animals at terminal sacrifice. The following measurements were conducted:

pH	Glucose
Proteins	Bilirubin
Occult blood	Urobilinogen

**Investigations *post mortem*:**

**Macroscopic examination:** Animals were euthanized by sodium pentobarbital anaesthesia followed by exsanguination. This involved examination of the animal's external surface; all orifices; the carcass; external surface of the brain, the cranial, thoracic, abdominal and pelvic cavities and their viscera; and cervical tissues and organs.

**Organ weights:** From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

Adrenal glands	Ovaries
Brain	Spleen
Heart	Testes with epididymides
Kidneys	Thymus
Liver	Lungs

**Tissue submission:** Tissues from approximately 40 organs, along with gross lesions were collected at necropsy and preserved in 10% neutral buffered formalin. Of these, selected tissues and the gross lesions were trimmed, processed, embedded in paraffin, sectioned and slides were prepared and stained with haematoxylin and eosin. These select tissues were microscopically examined from all animals in the high-dose groups and from five randomly selected animals per sex of the control groups.

Adrenal glands	Thyroid
Oesophagus	Trachea
Larynx	Heart
Spleen	Liver
Kidneys	Stomach
Duodenum	Jejunum
Colon	Pancreas
Gross lesions (except enlarged mandibular lymph nodes)	

**Microscopic examination:** All prepared slides were examined by a board-certified veterinary pathologist. The inflammatory and degenerative lesions were graded according to severity of the condition and the data were tabulated according to individual animal and summarized by group. The gross observations and microscopic diagnoses were correlated for each animal.

**Statistical evaluation:** Tests for homogeneity of variances and analysis of variance (ANOVA) were evaluated at the 5% one-tailed probability level. Control versus treated group means were evaluated using Dunnett's t-test at the 5% two-tailed probability level. When variances were heterogeneous data transformations were performed to determine significant differences between the treated and control groups.



## RESULTS

**Mortality:** No animals died during the study

**Clinical observations:**

Urine-stained fur was observed in the high-dose males (3/10) and in the two highest dose female groups (2/10 and 4/10, respectively), but not in the lower dose levels or controls. Salivation occurred soon after dosing during the first two weeks of the study in approximately 50% of the high-dose animals. After this, only occasional salivation occurred. Salivation was not observed in the lower dose groups nor in the controls. The urine staining of the fur and salivation appear to be related to 2,4,6-TCP treatment

**Bodyweight and weight gain:** No statistically significant differences were observed in the final body weights, which ranged from  $305.0 \pm 24.9$  to  $324.0 \pm 28.1$  g for female groups and  $540.8 \pm 35.6$  to  $580.0 \pm 33.9$  g for male groups.

**Food consumption:** No statistically significant ( $p < 0.05$ ) differences were found for food consumption; consumption ranged from  $19.2 \pm 2.42$  to  $20.3 \pm 2.52$  g/day for females and  $26.0 \pm 2.53$  to  $26.6 \pm 2.29$  g/day for males.

**Ophthalmoscopic examination:** The ophthalmoscopic examination during the 13th week revealed that several animals had uni- or bilateral multifocal retinopathy. The incidence of this condition was greater in 2,4,6-TCP-treated male groups (8/30 vs. 0/10 in the controls) but in an equal percentage of females (6/30 vs. 2/10). The incidences in males were dispersed across all treated groups with no dose-dependent relationship in either males or females. The condition was therefore not considered related to the 2,4,6-TCP treatment.

**Haematology:** No statistically significant changes were seen for any of the haematology parameters

**Blood clinical chemistry:** Significant ( $p < 0.05$ ) increases were observed for total protein in the high-dose males, albumin for the two high-dose male and the high-dose female groups, and ALT in high-dose males. A decrease in blood urea nitrogen was seen in the high-dose females, however, the levels at the mid dose were slightly elevated and thus the decrease in BUN in the high-dose group was considered spurious. The toxicological significance of the increase in ALT is unclear, since only three of the high-dose animals had values that were higher than the highest value in the control group. The increase in albumin and total protein in the serum could suggest either a change in the hydration status of the animals or altered hepatic function.

**Table 6.8.1-68: Clinical chemistry values in rats administered 2,4,6-TCP by gavage**

Dose group	Vehicle (corn oil)	2,4,6-TCP (mg/kg/day)		
	0	80	240	720
<b>Females</b>				
Albumin (g/dl)	$5.2 \pm 0.4$	$5.3 \pm 0.4$	$5.4 \pm 0.5$	$5.1 \pm 0.2^a$
BUN (mg/dl)	$13.0 \pm 1.4$	$12.0 \pm 2.4$	$13.0 \pm 2.5$	$10.0 \pm 2.5^a$
<b>Males</b>				
Total protein (g/dl)	$6.4 \pm 0.4$	$6.6 \pm 0.5$	$6.6 \pm 0.3$	$6.9 \pm 0.3^a$
Albumin (g/dl)	$4.6 \pm 0.2$	$4.7 \pm 0.2$	$4.9 \pm 0.2^a$	$5.3 \pm 0.2^a$
ALT (U/L)	$33.0 \pm 7.0$	$33.0 \pm 6.3$	$41.0 \pm 13.3$	$46.0 \pm 12.7^a$

<sup>a</sup> Statistically different ( $p < 0.05$ ) from controls

**Urinalysis:** A dose-related decrease in pH was found in both female and male dosed groups. This was especially evident at the high-dose groups in which the pH values were considerably lower (6.05 vs. 6.55 in the female controls and 6.15 vs. 6.95 in the male controls).

**Sacrifice and pathology:**

**Organ weights:** Statistically significant ( $p < 0.05$ ) increases in organ weights were observed for absolute weight of the adrenal glands in all treated female groups, relative weights of the adrenal glands in the high-dose females, and absolute and relative weights of the kidneys in the high-dose males. The absolute and relative liver weights were also increased in all treated groups in a dose-related manner, statistically significant ( $p < 0.05$ ) in the two highest dose male and female groups. Relative testes weights were increased in high-dose males. The absolute weights of the other organs in treated animals were comparable to those of the controls.

**Table 6.8.1-69: Absolute organ weights for rats administered 2,4,6-TCP by gavage for 90 days**

	Vehicle (corn oil)	2,4,6-TCP (mg/kg/day)		
Dose group	0	80	240	720
<b>Females</b>				
Liver	7.91 ± 0.58	8.63 ± 0.59	<b>9.07 ± 1.02<sup>a</sup></b>	<b>10.73 ± 1.14<sup>a</sup></b>
Adrenal glands	0.06 ± 0.01	<b>0.08 ± 0.01<sup>a</sup></b>	<b>0.08 ± 0.01<sup>a</sup></b>	<b>0.08 ± 0.01<sup>a</sup></b>
<b>Males</b>				
Kidneys	3.35 ± 0.30	3.31 ± 0.22	3.62 ± 0.31	<b>4.01 ± 0.48<sup>a</sup></b>
Liver	12.64 ± 1.12	12.98 ± 1.42	14.10 ± 1.96	<b>16.38 ± 1.54<sup>a</sup></b>
Testes	5.01 ± 0.18	4.95 ± 0.31	5.10 ± 0.26	5.10 ± 0.30

<sup>a</sup> Statistically different ( $p < 0.05$ ) from controls

**Table 6.8.1-70: Organ to total body weight (TBW) ratios for rats administered 2,4,6-TCP by gavage for 90 days**

	Vehicle (corn oil)	2,4,6-TCP (mg/kg/day)		
Dose group (mg/kg)	0	80	240	720
<b>Females</b>				
Liver/TBW	2.73 ± 0.21	2.88 ± 0.38	3.13 ± 0.35	<b>3.78 ± 0.47<sup>a</sup></b>
Adrenals/TBW	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	<b>0.03 ± 0.00<sup>a</sup></b>
<b>Males</b>				
Testes/TBW	0.91 ± 0.08	0.91 ± 0.08	0.95 ± 0.08	<b>1.00 ± 0.09<sup>a</sup></b>
Kidneys/TBW	0.61 ± 0.06	0.60 ± 0.04	0.68 ± 0.08	<b>0.78 ± 0.07<sup>a</sup></b>
Liver/TBW	2.29 ± 0.11	2.36 ± 0.15	<b>2.61 ± 0.19<sup>a</sup></b>	<b>3.20 ± 0.24<sup>a</sup></b>

<sup>a</sup> Statistically different ( $p < 0.05$ ) from controls

**Macroscopic findings:** Only a few gross lesions were seen at terminal sacrifice and most were represented by a single occurrence per group. Exceptions to this were the presence of dark areas and pitted mucosa of the glandular portion of the stomach which were seen in approximately 50% of animals in all groups, including controls. A distended cecum was found in four of the high-dose males but not in any other males or females. The findings in the stomach are common in rats following fasting and are not considered treatment-related. The relationship of distention of the cecum to 2,4,6-TCP treatment is not clear.

**Microscopic findings:** An extremely small number of lesions were observed and no differences were detected between control and compound-treated animals. All findings were considered incidental and unrelated to compound treatment. A slight but non-significant increase in the incidence of stomach erosion occurred with 5 of 10 animals in the high-dose female group and 2 of 10 in high-dose male group versus 2 of 5 in female controls and 0 of 5 in the male control group.

**Table 6.8.1-71: Summary of gross pathology findings in the stomach**

	Females		Males	
Dose group (mg/kg)	0	720	0	720
Stomach Erosion	2/5	5/10	0/5	2/10

## CONCLUSION:

In a publication from 1990, male and female Sprague-Dawley rats (10/sex/dose) were gavaged with 2,4,6-Trichlorophenol (2,4,6-TCP) administered in corn oil for 90 consecutive days at dose levels of 0, 80, 240, and 720 mg/kg bw/d. The study is broadly consistent with OECD guideline 408.

No mortality or significant effects were observed at any dose level for body weight, food consumption, ophthalmic lesions, haematology, gross pathology, or histopathology. Treatment-related effects were observed at the highest dose (720 mg/kg bw/d) and consisted of salivation, urine stains on the fur, increase in absolute and relative weights of the kidneys, liver, adrenal glands, and testes. At this dose, increases were seen in serum protein, albumin, and alanine aminotransferase (ALT), with a decrease in urinary pH. At 240 mg/kg bw/d there were increases in the absolute and relative weights of the liver and adrenal glands in females, relative liver weights in males, and an increase in serum albumin in males. No treatment-related effects were observed at 80 mg/kg bw/d.

Based on these findings, in this 90-day study in the rat, a LOAEL was identified at 240 mg/kg bw/d and the NOAEL was set at 80 mg/kg bw/d.

(Bercz J *et al*, 1990)

Following the EU peer-review process, it became evident that more robust repeated dose toxicity studies were needed to characterise the general toxicity profile of 2,4,6-TCP. On this basis, Syngenta recently submitted a 14-day range-finder study and a 28-day oral study in the rat.

These new studies have been fully evaluated by HSE and are presented outside the green commenting boxes, as yellow-highlighted text.

#### 14-day range-finder in the rat

Report reference	██████████, ██████████, 2021
Report year	2021
Report title	CA6519 - 14 Day Oral (Gavage) Dose Range-Finding Study in the Rat.
Report No	██████████
Document No	VV-894153
Guidelines	None
Deviations from current test guideline	Not applicable
Previous evaluation	No, not previously submitted
GLP	No
Test substance and purity	2,4,6-TCP (CA6519), batch no MES 663/2, 99% w/w
Acceptability	Yes

#### Methods

In a dose-range finding study for the subsequent 28-day study, groups of three male and three female Han Wistar rats were dosed with 0 (vehicle - corn oil), 250, 500, 750 or 1000 mg/kg bw/d 2,4,6-TCP, respectively. Animals were dosed once daily, by gavage for 14 days, until the day before necropsy. The following parameters were investigated: clinical observations, body weight, food intake, haematology, blood chemistry, organ weights and macroscopic pathology.

#### Results

*Mortality and clinical signs of toxicity*

At 1000 mg/kg bw/d, one male was found dead on Day 2 of dosing, with no prior clinical signs and one female was euthanised one hour after dosing on Day 3 due to a poor clinical condition (piloerection, prostration and tremors). At necropsy, the male showed incompletely collapsed and abnormally consolidated lungs and the female, dark liver, pale spleen, pale areas of the glandular mucosa of the stomach, red areas of the submandibular lymph nodes and distended uterus with clear fluid. The remaining four animals given 1000 mg/kg bw/d were euthanised on Day 4 (after receiving 3 doses) due to clinical signs of toxicity (piloerection, partially closed eyes, ploughing behaviour) and body weight losses (up to 17 and 9 g for males and females respectively). Food intake for both sexes given 1000 mg/kg bw/d was markedly lower than controls on Days 1 and 2. At necropsy, only one male showed macroscopic abnormalities (pale lungs with red areas, multiple raised areas of the non-glandular mucosa of the stomach).

There were no deaths or clinical signs of toxicity in any other dose group. Overall, deaths and clinical signs of toxicity were observed at the top dose of 1000 mg/kg bw/d.

*Body weight and food consumption*

Treated males generally gained less weight than controls throughout the dosing period, particularly during week 1, resulting in overall group mean body weight gain values of 21, 12 and 35 % lower than controls at 250, 500 and 750 mg/kg bw/d, respectively. Given the lack of dose-response, and taking into account the magnitude of the effect, only the decrease in body weight gain at 750 mg/kg bw/d in males is considered treatment-related and adverse. With the exception of a marginal body weight loss over Days 1 - 2 for females given 500 or 750 mg/kg bw/d, there were no effects on body weight in females up to 750 mg/kg bw/d.

Overall group mean food intake for males given 750 mg/kg bw/d was 14% lower than controls. In conclusion, there were effects on body weight gains and food consumption in males at 750 mg/kg bw/d.

*Haematology and clinical-chemistry*

There was no effect of 2,4,6-TCP on haematology parameters. Although there were differences in some parameters when compared with controls, because these were very minor and/or lacked a clear dose relationship and all individual values were within the historical background ranges, they were considered not to be related to treatment.

In males given 750 mg/kg bw/d, group mean plasma globulin concentration was marginally lower than controls, resulting in a slightly higher group mean albumin/globulin ratio, although most individual values (except one male) were within the historical control range and therefore not considered adverse. Group mean triglyceride levels in males and females given 750 mg/kg bw/d were higher than controls and group mean glucose concentrations were lower than controls, although again all individual values for these parameters were within the historical control range and therefore not considered adverse. Alkaline phosphatase (ALP) activities were slightly higher than the control mean in males and females given 750 mg/kg bw/d, although most individual values were within the historical control range and therefore not considered adverse. Alanine aminotransferase (ALT) activities were generally higher than controls in females from 250 mg/kg bw/d, but in the absence of a dose-response and with values falling within the historical control range, they were not considered treatment-related.

Other minor differences from the control means were not dose-related or were in the opposite direction to that usually associated with toxicity and were considered to be unrelated to treatment.

Overall, there were no treatment-related or adverse effects on haematology or clinical-chemistry parameters.

*Organ weights*

Administration of 500 or 750 mg/kg bw/d was associated with an increase in relative liver weight in males (by 13% at both dose levels) and females (by 7 and 20% at 500 and 750 mg/kg bw/d respectively). All individual values for males were within the historical control range but all individual values for females dosed at 750 mg/kg bw/d were above the range. Relative kidney weights were increased by 12% in males at 750 mg/kg bw/d. Overall, there were adverse effects on liver weight in females and on kidney weight in males at 750 mg/kg bw/d.

#### *Gross Pathology*

Other than those findings previously described for males and females dosed at 1000 mg/kg bw/d, there were no macroscopic abnormalities at lower dose levels, with the possible exception of reddened submandibular lymph nodes in one male given 750 mg/kg bw/d.

#### **Conclusion**

In a non-GLP dose-range finding study in rats administered 2,4,6-TCP by gavage for 14 days, the top dose level of 1000 mg/kg bw/d was highly toxic to the animals with deaths and clinical signs of toxicity, which resulted in all remaining animals being euthanised on Day 4. There were no adverse effects at 250 and 500 mg/kg bw/d but effects on body weight gain, food consumption, liver and kidney weights were seen at 750 mg/kg bw/d.

(██████████, ██████████, 2021)

#### **28-day oral toxicity study in the rat with pharmacokinetic investigations**

Report reference	██████████, 2021
Report year	2021
Report title	CA6519 - 28 Day Oral (Gavage) Toxicity Study in the Rat.
Report No	██████████
Document No	VV-897862
Guidelines followed in study	OECD 407 (2018)
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP	Yes
Test substance, batch and purity	2,4,6-TCP (CA6519), batch no MES 663/2, 99% w/w
Method of analysis	Acceptable method : Refer to Vol 3CA B5.1.2.3
Acceptability/Reliability	Yes

#### **Methods**

The objective of this GLP and guideline study was to assess the potential toxicity of 2,4,6-TCP when administered by gavage to the rat once daily for 28 days. In addition, the pharmacokinetic (PK) characteristics of 2,4,6-TCP were determined.

A total of 40 animals (██████WI(Han) strain) were allocated to the study in 4 groups (5 rats/sex/group) and were dosed with 0 (vehicle), 100, 250 or 500 mg/kg bw/d 2,4,6-TCP, respectively. Animals were dosed once daily, by gavage for 28 days, until the day before necropsy. The animals were monitored regularly for general health/mortality and moribundity as well as clinical observations. Body weights and food consumption were measured and recorded at predetermined intervals. Blood samples

were collected from all animals on the day of termination to evaluate haematology and clinical chemistry parameters. Blood samples were also collected at predetermined intervals on Days 2 and 22 for bioanalysis and pharmacokinetic evaluation. All animals were euthanised after at least 28 days of treatment and subjected to a detailed necropsy examination, with selected organs being weighed. Tissues from all controls and animals administered 500 mg/kg bw/d 2,4,6-TCP were subjected to a comprehensive histopathological evaluation.

## Results

### Pharmacokinetics

The pharmacokinetics of 2,4,6-TCP were characterised at all dose levels following exposure for 1 or 22 days at 24 hours after dosing. Following a single dose, maximal blood concentrations ( $C_{\max}$ ) of and systemic exposure ( $AUC_{0-24}$ ) to 2,4,6-TCP increased with dose in a generally supra-proportional manner in both sexes. The time to reach maximal concentration ( $T_{\max}$ ) was variable ranging from 2 to 4 hours for males and 2 to 6 hours for females.

Following repeat daily dosing, in males,  $C_{\max}$  and  $AUC_{0-24}$  continued to increase supra-proportionally with increasing dose. However, in females the increase in  $C_{\max}$  and  $AUC_{0-24}$  was not strictly dose proportional. There was no evidence of accumulation. There was an increase in blood concentrations of 2,4,6-TCP in males across all dose levels and in females at 250 and 500 mg/kg bw/d at approximately 6 to 8 hours. This may be due to enterohepatic re-circulation of 2,4,6-TCP. No 2,4,6-TCP was detected in any sample from the control animals.

### Intergroup comparison of pharmacokinetic parameters

Day of dosing	Pharmacokinetic parameter	Dose (mg/kg bw/d)					
		Male			Female		
		100	250	500	100	250	500
1	$C_{\max}$ (ng/mL)	2880	14900	53100	2810	14200	33200
	$T_{\max}$ (h)	2.0 – 2.0	2.0 – 4.0	2.0 – 2.0	2.0 – 6.0	2.0 – 2.0	2.0 – 4.0
	$AUC_{\text{tlast}}$ (ng.h/mL)	12400	76300	365000	22100	71200	246000
	$AUC_{0-24}$ (ng.h/mL)	16700	76300	365000	22100	71200	246000
	$AUC_{\text{inf}}$ (ng.h/mL)	16800	76400	366000	18900	71700	250000
	$C_{\max}/D$	28.8	59.4	106	28.1	56.8	66.3
	$AUC_{0-24}/D$	167	305	730	221	285	491
22	$C_{\max}$ (ng/mL)	3980	19000	54600	8170	14800	48300
	$T_{\max}$ (h)	2.0 – 4.0	2.0 – 2.0	2.0 – 2.0	2.0 – 6.0	2.0 – 2.0	2.0 – 4.0
	$AUC_{\text{tlast}}$ (ng.h/mL)	18000	76900	418000	46100	74400	377000
	$AUC_{0-24}$ (ng.h/mL)	20900	76900	418000	46100	74400	377000
	$AUC_{\text{inf}}$ (ng.h/mL)	18800	77200	419000	42600	64400	378000
	$C_{\max}/D$	39.8	75.8	109	81.7	59.2	96.6
	$AUC_{0-24}/D$	209	308	836	461	297	753

### Mortality and clinical signs of toxicity

There were no deaths at any dose level. Ploughing behaviour was present sporadically from Days 8 to 12 of the study in all, but one animal given 250 mg/kg bw/d, and the entire 500 mg/kg bw/d dose group. There were no clinical signs of toxicity at 100 mg/kg bw/d. These effects are considered to be treatment-related; however, in isolation, with no other clinical signs of toxicity or effects on FOB and MA

parameters, and considering their sporadic nature on particular treatment days, they are not regarded to be adverse.

#### *Body weight and food consumption*

There were no effects of treatment on body weights, body weight gains or food consumption.

#### *Haematology*

Fibrinogen concentration in top dose females was statistically significantly lower (by 20%) than controls. There was a statistically significant increase (by 52%) in neutrophil count in top dose males compared with controls, although all individual values were within the laboratory historical control range. Due to the relatively minor nature of these changes, the lack of effects in other related parameters, the absence of a clear dose-response and the sex-specificity of the response, they are considered unrelated to treatment. A dose-related decrease in platelet counts was seen at the top dose in males (by 12%) and in females (by 10%). However, given the low magnitude of the change which was within the laboratory historical control range, it was not considered treatment-related.

#### **Intergroup comparison of selected haematology parameters**

Parameter	Dose (mg/kg bw/d)									
	Males					Females				
	0	100	250	500	Lab HCD (mean-2SD – mean +2SD)	0	100	250	500	Lab HCD (mean-2SD – mean +2SD)
Fibrinogen (mg/dl)	271.1	282.0	297.8	282.3	-	212.6	189.5	193.1	170.5**	-
Neutrophil ((10 <sup>3</sup> /ul) count)	0.738	1.020	0.908	1.126 *	0.33-1.79	0.564	0.622	0.546	0.584	0.13-1.10
Platelets ((10 <sup>3</sup> /ul) count)	781.6	778.5	743.3	698.4	647-1173	751.8	721.0	659.0	660.3	639-1143

\* Statistically significant difference from control group mean, p<0.05 (Dunnett)

\*\* Statistically significant difference from control group mean, p<0.01 (Dunnett)

#### *Clinical-chemistry*

Plasma potassium concentration in females given 250 or 500 mg/kg bw/d was statistically significantly higher than controls (by 14% and 16%, respectively), however all individual values were within the laboratory historical control range (3.5-4.7). Plasma albumin concentration in females given 500 mg/kg bw/d was statistically significantly higher (by 7%) than controls, with the individual values for three animals above the upper limit of the laboratory historical control range (3.8-4.9). Due to their relatively small magnitude and inclusion in the historical control range, these changes are considered not to be adverse.

Statistically significant lower creatinine levels (by 16%) were seen in mid-dose and top-dose females. However, these decreases were in the opposite direction to that usually associated with toxicity, were not dose-related and were within the laboratory historical control range and therefore they are considered unrelated to treatment.

In females, triglyceride and glucose concentrations were statistically significantly higher compared with controls at 250 and 100 mg/kg bw/d, respectively, but not at the top dose. These changes were not dose-related, nor consistent between sexes, and all values were within the laboratory historical control range. Therefore, these effects are considered chance findings.

Plasma chloride concentrations in top-dose males were statistically significantly but marginally higher (by 1.6|%) than controls while in females, the chloride concentrations were statistically significantly but marginally lower (by 2%) than controls. Given the contradictory nature of this finding and, as all values for both sexes were within the laboratory historical control ranges, these differences were considered to be due to chance.

Overall, there were no adverse or treatment-related effects on clinical-chemistry parameters.

#### Intergroup comparison of selected blood chemistry parameters

Parameter	Dose (mg/kg bw/d)							
	Males				Females			
	0	100	250	500	0	100	250	500
Potassium (mmol/l)	4.62	4.86	4.65	4.69	3.61	3.90	4.13*	4.19*
Albumin (g/dl)	4.06	4.06	4.04	4.18	4.58	4.54	4.72	4.94*

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett)

#### Behavioural observations, FOB and MA parameters

At 500 mg/kg bw/d, time at rest was statistically significantly higher (by 39%) than controls in males during Period 3, but not in Periods 1, 2, 4, 5 and 6. In addition, this was an isolated incident and as such was considered to be unrelated to treatment. There was no statistically significant effect on grip strength in either sex or any other FOB or MA parameter.

#### Organ weights

Changes in organ weights were seen in the liver, kidney, thyroid and uterus.

In top-dose males, relative liver weight was statistically significantly higher than controls by 13 %. Relative liver weight in females was statistically significantly higher than controls by 12 % and 16 % at 250 and 500 mg/kg bw/d, respectively. All individual values for males were within the laboratory historical control range (3.4-4.9). Values for two top-dose females were marginally above the upper limit of the range (3.3-4.6). Generally, for liver weight, an increase  $\geq 15\%$  is considered adverse. Therefore, there was an adverse effect on liver weight only in top-dose females, where in addition, some individual values were above the laboratory historical control range.

Relative kidney weight was statistically significantly higher (by 10 % in males and by 14 % in females) than controls at the top dose. However, most or all individual values were within the laboratory historical control ranges (0.6-0.9 in males and 0.66-0.95 in females). Given the low magnitude of the increase which was within the historical control range, it is concluded that there was no adverse effect on kidney weight.

Relative thyroid weights were statistically significantly higher (by 32%) than controls in top-dose males. Although all individual values were well within the laboratory historical control range (0.003-0.0099), given the magnitude of the effect and its statistical significance, the increase in thyroid weight in top-dose males is considered treatment-related and adverse.

Relative uterus weight was substantially lower (by 38%) in top-dose females. Although not statistically significant, a dose-response was apparent. In addition, in the absence of historical control data for this organ, the decrease is considered treatment-related and adverse.

Overall, there were adverse effects on liver weight in females, on thyroid weight in males and on uterus weight in females at the top dose.



**Intergroup comparison of selected organ weights**

Parameter	Dose (mg/kg bw/d)							
	Males				Males			
	0	100	250	500	0	100	250	500
Liver ((g) relative to bw)	3.922	4.098	4.282	4.384***	3.875	3.740	4.319*	4.488**
Kidney ((g) relative to bw)	0.699	0.696	0.678	0.786**	0.758	0.749	0.820	0.865*
Thyroid ((g) relative to bw)	0.052	0.057	0.060	0.0069**	0.0072	0.0081	0.0096	0.0087
Uterus ((g) relative to bw)	-	-	-	-	0.4101	0.3728	0.3058	0.2555

\* Statistically significant difference from control group mean,  $p < 0.05$  (Dunnett)

\*\* Statistically significant difference from control group mean,  $p < 0.01$  (Dunnett)

\*\*\* Statistically significant difference from control group mean,  $p < 0.001$  (Dunnett)

**Macroscopy**

There were no treatment-related effects on macroscopic parameters.

**Microscopy**

The only histopathological findings considered to be treatment-related were seen in the nasal turbinates. Degeneration of the olfactory epithelium with an associated inflammatory exudate in the lumen was noted in two males and two females at the top dose. Typically, this type of lesion is associated with the presence of foreign material in the nasal cavity. In this study, ploughing behaviour was present sporadically from Days 8 to 12 in most animals given 250 mg/kg bw/d, and all animals given 500 mg/kg bw/d, which may have led to the inhalation of foreign material. Therefore, this effect is not a specific toxic effect of the substance and hence it is not considered relevant.

Overall, there were no relevant adverse histopathological findings.

**Conclusion**

In a GLP and guideline 28-day gavage study in which rats were given 0, 100, 250 or 500 mg/kg bw/d 2,4,6-TCP, the only relevant adverse effects were seen at the top dose on the weight of the liver (females), thyroid (males) and uterus (females). Based on these effects a **NOAEL of 250 mg/kg bw/d** can be identified from the study.

The applicant proposed a NOAEL of 500 mg/kg bw/d as the observed organ weight changes were regarded to be non-adverse in the absence of any associated histopathology. It is HSE's view, that despite the lack of histopathology, the adversity of these organ changes cannot be disregarded, given their magnitude.

(████████, 2021)

**GENOTOXICITY**

A summary of the published literature data on 2,4,6-TCP in relation to genotoxicity is provided in Table 6.8.1-72, by genotoxicity endpoint. New modern studies performed recently by Syngenta are also included (in yellow highlighted). Full study summaries are provided in the section below.

**Table 6.8.1-72: A summary of the genotoxicity studies on 2,4,6-TCP available in the published literature and modern studies generated by Syngenta**

Study	Study design	Result	Reference	Klimisch score
<b><i>In vitro</i> assays</b>				
<b><i>Bacterial systems</i></b>				
Bacterial mutation assay (Ames)	Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay  Incubated with the test item up to the maximal concentration of 5000 µg/plate with and without S9 mix	Negative	Chang S (2020)	1
Bacterial mutation assay (Ames)	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537 Dosed up to 10 mg/plate with and without S9 mix Preincubation assay	Negative	Haworth S; Lawlor T; Mortelmans K; Speck W; Zeiger E; ENVIRON. MUTAGEN., (1983),1: 3-142.	1
Bacterial mutation assay (Ames)	<i>Salmonella typhimurium</i> TA98, TA100 with and without S9 mix Preincubation assay	Negative	Onodera S; Yoshimatsu K; Saitoh H; Uchida A; JAPANESE JOURNAL OF TOXICOLOGY AND ENVIRONMENTAL HEALTH, (1998) 44(4), 289-299.	3
Bacterial mutation assay (Ames)	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537 Dosed up to 500 µg/plate with and without S9 mix  Plate incorporation assay	Negative	Rasanen L; Hattula M; BULL. ENVIRON. CONTAM. TOXICOL. (1977), 18(5), 565-71.	2
Bacterial mutation assay (Ames)	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA104 Dosed up to 1000 µg/plate with and without S9 Plate incorporation assay	Positive TA97 +S9 only, LEC 10 µg/plate TA98 +S9 only, LEC 10 µg/plate TA104 +S9 only, LEC 50 µg/plate	Strobel K; Grummt T; TOXICOLOGICAL AND ENVIRONMENTAL CHEMISTRY, (1987) 14(1-2), 143-56.	1
Induction of DNA repair genes in Salmonella typhimurium	<i>Salmonella typhimurium</i> TA1535/pSK1002 <i>umuC'</i> - <i>'lacZ</i> Dosed up to top 300 mg/L with and without S9 mix	Negative	Ono Y; Kobayashi U; Somiya I; Nunoshiba T; Oda Y; MIZU KANKYO GAKKAISHI, (1996), 19(11), 871-877.	3

Study	Study design	Result	Reference	Klimisch score
	<i>Salmonella typhimurium</i> TA1535/pSK1002 <i>umuC</i> '- <i>lacZ katG</i> deleted Dosed up to top 300 mg/L without S9 mix only	Weakly positive LEC = 300 mg/L		3
Rec-assay in <i>Bacillus subtilis</i>	M45 Rec- and WT H17 Rec+ of <i>Bacillus subtilis</i> Dosed up to 100 µg/disk without S9 mix 24h exposure	Positive LEC=50 µg/disk	Ozaki A; Yamaguchi Y; Fujita T; Kuroda K; Endo G; FOOD AND CHEMICAL TOXICOLOGY, (2004) 42(8), 1323-37.	1
<i>Yeast assays</i>				
<i>Saccharomyces cerevisiae</i>	<i>Saccharomyces cerevisiae</i> MP1 Dosed up to 400 mg/L without S9 mix 3 and 5 h treatment period	Negative (gene conversion assay) Negative (homozygosis by mitotic recombination or gene conversion assay) Weak positive (forward mutation assay) LEC = of 400 mg/L	Fahrig R; Nilsson C.A; Rappe C; ENVIRONMENTAL SCIENCE RESEARCH, (1978,) 12. Pentachlorophenol: Chem., Pharmacol., Environ. Toxicol., 325-38.	2
<i>Insect assay</i>				
<i>Drosophila melanogaster</i> sex linked recessive lethal mutation assay	Dosed up to 250 ppm in food Dosed up to 10000 ppm injected	Negative	Valencia R; Mason J.M; Woodruff R.C; Zimmering S; ENVIRONMENTAL MUTAGENESIS, (1985), 7(3), 325-48.	1
<i>Mammalian cells</i>				
Hprt gene mutation assay	Cell Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)  Treated up to 1000 µg/ml with and without metabolic activation	Negative up to precipitation/cytotoxicity	Sokolowski A (2020)	1
Hprt gene mutation assay	Chinese hamster lung V79 cells Dosed up to 60 µg/mg without S9 mix or incubated with hepatocytes 48 h treatment period	Positive without S9 only LEC = 30 µg/mg	Hattula M.L; Knuutinen J; CHEMOSPHERE (1985), 14(10), 1617-1625.	1
Hprt gene mutation assay	Chinese hamster lung V79 cells Dosed up to 100 µg/mL without S9 mix 24h treatment period	Negative	Jansson K; Jansson V; MUTATION RESEARCH, (1986), 171(2-3), 165-168.	1
Hprt gene mutation assay	Chinese hamster lung V79 cells Dosed up to 180 µg/mL without S9 mix 24h treatment period	Negative	Jansson K; Jansson V; MUTATION RESEARCH, (1992), 280(3), 175-179.	1

Study	Study design	Result	Reference	Klimisch score
Thymidine kinase gene mutation assay	L5178Y mouse lymphoma Dosed up to 200 µg/mL without S9 mix 4 h treatment period	Positive without S9 only LEC = 80 µg/mL	McGregor D.B; Brown A; Cattanach P; Edwards I; McBride D; Riach C; Caspary W.J; ENVIRONMENTAL AND MOLECULAR MUTAGENESIS, (1988), 12(1), 85-154.	1
In Vitro Micronucleus Assay	Micronucleus test inhuman lymphocytes in vitro.  Assessed at concentrations up to 1975µg/ml for potential to induce micronuclei in human lymphocytes in vitro in two independent experiments	Negative up to precipitation/cytotoxicity	Naumann S (2020)	1
Chromosome aberration assay	Chinese hamster lung V79 cells Dosed up to 700 µg/ml with and without S9 mix (3h exposure) Dosed up to 250 µg/ml without S9 mix (24h exposure) Exposure period: - with S9: 3h treatment, 17h recovery - without S9: 3h treatment + 17h recovery; 24h + 0, 4, 8, 12, or 24 h recovery	Positive: without S9 only  3h treatment + 17h recovery  24h treatment and 4-12 h recovery  Negative: 24h treatment and immediate harvest  24h treatment and 24h recovery	Armstrong M.J; Galloway S.M; Ashby J; MUTATION RESEARCH, (1993), 303(3), 101-108.	2
Chromosome aberration assay	Chinese hamster ovary cells Dosed up to 600 µg/ml with and 500 µg/ml without S9 mix Exposure period: - with S9: 3h treatment, 17h recovery - without S9: 3h treatment + 17h recovery; 24h + 0, 4, 8, 12, or 24 h recovery	Positive: with and without S9 3h treatment + 17h recovery  24h treatment and 4-12 h recovery  Negative: 24h treatment and immediate harvest  24h treatment and 24h recovery		2

Study	Study design	Result	Reference	Klimisch score
Chromosome aberration assay	Chinese hamster ovary cells Up to 500 µg/ml with and without S9 mix +S9 2h exposure -S9 18-26h exposure 2h treatment + 8-12h recovery or 18-26h recovery;	Negative	Galloway S.M; Armstrong M.J; Reuben C; Colman S; Brown B; Cannon C; Bloom A.D; Nakamura F; Ahmed M; Duk S; Rimpo J; Margolin B.H; Resnik M.A; Anderson B; Zeiger E; ENVIRONMENTAL AND MOLECULAR MUTAGENESIS; (1987), 10, (SUPPL 10) 1-175.	2
Chromosome aberration assay	Chinese hamster lung V79 cells Dosed up to 60 µg/ml without S9 mix 24h treatment period, with or without 24h recovery	Negative	Jansson K; Jansson V; Mutation research, (1992) Vol. 280, No. 3, pp. 175-9. Journal code: 0400763. ISSN: 0027-5107. L-ISSN: 0027-5107.	1
Chromosome aberration assay	Chinese hamster lung V79 cells Dosed up to 1500 µg/mL with and without S9 mix 6 h treatment period	Positive with S9 only LEC = 1000 µg/mL	Matsuoka A; Hayashi M; Sofuni T; KANKYO HEN'IGEN KENKYU, (1998) 20(3), 159-165.	2
<i>In vitro</i> comet assay	HL-60 cells Dosed up to 100 µg/mL without S9 mix 2 h treatment period	Positive LEC = 50 µg/mL	Ozaki A; Yamaguchi Y; Fujita T; Kuroda K; Endo G; FOOD AND CHEMICAL TOXICOLOGY, (2004), 42(8), 1323-1337.	2
Induction of aneuploidy	Chinese hamster lung V79 cells Dosed up to 250 µg/ml without S9 mix 24 h treatment period	Positive 24h treatment + 24h recovery	Armstrong M.J; Galloway S.M; Ashby J; MUTATION RESEARCH, (1993), 303(3), 101-108.	1
Induction of aneuploidy and micronuclei	Chinese hamster lung V79 cells Dosed up to 90 µg/ml without S9 mix 24h treatment with and without 24h recovery period	Positive LEC=30 µg/mL (24 h exposure w/o recovery)	Jansson K; Jansson V; MUTATION RESEARCH, (1992) 280(3), 175-9.	1
<b>Other assays</b>				
DNA strand breaking assay	purified PM2 DNA with and without S9 mix 24 h treatment period	Positive	Juhl U; Blum K; Witte I; CHEMICO-BIOLOGICAL INTERACTIONS, (1989), 69 (4), 333-344.	1
<b><i>In vivo</i> assays</b>				

Study	Study design	Result	Reference	Klimisch score
In Vivo Mutation of 2,4,6-TCP at the cII locus in Big Blue® Transgenic F344 Rats	2,4,6-TCP was administered for 28 days at: 0, 100, 250 and 500 mg/kg bw/d (Groups 1-4, respectively), to six male rats per group in Groups 1-3 and seven male rats in Group 4. Corn oil was used as the vehicle	2,4,6-TCP was concluded to be negative for the induction of cII mutants in liver, bone marrow and duodenum up to the MTD.	Bruce, S. (2021b)	1
Mouse spot test	Mice, C57BL/6JHan strain females crossed with males of the rotation bred T-stock Dosed up to 100 mg/kg bw by IP injection.	Positive <sup>11</sup> LEC = 100 mg/kg bw	Fahrig R; Nilsson C.A; Rappe C; ENVIRONMENTAL SCIENCE RESEARCH, (1978), 12, Pentachlorophenol: Chem., Pharmacol., Environ. Toxicol., 325-338.	3
Alkaline elution assay	Female Sprague-Dawley rats Dosed twice at 164 mg/kg bw 21 and 4h before termination Endpoints of interest: DNA damage (alkaline elution), liver and white blood cells Promotion of carcinogenesis (ornithine carboxylase activity) Hepatic cell death and damage (serum alanine aminotransferase)	Negative	Kitchin K.T; Brown J.L; TOXICOLOGICAL AND ENVIRONMENTAL CHEMISTRY, (1988) 16(3), 165-172.	3
<i>In vivo-in vitro</i> replicative DNA synthesis (RDS) test	B6C3F1 mice Dosed up to 2000 mg/kg bw. DNA synthesis analysed in hepatocytes	Negative	Miyagawa M; Takasawa H; Sugiyama A; Inoue Y; Murata T; Uno Y; Yoshikawa K MUTATION RESEARCH, (1995), 343(2-3), 157-183.	2
<i>In vivo</i> comet assay	ppY mice dosed at 500 mg/kg bw (MTD) for 3 and 8 h by oral gavage DNA strandbreaks were analysed in stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow	Positive LEC = 500 mg/kg bw (stomach, colon, liver, kidney, urinary bladder, lung, brain)	Sasaki Y.F; Sekihasi K; Izumiyama F; Nishidate E; Saga A; Ishida K; Tsuda S; CRITICAL REVIEWS IN TOXICOLOGY. (2000), 30(6), 629-799 <sup>12</sup> .	1
p53 zebrafish assay	Zebrafish Dosed up to 50 µg/L Livers were harvested after 1, 3, 6 or 10 days and analysed for p53 mutations	Positive	Yin D; Zhu H; Hu P; Zhao Q; ENVIRONMENTAL TOXICOLOGY AND CHEMISTRY, (2009), 28(3), 603-608.	2

<sup>11</sup> The methods and data were re-evaluated by Russell (1981) and considered uninterpretable due to the lack of statistical of the study. Russell L.B; Selby P.B; Von Halle E; Sheridan W; Valcovic L; (1981) Use of the mouse spot test in chemical mutagenesis: Interpretation of past data and recommendations for future work. Mutation Research, 86; 355-379.

<sup>12</sup> Sasaki *et al.*, (2000) was not identified in the literature search, but is included as it is a reliable publication on 2,4,6-TCP.

<b>Report:</b>	K-CA 5.8.1/20 Haworth S. <i>et al.</i> , (1983). Salmonella Mutagenicity Test Results for 250 Chemicals. Genetic Toxicology Department, Microbiological Associates, Bethesda, Maryland. Published: Haworth S, Lawlor T, Mortelmans K, Speck W and Zeiger E (1983). Salmonella Mutagenicity Test Results for 250 Chemicals. Environmental Mutagenesis 1:3-142. Syngenta File No. NA_13796.
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**STUDY TYPE:** Reverse Mutation Test Using Bacteria.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (practical)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

A *Salmonella typhimurium* reverse mutation assay was used to investigate the mutagenic potential of 2,4,6-trichlorophenol (2,4,6-TCP), according to the methods of Ames (Ames *et al.* 1975).

An unspecified concentration range was used (at least 5 concentrations), both in the presence and absence of a S9 metabolic activation system. Salmonella strains TA98, TA100, TA1535 and TA1537 were used.

Although no data is presented for 2,4,6-TCP, no increase in the number of revertant colonies was observed at any concentration of 2,4,6-TCP, neither in the presence nor absence of metabolic activation.

**Under the conditions of the study, 2,4,6-TCP did not cause an increase in the number of revertant colonies, neither in the presence nor absence of a S9 metabolic system, and was therefore considered to be non-mutagenic in the assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	B7X
<b>Purity:</b>	Practical
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	
<b>Solvent control (final concentration):</b>	Not specified; either distilled water, DMSO, ethanol (95%) or acetone – used in order of preference and based on solubility.
<b>Positive control:</b>	Nonactivation: 4-Nitro-o-phenylenediamine 5.0 µg/plate (TA98) Sodium azide 1.0 µg/plate (TA100, TA1535) 9-aminoacridine 50.0 µg/plate (TA1537)
	Activation: 2-Aminoanthracene 1.0 µg/plate (TA98 and TA100) 2.5 µg/plate (TA1535 and TA1537)

**Mammalian metabolic system: S9 derived**

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None	X	Hamster		Other
			Other β-naphthoflavone		Other		

Liver S-9 fractions were routinely prepared from male Sprague-Dawley rats and male Syrian hamsters that were injected, ip, with Aroclor 1254 (200 mg/ml in corn oil) at 500 mg/kg. Five days after injection, the animals were sacrificed and the livers were removed aseptically. The animals were fasted for 12-24 hr immediately preceding sacrifice. Liver homogenates were prepared aseptically at 4°C. Excised livers were rinsed with 0.15 M KCl, then minced and homogenized (3 ml of 0.15 M KCl/g wet tissue) in a Potter-Elvehjem apparatus with a teflon pestle.

The homogenate was centrifuged for 10 min at 9,000g at 4°C. The supernatant (S-9) was decanted and distributed into freezing ampules and stored at -70°C. The microsomal enzyme reaction mix (S-9 mix) was prepared immediately prior to each assay. Unused S-9 mix was discarded and not refrozen. One milliliter of S-9 mix has the following composition: S-9, 0.10 ml; 0.04 M MgCl<sub>2</sub>, 0.02 ml; 1.65 M KCl, 0.02 ml; 0.04 M β-nicotinamide adenine dinucleotide phosphate (NADP), 0.10 ml; 0.05 M glucose-6-phosphate, 0.10 ml; 1.0 M NaH<sub>2</sub>PO<sub>4</sub> (pH 7.4), 0.10 ml; and distilled water, 0.56 ml.

**Test organisms:***S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

*E. coli* strains

	WP2 (pKM101)		WP2 <i>uvrA</i> (pKM101)						
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Properly maintained?

☒ Yes☐ NoChecked for appropriate genetic markers (*rfa* mutation, R factor)?☒ Yes☐ No**Test compound concentrations used**

Nonactivated conditions: Not specified, at least 5 concentrations

Activated conditions: Not specified, at least 5 concentrations



Three plates were used, and the experiment was repeated no less than 1 week after completion of the initial test.

### **Study Design and Methods:**

**In-life dates:** Not specified

### **TEST PERFORMANCE**

#### **Preliminary Cytotoxicity Assay:**

To select the dose range for the mutagenesis assay, 2,4,6-TCP was checked for toxicity to TA100 up to a concentration of 10 mg/plate or the limit of solubility, both in the presence and absence of S-9 mix. One or more parameters were used as an indication of toxicity: viability on complete medium and reduced numbers of revertant colonies per plate and/or thinning or absence of the bacterial lawn. If toxicity was not apparent in the preliminary toxicity determination, the highest dose tested was 10 mg/plate; otherwise the upper limit of solubility was used. If toxicity was observed, the doses of test chemical were chosen so that the high dose exhibited some degree of toxicity.

#### **Type of Bacterial assay:**

- \_\_\_ standard plate test
- X pre-incubation (60 minutes)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

#### **Protocol:**

0.5 mL of S-9 mix or 0.1M PO<sub>4</sub> buffer was dispensed into an appropriate number of 13 × 100 mm culture tubes maintained at 37°C in a dry-bath. Then, 0.05 mL of cells and 0.05 mL of solvent or chemical dilution were added to each tube. The mixture was vortexed and allowed to incubate for 20 min at 37°C.

Following the preincubation period, 2.0 mL of molten top agar (45°C) supplemented with 0.5 mM L-histidine and 0.5 mM d-biotin was pipetted into the tubes, which were immediately vortexed, and their contents poured onto 25 mL of minimal glucose bottom agar in a 15 x 100-mm plastic petri dish. After the overlay solidified, the plates were inverted and incubated at 37°C for 48 h. At least five doses of test chemical, in addition to the concurrent solvent and positive controls, were tested on each strain in the presence of S-9 mix or buffer. Three plates were used, and the experiment was repeated no less than 1 week after completion of the initial test.

#### **Statistical analysis:**

Statistical analysis was not incorporated into the initial data evaluations.

#### **Evaluation criteria:**

Prior to statistical analysis no formal rules were used; however, a positive response was indicated by a reproducible, dose-related increase, whether it be twofold over background or not. The matrix of test strains and activation systems used allowed the investigators to detect trends or patterns that might not be as evident if only one strain and activation system were examined. In addition to the standard “positive” and “negative” categories, there is also “questionable” (or “inconclusive”). This applied to low-level responses that were not reproducible within the laboratory or to results that showed a definite trend but with which the investigator did not feel comfortable in making a “+” or “-” decision. It also included tests in which an elevated revertant colony yield occurred at only a single dose level.

### **RESULTS**

**Preliminary cytotoxicity assay:** Data not presented

**Mutagenicity assay:** Data not presented

Over an unspecified concentration range, 2,4,6-TCP did not cause an increase in the number of revertant colonies in the strains of Salmonella used, neither in the presence nor absence of metabolic activation.

The positive controls for each experiment induced the expected responses, indicating the strains were responding satisfactorily in each case. Revertant colony numbers for the solvent control plates were within acceptable ranges.

**CONCLUSION:**

In a publication from 1983, 2,4,6-TCP was negative in an Ames test with and without metabolic activation. HSE notes that only four Salmonella strains (TA98, TA100, TA1535 and TA1537) were used and that, without information on the concentrations employed and the level of cytotoxicity observed, it is unclear whether the substance was tested up to cytotoxicity, the limit of solubility or the limit concentration.

**REFERENCES:**

Ames B, McCann J and Yamasaki E (1975). *Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res.*, 31:347-364.

(Haworth S *et al*, 1983)

<b>Report:</b>	K-CA 5.8.1/21 Onodera S. (1998). Behavior of Mutagenic Formation from Phenolic Compounds in Water Disinfection with Chlorine and their Mutagenic Potential Formation. Tokyo University of Science, Tokyo, Japan. Published: Onodera S, Yoshimatsu K, Saitoh H, Uchida A (1998). Behavior of mutagenic formation from phenolic compounds in water disinfection with chlorine and their mutagenic potential formation. Japanese Journal of Toxicology and Environmental Health 44:289-299. Syngenta File No. NA_13793.
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**STUDY TYPE:** Reverse Mutation Test Using Bacteria.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**The original publication was in Japanese and a translation to English obtained.**

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

A *Salmonella typhimurium* reverse mutation assay was used to investigate the mutagenic potential of 2,4,6-trichlorophenol (2,4,6-TCP).

An unspecified concentration range was used, both in the presence and absence of a S9 metabolic activation system. *Salmonella* strains TA98 and TA100 were tested.

Although no data is presented for 2,4,6-TCP, no increase in the number of revertant colonies was observed at any concentration of 2,4,6-TCP, neither in the presence nor absence of metabolic activation.

**Under the conditions of the study, 2,4,6-TCP did not cause an increase in the number of revertant colonies, neither in the presence nor absence of a S9 metabolic system, and was therefore considered to be non-mutagenic in the assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated.
<b>Lot/Batch number:</b>	Not indicated.
<b>Purity:</b>	Commercial special grade.
<b>CAS#:</b>	Not indicated.
<b>Stability of test compound:</b>	Not indicated
<b>Control Materials:</b>	
<b>Negative:</b>	Not indicated.
<b>Solvent control (final concentration):</b>	DMSO (100 µL)
<b>Positive control:</b>	Nonactivation: 2-Nitrofluorene 2.0 µg/plate (TA98) Sodium azide 1.0 µg/plate (TA100)
	Activation: Benzo(a)pyrene 5.0 µg/plate (TA98 and TA100)

### Mammalian metabolic system: S9 derived

Preparation not described.

### Test organisms:

*S. typhimurium* strains

TA97	X	TA98	X	TA100		TA102		TA104
TA1535		TA1537		TA1538		list any others		

*E. coli* strains

WP2 (pKM101)		WP2 <i>uvrA</i> (pKM101)						
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Properly maintained?

☒

Yes

☐

No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?

☒

Yes

☐

No

### Test compound concentrations used

Nonactivated conditions: Not specified.

Activated conditions: Not specified.

Three plates were used.

**Study Design and Methods:**

**In-life dates:** Not specified

**TEST PERFORMANCE****Preliminary Cytotoxicity Assay:**

Not specified.

**Type of Bacterial assay:**

- \_\_\_ standard plate test
- X pre-incubation (20 minutes)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Protocol:**

The mutagenicity of test solutions was investigated using histidine dependent *Salmonella typhimurium* TA98 and TA100 by the pre-incubation system of Yahagi (1) in systems with or without the addition of rat liver metabolic activation enzymes (S9 mix).

**Statistical analysis:**

Statistical analysis was not performed.

**Evaluation criteria:**

Prior to statistical analysis no formal rules were used; however, a positive response was indicated by a reproducible, dose-related increase, whether it be twofold over background or not. The matrix of test strains and activation systems used allowed the investigators to detect trends or patterns that might not be as evident if only one strain and activation system were examined. In addition to the standard “positive” and “negative” categories, there is also “questionable” (or “inconclusive”). This applied to low-level responses that were not reproducible within the laboratory or to results that showed a definite trend but with which the investigator did not feel comfortable in making a “+” or “-” decision. It also included tests in which an elevated revertant colony yield occurred at only a single dose level.

**RESULTS**

**Preliminary cytotoxicity assay:** Data not presented

**Mutagenicity assay:** Data not presented

An unspecified concentration range was used, both in the presence and absence of a S9 metabolic activation system. *Salmonella* strains TA98 and TA100 were tested.

Although no data is presented for 2,4,6-TCP, no increase in the number of revertant colonies was observed at any concentration of 2,4,6-TCP, neither in the presence nor absence of metabolic activation.

The positive controls for each experiment induced the expected responses, indicating the strains were responding satisfactorily in each case. Revertant colony numbers for the solvent control plates were within acceptable ranges.

**CONCLUSION:**

In a publication from 1998, 2,4,6-TCP was negative in an Ames test with and without metabolic activation. HSE notes that only two *Salmonella* strains (TA98 and TA100) were used and that, without information on the concentrations employed and the level of cytotoxicity observed, it is unclear whether the substance was tested up to cytotoxicity, the limit of solubility or the limit concentration.

**REFERENCES:**

Yahagi T. (1975). *Tanpakushitsu, Kakusan, Kouso*, 20:1178-1189.

(Onodera S *et al*, 1998)

<b>Report:</b>	K-CA 5.8.1/22 Räsänen L. <i>et al.</i> , (1977). The Mutagenicity of MCPA and its Soil Metabolites Chlorinated Phenols, Catechols and Some Widely Used Slimicides in Finland. Department of Cell Biology, University of Jyväskylä, SF-40100 Jyväskylä 10, Finland. Published: Räsänen L, Hattula M, Arstila A (1977). The Mutagenicity of MCPA and Its Soil Metabolites Chlorinated Phenols, Catechols and Some Widely Used Slimicides in Finland. Bulletin of Environmental Contamination & Toxicology, vol. 18, No.5. 565-571. Syngenta File No. NA_13795.
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**STUDY TYPE:** Reverse Mutation Test Using Bacteria.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (Fluka purum (>95%))

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 2**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

A *Salmonella typhimurium* reverse mutation assay was used to investigate the mutagenic potential of 2,4,6-trichlorophenol (2,4,6-TCP), according to the methods of Ames (Ames *et al.* 1975).

A concentration range of 0.5 – 500 µg/plate was used, both in the presence and absence of a S9 metabolic activation system. *Salmonella* strains TA98, TA100, TA1535 and TA1537 were used.

No increase in the number of revertant colonies was observed at any concentration of 2,4,6-TCP, neither in the presence nor absence of metabolic activation.

A reduction in the number of revertant colonies was seen at the highest dose of 500 µg/plate 2,4,6-TCP, indicating the compound was toxic to the bacteria at this concentration.

Under the conditions of the study, 2,4,6-TCP did not cause an increase in the number of revertant colonies, in both the presence and absence of a S9 metabolic system, and was therefore considered to be non-mutagenic in the assay.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Fluka purum (>95%)
<b>CAS#:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	None
<b>Solvent control (final concentration):</b>	DMSO
<b>Positive control:</b>	Nonactivation: MNNG 5.0 µg/plate (TA1535) 9-aminoacridine 100.0 µg/plate (TA1537)
	Activation: 2-Aminofluorine 10 µg/plate (TA98) Benzo(a)pyrene 10.0 µg/plate (TA100)

### Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbital		Mouse		Lung
			None		Hamster		Other
			Other β-naphthoflavone		Other		

Male Wistar rats, 2-3 months old, served as the source of the liver homogenate. The rats were induced by Aroclor 1254 5 days before the sacrifice and the food was removed one day before the sacrifice. The homogenate was made as described by Ames (Ames et al. 1975) and it was denoted by S9. The final S9 mix (liver homogenate + cofactor solution) contained per ml 0.1 ml S9, 8 µmoles MgCl<sub>2</sub>, 33 µmoles KCl, 5 µmoles glucose-6-phosphate, 4 µmoles NADP and 100 moles phosphate buffer (pH was 7.4).

### Test organisms:

#### *S. typhimurium* strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

#### *E. coli* strains

	WP2 (pKM101)		WP2 <i>uvrA</i> (pKM101)						
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Properly maintained?

☒

Yes

☐

No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?

☒

Yes

☐

No

**Test compound concentrations used**

Nonactivated conditions: 0.5, 5, 50 and 500 µg/plate

Activated conditions: 0.5, 5, 50 and 500 µg/plate

**Study Design and Methods:**

**In-life dates:** Not indicated

**TEST PERFORMANCE**

**Preliminary Cytotoxicity Assay:** Not performed.

**Type of Bacterial assay:**

- X standard plate test (both experiments –S9, initial experiment +S9)
- \_\_\_ pre-incubation (60 minutes) (second experiment +S9)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Protocol:**

The test was carried out as described by Ames (Ames et al.1975).

The media used was as follows: Vogel-Bonner–stock solution, minimum-glucose agar, nutrient agar, broth solution, top agar, 0.5 mM histidine-biotine solution, 0.2 M phosphate buffer and the cofactor solution

The compounds were tested over a wide range of concentrations (0.5, 5, 50 and 500 µg/plate) both in the presence and absence of S9 mix.

The revertant colonies were calculated after an incubation of 48 hours at 37°C.

**Statistical analysis:** None

**Evaluation criteria:** Not provided

**RESULTS**

**Preliminary cytotoxicity assay:** Not performed.

**Mutagenicity assay:**

Over a concentration range of 0.5 – 500 µg/plate 2,4,6-TCP, in both the presence and absence of metabolic activation, no increase in the number of revertant colonies was observed (data only presented for 5 µg/plate, see Table below).

At the highest dose tested of 500 µg/plate, a decrease in the number of revertant colonies was observed, due to the toxic effect of the test compound on the bacteria (data not presented).

The positive controls for each experiment induced the expected responses, indicating the strains were responding satisfactorily in each case.

Reversion of the bacterial tester strains with the test compounds and positive control mutagens (number of colonies calculated) at the level of 5 µg of the compound.  
 [(+) = S-9Mix added, (-) = without S-9Mix.]

Compounds tested	µg/plate	TA98		TA100		TA1535		TA1537	
		+	-	+	-	+	-	+	-
2,3,5-trichlorophenol	"	64/52	62/36	144/168	188/142	24/26	20/30	12/18	15/16
2,3,6- "	"	67/63	53/63	368/332	308/196	16/18	59/61	9/6	19/21
2,4,5- "	"	12/52	30/36	96/168	192/142	22/26	37/30	16/8	10/6
2,4,6- "	"	72/52	32/36	112/168	140/142	36/26	44/30	13/8	12/6
2-aminofluorine	10	3 480/63		1 244/332		13 600/46		8 200/9	
benzo(a)pyrene	10								
MNNG	5								
9-aminoacridine	100								

<sup>x</sup>Revertant colonies in test plates/revertant colonies in control plates

## CONCLUSION:

In a publication from 1977, 2,4,6-TCP was negative in an Ames test with and without metabolic activation up to a concentration causing cytotoxicity. HSE notes that only four *Salmonella* strains (TA98, TA100, TA1535 and TA1537) were used.

## REFERENCES:

Ames B, McCann J and Yamasaki E (1975). *Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res., 31:347-364.*

(Räsänen L *et al*, 1977)

<b>Report:</b>	K-CA 5.8.1/23 Strobel K. and Grummt T. (1987). Aliphatic and Aromatic Halocarbons as Potential Mutagens in Drinking Water. Research Institute for Hygiene and Microbiology, DDR-9933 Bad Elster, German Democratic Republic. Published: Strobel K and Grummt T (1987). Aliphatic and aromatic halocarbons as potential mutagens in drinking water. Toxicological and Environmental Chemistry 14:143-156. Syngenta File No. NA_13775.
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**STUDY TYPE:** Reverse Mutation Test Using Bacteria

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study



## EXECUTIVE SUMMARY

A *Salmonella typhimurium* reverse mutation assay was used to investigate the mutagenic potential of 2,4,6-trichlorophenol (2,4,6-TCP), according to the methods of Ames (Ames *et al.* 1975).

A concentration range of 0.01 – 1 mg/plate was used, both in the presence and absence of a S9 metabolic activation system. *Salmonella* strains TA98, TA100, TA97 and TA104 were used.

2,4,6-TCP induced an increase in the revertant colony frequency in *Salmonella* strains TA97 and TA104, in the presence of metabolic activation only. This data indicates the potential for 2,4,6-TCP to induce frameshift mutations.

A reduction in the number of revertant colonies was seen at the higher concentrations of 2,4,6-TCP, indicating the compound was toxic to the bacteria.

**Under the conditions of the assay reported, 2,4,6-TCP caused an increase in the number of revertant mutant colonies, in the presence of S9 metabolic activation only. 2,4,6-TCP was therefore mutagenic in the assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>CAS#:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	
<b>Solvent control (final concentration):</b>	DMSO
<b>Positive control:</b>	Nonactivation: Nor-HN <sub>2</sub> Not indicated (TA100) Dinitro-ortho-cresol (DNOC) 0.1 mg/plate (TA98, TA97) Methyl methanesulfonate (MMS) 0.1 mg/plate (TA104)
	Activation: 2-acetylaminofluorene (AAF) 0.05 mg/plate (TA98, TA97) 2-aminofluorene (AF) 0.1 mg/plate (TA100, TA104)

### Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbital		Mouse		Lung
			None		Hamster		Other
			Other β-naphthoflavone		Other		

S9 mix was prepared out of liver microsomes of rats which had undergone the usual Aroclor 1254-pretreatment.

### Test organisms:

*S. typhimurium* strains

X	TA97	X	TA98	X	TA100		TA102	X	TA104
	TA1535		TA1537		TA1538		list any others		

*E. coli* strains

	WP2 (pKM101)		WP2 <i>uvrA</i> (pKM101)						
--	--------------	--	--------------------------	--	--	--	--	--	--

Properly maintained?

☐  
☐

Yes

Yes

☐  
☐

No

No

Checked for appropriate genetic markers (*rfa* mutation, R factor)?**Test compound concentrations used**

Nonactivated conditions: 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 mg/plate

Activated conditions: 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 and 1.0 mg/plate

**Study Design and Methods:****In-life dates:** Not indicated**TEST PERFORMANCE****Preliminary Cytotoxicity Assay:** Not performed.**Type of Bacterial assay:**

- X standard plate test (both experiments –S9, initial experiment +S9)
- \_\_\_ pre-incubation (60 minutes) (second experiment +S9)
- \_\_\_ “Prival” modification (i.e. azo-reduction method)
- \_\_\_ spot test
- \_\_\_ other

**Protocol:**

The plate incorporation assays were performed as described by Ames (Ames et. al, 1975). *Salmonella typhimurium* tester strains TA97, TA98, TA100 and TA104 were employed. DMSO was used as the solvent for all test compounds. Test compounds were added on top agar in a volume of 0.1 mL. Both negative and positive controls were included in each assay. The values listed are the means obtained from duplicate or triplicate experiments. S-9 mix was prepared out of liver microsomes of rats which had undergone the usual Aroclor 1254-pretreatment.

**Statistical analysis:** None**Evaluation criteria:** Not provided**RESULTS****Preliminary cytotoxicity assay:** Not performed.**Mutagenicity assay:**

Under the conditions of the study, 2,4,6-TCP induced an increase in the revertant colony frequency in *Salmonella* strains TA97 and TA104, in the presence of metabolic activation only. This data indicates the potential for 2,4,6-TCP to induce frameshift mutations (Table 6.8.1-73a).

Table 6.8.1-73a: Mutagenicity of 2,4,6 trichlorophenol (Ames test)

		his <sup>+</sup> revertant colonies							
Compound	Dose mg/plate	TA98		TA100		TA97		TA104	
		–S9	+S9	–S9	+S9	–S9	+S9	–S9	+S9
2,4,6-trichlorophenol	1.0	tox.	tox.	tox.	tox.	tox.	tox.	tox.	tox.
	0.5	tox.	tox.	tox.	tox.	tox.	636 ± 31	342 ± 21	656 ± 36
	0.25	6 ± 2	55 ± 8	tox.	104 ± 21	tox.	800 ± 41	468 ± 31	580 ± 37
	0.1	9 ± 3	63 ± 9	106 ± 18	160 ± 18	109 ± 21	796 ± 33	491 ± 41	734 ± 41
	0.05	12 ± 4	102 ± 11	102 ± 12	204 ± 24	75 ± 15	780 ± 29	432 ± 29	828 ± 42
	0.025	19 ± 4	104 ± 13	102 ± 12	160 ± 18	96 ± 18	762 ± 24	314 ± 25	640 ± 39
	0.01	16 ± 4	90 ± 9	134 ± 14	198 ± 24	98 ± 18	784 ± 22	328 ± 24	632 ± 28
DMSO		16 ± 4	16 ± 4	160 ± 31	160 ± 31	140 ± 21	140 ± 21	410 ± 15	410 ± 15
Nor-HN <sub>2</sub>				968 ± 41					
DNOC	0.1	160 ± 46				430 ± 62			
AAF	0.05		470 ± 32				1320 ± 34		
AF	0.1				1298 ± 36				2461 ± 110
MMS	0.1							1268 ± 52	

Toxicity to the bacteria was observed at the higher concentrations, in all strains used, summarised in the Table 6.8.1-73b.

**Table 6.8.1-73b: Summary of cytotoxicity response to strains of Salmonella**

	Salmonella strain							
Dose (mg/plate)	TA98		TA100		TA97		TA104	
+/-S9	-	+	-	+	-	+	-	+
0.01	/	/	/	/	/	/	/	/
0.025	/	/	/	/	/	/	/	/
0.05	/	/	/	/	/	/	/	/
0.1	/	/	/	/	/	/	/	/
0.25	/	/	Tox	/	Tox	/	/	/
0.5	Tox	Tox	Tox	Tox	Tox	/	/	/
1.0	Tox	Tox	Tox	Tox	Tox	Tox	Tox	Tox

/ = not cytotoxic, Tox = cytotoxic

The positive controls for each experiment induced the expected responses, indicating the strains were responding satisfactorily in each case. Revertant colony numbers for the solvent control plates were within acceptable ranges.

## CONCLUSION:

In a publication from 1987, 2,4,6-TCP was positive in an Ames test in strains TA97 and TA104 in the presence of metabolic activation. Cytotoxicity was observed at the top concentration of 1 mg/plate. HSE notes that only four Salmonella strains (TA98, TA100, TA97 and TA104) were used. HSE also notes that the results of this study are inconsistent with the findings of other Ames tests.

## REFERENCES:

Ames B, McCann J and Yamasaki E (1975). *Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res., 31:347-364.*

(Strobel K and Grummt T 1987)

<b>Report:</b>	K-CA 5.8.1/24 Ono Y. <i>et al.</i> , (1996). DNA Damaging Properties of Active Oxygen from Organochlorine Compounds and Nitroarenes. University of Okayama, Okayama, Japan. Published: Ono Y, Kobayashi U, Somiya I, Nunoshiba T, Oda Y (1996). DNA damaging properties of active oxygen from organochlorine compounds and nitroarenes. Mizu Kankyo Gakkaishi, (1996) 19:871-877. Syngenta File No. NA_13792.
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**STUDY TYPE:** Reverse Mutation Test Using Bacteria.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**The original publication was in Japanese and a translation to English obtained.**

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE:** 3

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

Test compounds described in the literature as non-mutagenic carcinogens were analysed for their ability to cause oxidative DNA damage. 2,4,6-trichlorophenol (2,4,6-TCP) was one of the compounds tested.

2,4,6-TCP was tested for its ability to induce the DNA repair gene *umuC* in a transformed *Salmonella typhimurium* TA1535/ pSK1002 *umuC*'-'*lacZ* strain in the presence and absence of the bacterial catalase HP1. The ability of 2,4,6-TCP to produce  $O_2^-$  was analysed in an *E.coli* detector strain.

2,4,6-TCP did not induce the DNA repair gene *umuC* in the transformed *Salmonella typhimurium* TA1535/pSK1002 *umuC*'-'*lacZ* . Only in the catalase deleted strain (OY413 strain), 2,4,6-TCP tested weakly positive. 2,4,6-TCP induced expression of the  $O_2^-$  sensitive gene *soxS* in the *E.coli* detector strain.

**In conclusion, 2,4,6-TCP induced the production of  $O_2^-$  in an *E.coli* detector strain. 2,4,6-TCP tested negative for the induction of the repair gene *umuC* in *Salmonella typhimurium* TA1535. Only when the bacterial strain was tested without catalase expression, 2,4,6-TCP was tested weakly positive for the induction of the DNA repair gene *umuC*.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated.
<b>Lot/Batch number:</b>	Not indicated.
<b>Purity:</b>	Not indicated.
<b>CAS#:</b>	Not indicated.
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	None
<b>Solvent control (final concentration):</b>	Methanol
<b>Positive control:</b>	None

**Mammalian metabolic system: S9 derived**

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol and 5,6-benzoflavone		Mouse		Lung
			None		Hamster		Other
			Other $\beta$ -naphthoflavone		Other		

**Test organisms:**

- *Umu* test: *Salmonella typhimurium* TA1535/pSK1002 *umuC'*-*'lacZ*
- *Umu* test in catalase deficient mutant (OY413) strain: *Salmonella typhimurium* TA4113/pSK1002 *umuC'*-*'lacZ* deficient in *katG*
- *soxRS* induction detection method (TN strain): *Escherichia coli*/lambda-TN 1520 *soxS'*-*lacZ*

**Test compound concentrations used**

*Umu* test: 300 mg/L, with and without S9

*Umu* test in catalase deficient mutant strain (OY strain): 100 and 300 mg/L, without S9 only

*soxRS* induction detection method (TN strain): 42 mg/L, without S9 only

**TEST PERFORMANCE****Preliminary cytotoxicity assay:**

Not specified.

**Protocol:*****umu* test:**

Test compounds were tested for their ability to induce the DNA repair gene *umu* in *Salmonella typhimurium* TA1535/pSK1002 *umuC'*-*'lacZ* and in *Salmonella typhimurium* TA1535/pSK1002 *umuC'*-*'lacZ* where the catalase gene *katG* was deleted.

The overnight culture of the tester bacterial strains were diluted 50-fold into TGA medium and incubated at 37°C until the bacterial density reached OD<sub>600</sub> = 0.25-0.3. The bacterial culture was subdivided into 2.4-mL portions in test tubes and 0.1 mL of the test compound was added to each tube. Then, either 0.5 mL of 0.1 M phosphate buffer (pH 7.4) or S9 mixture containing 50µL of S9 microsomal fraction for metabolic activation was added. After 2 h of incubation at 37°C with shaking, the level of  $\beta$ -galactosidase activity in the cells was assayed. The bacterial density was measured at OD<sub>600</sub>. Fractions (0.2 mL) of the culture were diluted with 1.8 mL of Z buffer, and the bacterial cells were made permeable to the chromogenic substrate for  $\beta$ -galactosidase by adding one drop of toluene and mixing vigorously. After the toluene was evaporated by shaking at 37°C for 40 min with the top open, the enzyme reaction was initiated by the addition of 0.2 mL of 2-nitrophenyl- $\beta$ -D-galactopyranoside solution (4 mg/mL in 0.1 M phosphate buffer, pH 7.0) at 28°C. After 10-20 min, the reaction was stopped by adding 1 mL of 1 M Na<sub>2</sub>CO<sub>3</sub>, and the absorbance at OD<sub>420</sub> and OD<sub>550</sub> was measured. The unit of enzyme activity was calculated.

***soxRS* induction detection method (TN strain)**

*Escherichia coli* expressing the *soxS'-lacZ* gene inserted into the chromosome where treated with 2,4,6-TCP. *SoxS* is a gene induced by  $O_2^-$ . The experimental methods used were almost the same as for the *umu* test. In this method, streptomycin (20 mg·L<sup>-1</sup> medium) was used as antibiotic and the medium used for culturing and pre-culturing was LB medium in both cases. Pre-culturing was performed for about 1 h until OD<sub>600</sub> = 0.1. After pre-culturing, 0.5 mL of sodium phosphate buffer was added instead of S9 mix. The reaction time was 1 h and β-galactosidase activity was measured after the end of the reaction.

**Statistical analysis:**

Statistical analysis was not performed.

**Evaluation criteria:** The genotoxicity potency was calculated according to the following formula:

$$\text{Genotoxic potency} = (A-B)/B$$

A = enzyme activity of test substance

B = enzyme activity of control.

A positive results was defined as the concentrations for which the calculation (A-B)/B was in excess of the assessment criterion of 1.0

**RESULTS**

**Preliminary cytotoxicity assay:** Data not presented

***Umu* test:** 2,4,6-TCP was tested for its ability to induce the DNA repair gene *umuC* in the detector strain *Salmonella typhimurium* TA1535 expressing a *umuC'-lacZ* fusion gene. 2,4,6-TCP was tested negative in this test.

**Catalase deficient mutant strain (OY strain) test:** 2,4,6-TCP was tested for its ability to induce DNA damage involving H<sub>2</sub>O<sub>2</sub> in a *Salmonella typhimurium* TA1535 strain expressing the *umuC'-lacZ* fusion gene and deletion of the catalase gene *katG*. 2,4,6-TCP was tested weakly positive in this assay.

***soxRS* induction detection method:** 2,4,6-TCP was tested for its potential to induce  $O_2^-$  formation. 2,4,6-TCP induced  $O_2^-$  production in the TN strain with a (A-B)/A ration of 4.69.

**CONCLUSION:**

In a publication from 1996, 2,4,6-TCP induced the production of  $O_2^-$  in an *E.coli* detector strain. 2,4,6-TCP tested negative for the induction of the repair gene *umuC* in *Salmonella typhimurium* TA1535. Only when the bacterial strain was tested without catalase expression, 2,4,6-TCP was weakly positive for the induction of the DNA repair gene *umuC*. HSE notes that this is a non-standard test providing little information on the mutagenicity of 2,4,6-TCP in bacteria.

(Ono Y *et al*, 1996)

<b>Report:</b>	K-CA 5.8.1/25 Ozaki A, <i>et al.</i> , (2004) Chemical Analysis and Genotoxicological Safety Assessment of Paper and Paperboard used for Food Packaging. Osaka City Institute of Public Health and Environmental Sciences, 8-34 Tojo-cho, Tennouji-ku, Osaka 543-0026, Japan. Published. Ozaki A, Yamaguchi Y, Fujita T, Kuroda K and Endo, G (2004). Chemical analysis and genotoxicological safety assessment of paper and paperboard used for food packaging. Food and Chemical Toxicology; 42: 1323-1337. Syngenta File No. NA_13777.
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**STUDY TYPE:** *in vitro* Comet Assay and Rec-Assay

**TEST MATERIAL (PURITY):** 2,4,6-TCP (>97%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE:** 2

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

2,4,6-Trichlorophenol (2,4,6-TCP) was found to be one of the potential contaminants in recycled and virgin papers.

The genotoxic potential of 2,4,6-TCP was investigated using a *Rec*-assay in *Bacillus subtilis* and via an *in vitro* comet assay in HL-60 cells.

The study also performed a *Rec* assay using *Bacillus subtilis* with 2,4,6-TCP. It was found that 2,4,6-TCP did not have a cytotoxic effect on the cells and also that the killing zone in M45 *Rec*<sup>-</sup> was larger compared to H17 *Rec*<sup>+</sup>, leading to the authors to conclude that this was a positive response for damage to DNA.

In the comet assay, there was a significant increase ( $p < 0.01$ ) in the number of highly damaged cells with respect to the negative control at 50 and 100 µg/mL. The authors concluded that this is a genotoxic response.

**Under the conditions reported in this paper, 2,4,6-TCP gave a positive genotoxic response in both the *Rec*-assay in *Bacillus subtilis* and *in vitro* comet assay in HL-60 cells.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	> 97 %
<b>CAS#:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated

### *Rec* Assay

<b>Control Materials:</b>	
<b>Negative control:</b>	DMSO
<b>Positive control:</b>	2-(2-furyl)-3-nitro-2-furyl acrylamide (0.03 µg/disc)

**Mammalian metabolic system:** Not reported

### Test organisms:

Recombinationless strain: *Bacillus subtilis* M45 *Rec*<sup>-</sup>

Wild type strain: *Bacillus subtilis* H17 *Rec*<sup>+</sup>  
Origin of strains: National institute of Genetics

**Test compound concentrations used:** 100, 50 and 6 µg/disc

### Study Design and Methods:

#### Protocol:

A recombinationless strain, M45 *Rec*<sup>-</sup> and the wild strain, H17 *Rec*<sup>+</sup> of *Bacillus subtilis* were used. Briefly plates were prepared by adding to B-2 (10 g beef extract, 10 g polypeptone, 5 g NaCl, 1000 mL water; adjusted to pH 7.0) agar (1.5%) 2 x 10<sup>5</sup> spores/mL of strain H17 and M45, preparing 10 mg of the resulting spore agar at 42°C, pouring it into a level plastic petri dish and allowing it to solidify. A paper disc, impregnated with 30 µL of the chemical or sample solution was then placed on the surface. After 24 h of incubation at 37°C, the diameters of the inhibition zones were measured. Chemicals were dissolved in DMSO. To prepare samples, 1 mL of *n*-hexane solution before trimethylsilylation, was dried under a nitrogen stream and dissolved in 100 µl of DMSO.

**Statistical analysis:** No statistical analysis was reported for the *Rec* assay.

#### Comet assay

##### Control Materials:

Negative control:	DMSO (10 µL)
Positive control:	MNNG

**Mammalian metabolic system:** Not reported

#### Test cell line:

Cells: HL-60 from human promyelocytic leukemia cell line  
Origin of strains: Not indicated

**Test compound concentrations used:** 50 and 100 µg/mL

### Study Design and Methods:

#### Protocol:

HL-60 cells were grown in RPMI-1640 media supplemented with 7% fetal bovine serum and cultured in an incubator at 37°C in a 5% humidified CO<sub>2</sub> atmosphere. The cells (1 mL) were grown in 12-well plates at a density of 1x10<sup>6</sup> cells/well for 18 h. The standard compound (10 µL) in DMSO was then added and incubated. Control cells received an equal volume of DMSO alone. After 2 h, a 200 µL aliquot was removed to measure acute cytotoxicity using trypan blue dye exclusion (TBDE) test. The remaining suspension from each well was centrifuged at 4°C, washed with PBS and suspended in a small volume of PBS. An aliquot of 10 µL of 0.5% low-melting agarose kept at 42°C and rapidly spread on microscope slides, which were incubated at 4°C for 10 min to accelerate the gelling of the agarose and then transferred to a pre-chilled lysis solution (2.5M NaCl, 100 mM EDTA, pH 7.5, 10% DMSO and 1 % Triton-X-100, added fresh) for 30 min at 4 °C. A denaturation step was then performed in alkali solution (0.3M NaOH, 1mM EDTA) for 20 min and electrophoresis was conducted for the next 25 min at 25V, 300mA. The slides were then transferred to 0.4M Tris, pH 7.5 for 15 min to neutralise after which they were fixed in ice-cold 100 % ethanol for 5 min and air-dried. For observation, samples were stained with SYBR green diluted 1:10000 in TE buffer (1mM EDTA, 10mM Tris, pH 7.5). All steps were conducted under dimmed light to prevent additional DNA damage. The slides were observed at 400 x magnification under a confocal laser scanning microscope. Images of 200 randomly selected cell



samples were classified into four categories for qualitative evaluation: undamaged cells without tail (UCs), cells with tiny tail (slightly damaged cells: SDCs), cells with clear tail (damaged cells: DCs) and cells with teardrop-shaped tail (highly damaged cells (HDCs) (Fig. 6.8.1-3).

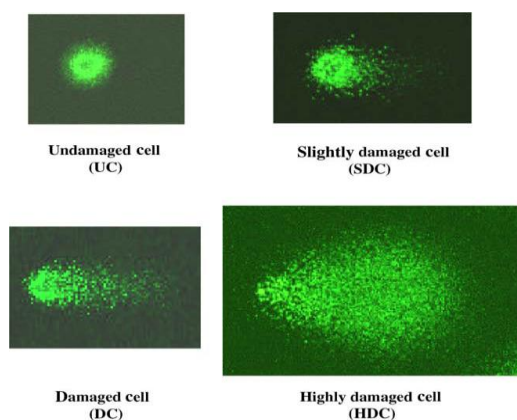


Fig. 6.8.1-3: Photomicrographs of UC, SDC, DC and HDC observed after comet assay

**Statistical analysis:** Statistical analysis was performed of each type of comet cell. For assessment a  $\chi^2$  test was performed. A  $p$ -value  $<0.01$  with respect to the solvent control was considered significant.

## RESULTS AND DISCUSSION

### *Rec<sup>-</sup>* assay

2,4,6-TCP was found to induce a larger killing zone in the M45 *Rec<sup>-</sup>* compared to M45 *Rec<sup>+</sup>* at 100 and 50  $\mu\text{g}/\text{disc}$  with differences in killing zones of 6mm and 3 mm respectively. The authors interpreted this as a positive result and that under the conditions of this assay 2,4,6-TCP induced DNA damage in *Bacillus subtilis*.

### *Rec<sup>-</sup>*-assay



### Comet assay

Under the conditions of the comet assay described in this study 2,4,6 TCP was found to significantly increase the number of HDC at 50  $\mu\text{g}/\text{mL}$  dose (1.1% in negative control vs. 16.3%;  $p<0.01$ ) and the number of HDC at 100  $\mu\text{g}/\text{mL}$  (1.1% in negative control vs. approximately 40%;  $p<0.01$ ). Increases in HDC incidence were associated with a concentration dependant, but not statistically significant, elevation in cell death. No significant change in SDC or DC incidence was observed at any concentration (Fig. 6.8.1-4).

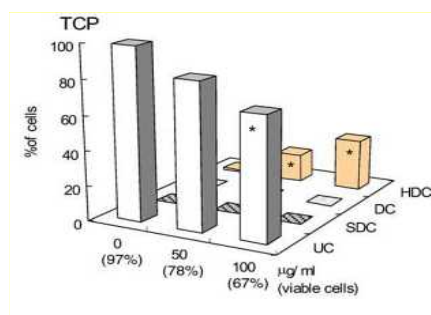


Fig. 6.8.1-4: Comet assay and trypan blue dye exclusion (TBDE) test in HL-60 cells exposed to standard compounds. The percentage of comet cells in each treatment based on random counting of 200 cells is classified

into four grades: undamaged cells (UC), slightly damaged cells (SDC), damaged cells (DC) and highly damaged cells (HDC). \*  $p < 0.01$ , compared to solvent control by  $\chi^2$  test.

## CONCLUSION:

In a publication from 2004, the genotoxic potential of 2,4,6-TCP was investigated using a *Rec*-assay in *Bacillus subtilis* and *via* an *in vitro* comet assay in HL-60 cells. It was found that 2,4,6-TCP did not have a cytotoxic effect on the cells and also that the killing zone in M45 *Rec*<sup>-</sup> was larger compared to H17 *Rec*<sup>+</sup>, leading to the authors to conclude that this was a positive response for damage to DNA. In the comet assay, there was a significant increase ( $p < 0.01$ ) in the number of highly damaged cells with respect to the negative control at 50 and 100 µg/mL. The authors concluded that this is a genotoxic response.

Overall, 2,4,6-TCP gave a positive genotoxic response in both the *Rec*-assay in *Bacillus subtilis* and *in vitro* comet assay in HL-60 cells. HSE notes that these are non-standard tests and that insufficient information is available in the publication to judge the reliability of the reported findings.

(Ozaki A *et al*, 2004)

<b>Report:</b>	K-CA 5.8.1/26 Fahrig R. <i>et al.</i> , (1978). Genetic Activity of Chlorophenols and Chlorophenol Impurities. Zentrallaboratorium für Mutagenitätsprüfung der Deutschen Forschungsgemeinschaft, 78 Freiburg i. Br., West Germany. Published: Fahrig R, Nilsson C and Rappe C (1978). Genetic Activity of Chlorophenols and Chlorophenol Impurities. Environmental Science Research, Vol. 12, pp 325-338. Syngenta File No. NA_13765.
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**STUDY TYPE:** Genetic toxicity assay in *Saccharomyces cerevisiae* and Mammalian Spot test *in vivo*.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (99%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 2 for *Saccharomyces cerevisiae* assay; 3 for Mammalian spot test**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

2,4,6-Trichlorophenol (2,4,6-TCP) was tested *in vitro* with the multipurpose strain MP-1 of *Saccharomyces cerevisiae*, (suitable for simultaneous detection of forward mutations, intergenic recombinations (mitotic crossing over), and intragenic recombinations (mitotic gene conversion)) and *in vivo* in a mouse spot test.

2,4,6-TCP showed very weak but statistically significant activity in the *S. cerevisiae* mutation system at a concentration of 400 mg/L. 2,4,6-TCP did not cause an increase in intergenic or intragenic recombination. Evaluation criteria for this positive response were not included in the text.

After intraperitoneal injection of 2,4,6-TCP to dams on day 10 of gestation, a positive increase in the number of coloured spots on the coats of offspring was observed, indicative of a mutagenic effect. Evaluation criteria for this positive response were not included in the text.

**Under the conditions of the studies reported, 2,4,6-TCP was active in the *S.cerevisiae* mutation assay *in vitro*, as well as the mouse spot test *in vivo*. 2,4,6-TCP therefore demonstrated mutagenic potential both *in vitro* and *in vivo*.**

## MATERIALS AND METHODS

### Materials:

Test Material:	2,4,6-TCP
Description:	Not indicated
Lot/Batch number:	Not indicated
Purity:	99%
CAS#:	88-06-2
Stability of test compound:	Not indicated

**Vehicle control:** Not included, 2,4,6-TCP added directly to test medium

### Study Design and Methods:

**In-life dates:** Not indicated

### *In Vitro* Test with *Saccharomyces cerevisiae* MP-1:

#### Yeast strain

The diploid multipurpose strain MP-1 was used which allows for screening of intergenic recombination (mitotic crossing over), intra genic recombination (mitotic gene conversion) and forward mutation.

#### Media

Synthetic complete medium: The medium is prepared by adding to Bacto yeast nitrogen base without amino acids the following ingredients per liter of medium: L-Arginine-HCl (10 mg), L-Aspartic acid (10 mg), L-Glutamic acid (100 mg), L-Leucine (60 mg), L-Lysine-HCl (10 mg), L-Methionine (10 mg), L-Phenylalanine (50 mg), L-Serine (20 mg), L-Tyrosine (30 mg), L-Valine (30 mg) and Uracil (10 mg).

According to the system used, the following ingredients are added or not added per liter of medium: Adenine sulfate (10 mg), L-Histidine-HCl (10 mg), L-Tryptophan (10 mg), Actidione (1 mg).

Liquid complete medium: The liquid medium YEP is composed of 2% Bacto peptone, 1% yeast extract and 2% glucose.

#### Culturing of Yeast Cells

About  $10^3$  yeast cells are inoculated in a 300 mL Erlenmeyer flask containing 100 ml YEP, set on a shaker and allowed to grow for 3 days at 25°C into the stationary phase. For the detection of the spontaneous frequency of intragenic recombination, the cultures of strains are stored at 4°C for 3-4 days. During this time their spontaneous frequency is determined by spreading 0.1 mL from the YEP/yeast suspension on solid media selective for intragenic recombinants. Only cultures with a low spontaneous frequency are used for the experiments. The cultures needed for one experiment are mixed together in order to obtain a similar spontaneous frequency of genetic alterations in each of the experiments.

### *In Vitro* Test

The cell cultures are washed twice with distilled water and the cell titers are adjusted to  $3 \times 10^8$  cells per mL of 0.1 M phosphate buffer, pH 7. These cell suspensions are incubated in a test tube on a shaker at 25°C with different concentrations of the test substances. At defined time intervals treatments are stopped, and  $4 \times 0.1$  mL of the suspensions containing  $3 \times 10^7$  cells are spread on four plates of a solid nutrient-deficient media, selective for intragenic recombinants and mutants, respectively. Similarly, after 5 dilutions 1:10 in distilled water suspensions of  $3 \times 10^2$  cells are plated out on ten plates of complete medium to attain the number of survivors (white colonies) and intergenic recombinants (red colonies or sectors). These cultures are incubated at 25°C, and the survivor and recombinant colonies are counted after 4 days. Actidione-resistant colonies are incubated 8 days.

Treatment time was 3, 5 hours for all experiments

**Test compound concentrations used:**

2,4,6-TCP                                      400 mg/L

**Statistical Analysis and Evaluation Criteria:** Not indicated

***In vivo* Mouse Spot Test**

<b>Control Materials:</b>			
<b>Negative control (if not vehicle) :</b>	N/A	<b>Final Volume:</b> N/A	<b>Route:</b> N/A
<b>Vehicle:</b>	Dimethylformamide (DMF)	<b>Final Volume:</b> 0.1 mL/ 10 g bw	<b>Route:</b> IP
<b>Positive control :</b>	N/A	<b>Final Doses:</b> N/A	<b>Route:</b> N/A

<b>Test Animals:</b>	
<b>Species</b>	Mouse
<b>Strain</b>	Embryos of the genotype <i>a/a</i> ; <i>b/+</i> ; <i>c<sup>ch</sup>p/+</i> ; <i>d se/+</i> ; <i>s/+</i> (black coat, dark eyes) are produced by mating about ten weeks old virgin females of the inbred C57BL/6JHan strain ( <i>a/a</i> ; otherwise wild type) to fertile males of the rotation bred T-stock ( <i>a/a</i> = nonagouti; <i>b/b</i> = brown; <i>c<sup>ch</sup>p/c<sup>ch</sup>p</i> = chinchilla and pink-eyed dilution; <i>d se/d se</i> = dilute and short ear; <i>s/s</i> = piebald spotting).
<b>Age/weight at dosing</b>	Not indicated
<b>Source</b>	Bred in house
<b>Housing</b>	Not indicated
<b>Acclimatisation period</b>	Not indicated
<b>Diet</b>	Not indicated
<b>Water</b>	Not indicated
<b>Environmental conditions</b>	Not indicated

These embryos which are heterozygous for four different recessive coat-colour genes are treated in utero during the tenth day of foetal development by injection of the mutagen into the peritoneal cavity of the mother animal. If this treatment leads in a pigment precursor cell to an alteration of the wild type allele of one of the genes under study or to its loss, a colour spot in the adult coat can be seen.

**Test compound administration:**

	Dose Level	Final Volume	Route
2,4,6-TCP	50 and 100 mg/kg	0.1 mL/10g bw	IP

2,4,6-TCP was administered intraperitoneally in 10% dimethylformamide (DMF). The concentration of 2,4,6-TCP in the stock solutions allows the intraperitoneal injection of 0.1 mL of the solution per 10 g body weight.

### Experimental Design

Treatment	Dose	Time of dosing	Number of Dams	No. of offspring examined for coat spots
Vehicle control (DMF)	0	10 <sup>th</sup> day of gestation	152	967
2,4,6-TCP	50 mg/kg		36	181
2,4,6-TCP	50 mg/kg		38	159
2,4,6-TCP	100 mg/kg		42	175

### Time of Observation

The first observation for colour spots on the coat of offspring is made at the end of the second week of age, because at that time dark grey dorsal spots can be clearly distinguished from the black fur. At a later age, these spots can be missed. On the other hand, spots in ventral regions are sometimes visible only at a later age, probably owing to the hair not yet being full length at two weeks of age. It is, therefore, checked for colour spots twice a week between two and five weeks of age.

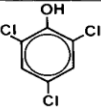
**Statistical Analysis and Evaluation Criteria:** Not indicated

## RESULTS

### Induction of Forward Mutation, Intragenic and Intergenic Recombination in *S. cerevisiae* MP-1 *in Vitro*

2,4,6-TCP showed very weak but statistically significant activity in the mutation system at a concentration of 400 mg/L. 2,4,6-TCP did not cause an increase in intergenic or intragenic recombination. Evaluation criteria for this positive response were not included in the text.

**Table 6.8.1-74a: Induction of forward mutation, intragenic and intergenic recombination in *S. cerevisiae* MP-1 in vitro with 2,4,6 trichlorophenol at a treatment time of 3.5 hours**

Substance	Genetic alteration	Experiment				Control		
		N <sup>a</sup>	Concentration (mg/l)	Survival <sup>b</sup> (%)	Colonies <sup>c</sup> of genetically altered cells per survivors	N <sup>a</sup>	Colonies <sup>c</sup> of genetically altered cells per survivors	(P)
	mutation	4	400	95 ± 16 (12487)	10.29 ± 2.75 (1249)	4	5.63 ± 0.91 (719)	< 0.02
	intergen. rec.				0.44 ± 0.07 (54)		0.44 ± 0.06 (58)	> 0.9
	intra-gen. rec.				10.63 ± 3.16 (1298)		7.59 ± 1.12 (974)	> 0.1

<sup>a</sup>Number of experiments  
<sup>b</sup>Survival control = 100%  
<sup>c</sup>Mutants, convertants (intragenic recombination)/10<sup>7</sup> survivors, recombinants (intergenic recombination)/10<sup>8</sup> survivors.  
 The numbers in parenthesis give the actual numbers of colonies counted.

### Spot Test with Mice *in vivo*

After intraperitoneal injection of 2,4,6-TCP to dams on day 10 of gestation (genetic cross described above), a positive increase in the number of coloured spots on the coats of offspring was observed, indicative of a mutagenic effect. Evaluation criteria for this positive response were not included in the text.

**Table 6.8.1-74b: Evaluation of mouse spot test results**

Chemical	Dose (mg/kg)	Females treated	Offspring survived to observation	Animals with spots of questionable genetic relevance			Animals with spots of genetic relevance			
				White	White-grey	Other	Light Grey	Grey	Light brown	Brown
Control	0	152	967	4	1	-	-	-	1	-
2,4,6-TCP	50	36	181	2	1	-	1	-	-	-
	50	38	159	-	3	-	1	-	-	-
	100	42	175	-	-	-	-	1	-	-

It is not clear which genetic alteration is responsible for the expression of a heterozygous recessive gene(s).

A reduction of litter-size and an increased loss of litters before and after birth were also observed.

#### CONCLUSION:

In a publication from 1978, 2,4,6-TCP was tested in vitro with the multipurpose strain MP-1 of *Saccharomyces cerevisiae*, (suitable for simultaneous detection of forward mutations, intergenic recombinations (mitotic crossing over), and intragenic recombinations (mitotic gene conversion)) and in vivo in a mouse spot test. 2,4,6-TCP showed very weak but statistically significant activity in the *S. cerevisiae* mutation system at a concentration of 400 mg/L. 2,4,6-TCP did not cause an increase in intergenic or intragenic recombination. Evaluation criteria for this positive response were not included in the text.

After intraperitoneal injection of 2,4,6-TCP to dams on day 10 of gestation, a positive increase in the number of coloured spots on the coats of offspring was observed, indicative of a mutagenic effect. Evaluation criteria for this positive response were not included in the text.

Overall, 2,4,6-TCP was active in the *S.cerevisiae* mutation assay in vitro, as well as the mouse spot test in vivo. 2,4,6-TCP therefore demonstrated mutagenic potential both in vitro and in vivo. HSE notes that these are non-standard tests and that insufficient information is available in the publication to judge the reliability of the reported findings.

(Fahrig R *et al.*, 1978)

<b>Report:</b>	K-CA 5.8.1/27 Russell L.B. <i>et al.</i> , (1981). Use of the mouse spot test in chemical mutagenesis: Interpretation of past data and recommendations for future work. Biology and information Division, Oak Ridge national Laboratory, Oak Ridge, TN 37830. Published: Russell L.B; Selby P.B; Von Halle E; Sheridan W; Valcovic L; (1981). Use of the mouse spot test in chemical mutagenesis: Interpretation of past data and recommendations for future work. Mutagenesis Research 86:355-379. Syngenta File No. NA_13778.
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**STUDY TYPE:** Re-evaluation of existing data.

**TEST MATERIAL (PURITY):** Not applicable.

**GLP:** Not applicable for an evaluation of existing data.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

#### EXECUTIVE SUMMARY

Evaluations of existing data on the mouse spot test (including data on 2,4,6-TCP reported in Fahrig *et al.*, (1978)) identified inadequacies in study design and reporting of experiments and conclude that a carefully specified protocol and consistent reporting is required.

(Russell L.B.. *et al.*, 1981)

<b>Report:</b>	K-CA 5.8.1/28 Valencia. <i>et al.</i> , (1985). Chemical Mutagenesis Testing in Drosophila. III. Results of 48 Coded Compounds Tested for the National Toxicology Program. Department of Zoology, University of Wisconsin, Madison. Published: Valencia R, Mason J, Woodruff R, Zimmering S (1985). Chemical Mutagenesis Testing in Drosophila. III. Results of 48 Coded Compounds Tested for the National Toxicology Program. Environmental Mutagenesis 7:325-348. Syngenta File No. NA_13773.
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**STUDY TYPE:** Mutagenesis test in Drosophila.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (Practical)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

#### EXECUTIVE SUMMARY

To test the potential for 2,4,6-trichlorophenol (2,4,6-TCP) to induce sex linked recessive lethals (SLRLs), 2,4,6-TCP was administered to Drosophila both in the diet (250 ppm) and injection (10000 ppm).

2,4,6-TCP did not increase the number of SLRLs when administered via either route.

**Under the conditions of the study reported, 2,4,6-TCP did not induce sex linked recessive lethals in Drosophila, after administration via feed or injection. 2,4,6-TCP was therefore non-mutagenic in the test described.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Practical
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	Ethanol
<b>Solvent control (final concentration):</b>	10%
<b>Positive control:</b>	None

### Test organisms: *Drosophila*

The *Drosophila* stocks, genetic crosses, and testing protocols have been described previously (Woodruff *et al*, 1984 and Zimmering *et al*, 1985).

### Test compound concentrations used:

Dietary administration	250 ppm
Injection	10000 ppm

### Study Design and Methods:

**In-life dates:** Not indicated

### Test performance:

#### Dose-Finding Studies

Careful range-finding studies were carried out with 2,4,6-TCP, including tests for solubility, toxicity, ingestion, and male sterility. Any one or more of these factors was used to limit the concentration of the test material administered to the flies. The solvent of choice was 10% ethanol.

Initial solutions were diluted with aqueous 5% sucrose for feeding and with aqueous 0.7% NaCl for injection. Toxicity tests were performed both for feeding and injection treatments using a series of concentrations of the chemical. If toxicity was observed, an attempt was made to choose a concentration that induced mortality of about 30% after 72 h of feeding or 24 h after injection.

#### Treatments

The males to be treated were collected from a Canton-S wild-type stock within 8 h of emergence and kept on regular culture medium until exposure. Males to be fed were shaken into vials containing glass fiber filter material that was soaked with the feeding solution or mixture. At 24 hr and again at 48 h, the flies were transferred to vials with freshly prepared feeding mixture and were mated at 72 h. Males to be injected were held on regular food for 1-3 days. They were then injected with 0.7% NaCl solution containing the test chemical. They were held for 24 h to recover and then mated.

#### Test Procedures

For the SLRL assay, treated and control males were mated individually to three harems of *Basc* virgin females to produce three broods of 3, 2, and 2 days. Thus, primarily post-meiotic germ cells were tested. To reduce the chances of recovering several lethals from the same male, no more than 40 F<sub>1</sub> females were mated individually from each brood of each male. Thus, no more than 120 chromosomes were



tested from each P<sub>1</sub> male. F<sub>2</sub> cultures were scored as presumptive lethals if the number of wild-type males recovered was 0, 1, or fewer than 5 % of the number of *Basc* males (or *Basc* + females). Presumptive lethals were confirmed by repeating the matings and scoring the F<sub>3</sub>. Semi-lethals (5% or fewer of the expected number of wild-type males) were counted as lethals, but fractional lethals, identified in the confirmation crosses, were not counted, since the experiments were not designed to detect all of them.

### Statistical Methods:

In the SLRL assay, when more than two lethals were observed from a single P<sub>1</sub> male, the multiple was compared to the expectation based on a Poisson distribution. When the expectation was less than 0.01, the multiple was assumed to be due to spontaneous mutation in a gonial cell, resulting in a “cluster” of sperm cells bearing the same mutation. In these cases, the lethal and non-lethal tests for that P<sub>1</sub> male were removed from the data. The corrected data were then tested for significance using the normal test as suggested by Margolin (Margolin *et al*, 1983). A SLRL result was called negative if  $P \geq 0.06$ , equivocal if  $P = 0.04-0.06$ , either questionable or positive if  $P = 0.01-0.04$  (depending on the control frequency), and positive if  $P \geq 0.01$ . Generally, a test was considered positive if the frequency of lethals in the treated series exceeded 0.2% over the control frequency.

## RESULTS

After administration of 2,4,6-TCP via feed and injection to *Drosophila*, no increase in the number of sex linked recessive lethals was observed up to concentrations of 250 ppm (feed) and 10000 ppm (injection).

**Table 6.8.1-75: Results of 2,4,6-TCP administration to drosophila and induction of recessive lethals**

Dose ppm	ROA <sup>a</sup>	Mortality <sup>b</sup>	Sterility <sup>c</sup>	Lethals : brood			Tests : brood			Total lethals	Total tests	Lethals
250	Feeding	4	85	1	3	5	3520	3266	3226	9	10012	0.09
0				3	2	1	2549	2482	2320	6	7351	0.08
10000	Injection	0	0	1	1	5	3782	3574	3535	7	10891	0.06
0				3	2	0	3074	2817	2598	5	8489	0.06
Clusters: one cluster of 66 in the treated injection experiment												

<sup>a</sup>ROA, route of administration.

<sup>b</sup> Mortality and sterility are shown as induced frequencies, ie, the control frequencies have been deducted.

<sup>c</sup>The lethal data are shown as observed along with the concurrent controls. Both treated and control data have been corrected for spontaneous clusters as indicated below the data.

### CONCLUSION:

In a publication from 1985, 2,4,6-TCP was tested for its ability to induce sex linked recessive lethals (SLRLs) in *Drosophila* both in the diet (at 250 ppm) and by injection (10000 ppm). 2,4,6-TCP did not increase the number of SLRLs when administered via either route. HSE notes that these *Drosophila* tests are no longer accepted for regulatory purposes.

### REFERENCES:

Margolin B, Collings B, Mason J (1983). *Statistical analysis and sample size determinations for mutagenicity experiments with binomial responses. Environ Mutagen* 5:705-716.

Woodruff R, Mason J, Valencia R, Zimmering S (1984). *Chemical mutagenesis testing in Drosophila. I. Comparison of positive and negative control data for sex-linked recessive lethal mutations and reciprocal translocations in three laboratories. Environ Mutagen* 6:189-202.

Zimmering S, Mason J, Valencia R, Woodruff R (1985). Chemical mutagenesis testing in *Drosophila*. II. Results of 20 coded compounds tested for the National Toxicology Program. *Environ Mutagen* 7: 87-100.

(Valencia R *et al*, 1985)

<b>Report:</b>	K-CA 5.8.1/29 Hattula M. and Knuutinen J. (1985). Mutagenesis of Mammalian Cells in Culture by Chlorophenols, Chlorocatechols and Chloroguaiacols. Department of Biology, University of Jyväskylä, SF-40100 Jyväskylä, Finland. Published: Hattula M and Knuutinen J (1985). Mutagenesis of mammalian cells in culture by chlorophenols, chlorocatechols and chloroguaiacols. <i>Chemosphere</i> 14: 1617-1625. Syngenta File No. NA_13766
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**STUDY TYPE:** *In Vitro* Mammalian Cell Gene Mutation Test

**TEST MATERIAL (PURITY):** 2,4,6-TCP (>99.95%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The mutagenic potential of 2,4,6-trichlorophenol (2,4,6-TCP) was investigated *in vitro* using a version of the Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) assay. The induction of 6-thioguanine resistant mutants was investigated in V79 cells using a direct method, and a hepatocyte mediated method.

In the direct method, a concentration range of 0 – 60 µg/mg 2,4,6-TCP was used. No metabolic activation system was used. 2,4,6-TCP caused an increase in the number of 6-thioguanine resistant mutants, indicative of mutagenic potential.

In the hepatocyte mediated method, at a single dose of 30 µg/ml 2,4,6-TCP, no increase in the number of 6-thioguanine resistant mutants was observed.

The positive controls induced an appropriate mutagenic response.

**Under the experimental conditions reported, 2,4,6-TCP showed mutagenic potential in V79 cells *in vitro*, when tested in the absence of metabolic activation.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	>99.95%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	
<b>Solvent control (final concentration):</b>	Acetone (Not specified)
<b>Positive control:</b>	MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), 0.5 and 1.0 µg/mg
	DMN (N-nitrosodimethylamine), 0.3, 1 and 3 mM

### Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells	X	Primary rat hepatocytes
<b>Media:</b> DMEM Leibovitz L-15 medium (Hepatocyte mediated assay)			
Properly maintained?	X	Yes	No
Periodically checked for Mycoplasma contamination?		Yes	No
Periodically checked for karyotype stability?		Yes	No
Periodically "cleansed" against high spontaneous background?		Yes	No

X indicates those that apply

<b>Locus Examined:</b>	<b>Thymidine kinase (TK)</b>	<b>Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)</b>	<b>Na<sup>+</sup>/K<sup>+</sup> ATPase</b>
Selection agent:	Bromodeoxyuridine (BrdU)	8-azaguanine (8-AG)	ouabain
	Fluorodeoxyuridine (FdU)	X 6-thioguanine (6-TG)	
	Trifluorothymidine (TFT)		

X indicates those that apply

### Test compound concentrations used:

Direct method	Absence of S9 mix	10, 20, 30, 45 and 60 µg/mg
Hepatocyte mediated method	Absence of S9 mix	30 µg/mL

### Study Design and Methods:

**In-life dates:** Not indicated

### Test performance:

#### Direct method:

In the direct method 10<sup>6</sup> V79 cells in 10 mL medium were seeded in a tissue culture dish (10 cm diameter) and the test compound was added 24 hr after the V79 cells in 1 ml of medium. The maximum amount of

acetone which was used as a solvent of the test compounds was 50 µl and it was added to controls, too. The cultures were incubated for 2 days and the cells were dissociated with trypsin-EDTA (0.05 and 0.1%) and seeded at 200 cells per 60 mm tissue culture dish in 5 mL of medium to determine the cloning efficiency and  $10^5$  cells per 10 mm dishes. 6 days later the cells were dissociated and reseeded for cloning efficiency, 200 cells per 60 mm dish, and  $2 \times 10^4$  cells per 60 mm dish in 4 mL of medium for determination of the number of 6-thioguanine resistant mutants. 6-thioguanine (final concentration 40 µM), was added in 1 mL of medium. The colonies were counted after Giemsa staining. Cloning efficiency was determined by counting the number of colonies in five dishes per point 7-8 days after cell seeding. The frequency of 6-thioguanine resistant mutants was determined by counting 16 dishes per point 12-14 days after cell seeding. MNNG (N-methyl-N'-nitro-N-nitrosoguanidine) was used as a positive control.

#### Hepatocyte-mediated assay

Primary hepatocytes of 2-3 months old male Sprague-Dawley rats were used. Only one concentration of 2,4,6-TCP was examined by this method, DMN (N-nitrosodimethylamine) was used as a positive control. V79 cells were seeded at  $5 \times 10^5$  cells/25 cm<sup>2</sup> T-flask. After 18 hr  $2 \times 10^6$  primary rat hepatocytes were seeded on the V79 cells in 4 ml Leibovitz L-15 medium containing 2 mM glutamine, 10% fetal calf serum, 100 IU penicillin/ml and streptomycin 100 µg/mL. After 3 h the medium was changed and 2,4,6-TCP was added in 4 mL of fresh medium. The cells were dispersed 18 hr later with 0.05% trypsin and 0.02% EDTA. The experiment was then conducted as in the direct method.

#### Statistical Methods:

None specified

#### Evaluation Criteria:

Not indicated

### RESULTS

**Preliminary toxicity assay:** Not detailed

#### Direct method

2,4,6-TCP tested positive for the induction of 6-thioguanine resistant mutants in V79 cells. The increase seen was dose dependant up to 30 µg/mL, at which point, the number of resistant mutants fell in line with the cloning efficiency at increasing doses.

**Table 6.8.1-76a: 2,4,6-TCP induction of 6-thioguanine resistant mutants in V79 cells**

	Concentration µg/mg	Cloning efficiency %	6-thioguanine resistant mutants per $10^6$ survivors
Control (negative)	0	61.7	0
2,4,6-TCP	10	58.0	1
	20	55.0	13
	30	55.1	53
	45	47.5	25
	60	40.5	11
Control (positive)	0.5	54.4	471
MNNG	1.0	45.5	799

MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

#### Hepatocyte-mediated assay

At a concentration of 30 µg/mL, 2,4,6-TCP tested negative for the induction of 6-thioguanine resistant mutants in V79 cells co-cultured with primary hepatocytes.

**Table 6.8.1-76b: Induction of 6-thioguanidine resistant mutants in the hepatocyte-mediated assay by 2,4,6 TCP and DMN as a positive control**

	Concentration	Cloning efficiency %	6-thioguanine resistant mutants per 10 <sup>6</sup> survivors
Control	µg/ml	54.7	34
246TCP	30	43.3	0
DMN	mM		
	0.3	49.3	102
	1.0	53.1	300
	3.0	37.6	347

DMN = N-nitrosodimethylamine

## CONCLUSION:

In a publication from 1985, the mutagenic potential of 2,4,6-TCP was investigated in vitro using a version of the hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) assay. The induction of 6-thioguanine resistant mutants was investigated in V79 cells with and without metabolic activation. Without metabolic activation, a concentration range of 0 – 60 µg/mg 2,4,6-TCP was used. 2,4,6-TCP caused an increase in the number of 6-thioguanine resistant mutants, indicative of mutagenic potential. With metabolic activation, at a single concentration of 30 µg/ml 2,4,6-TCP, no increase in the number of 6-thioguanine resistant mutants was observed. The positive controls induced an appropriate mutagenic response.

Overall, 2,4,6-TCP showed mutagenic potential in V79 cells in vitro, when tested in the absence of metabolic activation. HSE notes that the study was broadly comparable to OECD TG 476.

(Hattula MI and Knuutinen J, 1985)

<b>Report:</b>	K-CA 5.8.1/30 Jansson K. and Jansson V. (1986). Inability of Chlorophenols to Induce 6-Thioguanine-Resistant Mutants in V79 Chinese Hamster Cells. Department of Cell Biology, University of Jyväskylä, Vapaudenkatu 4, SF-40100 Jyväskylä Finland. Published: Jansson K and Jansson V (1986). Inability of chlorophenols to induce 6-thioguanine-resistant mutants in V79 Chinese hamster cells. Mutation Research 171:165-168. Syngenta File No. NA_13769
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**STUDY TYPE:** *In Vitro* Mammalian Cell Gene Mutation Test

**TEST MATERIAL (PURITY):** 2,4,6-TCP (>99.5%).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

In a mammalian cell gene mutation assay, using hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) as the target gene, V79 cells cultured *in vitro* were exposed to 2,4,6-trichlorophenol (2,4,6-TCP) dissolved in acetone at concentrations of 12.5–100 µg/mL, in the absence of metabolic activation only.

Marked dose-related, test-item induced cytotoxicity was observed as indicated by relative survival vs. control cultures (survival reduced to 53% of control at 100 µg/mL).

Over the concentration range tested, no increase in the number of 6-thioguanine resistant mutants was observed. Relevant vehicle and positive controls responded as expected.

**Under the conditions of the study reported, 2,4,6-TCP did not increase the number of 6-thioguanine resistant mutants in V79 cells *in vitro*, in the absence of metabolic activation. 2,4,6-TCP was therefore non mutagenic.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	>99.5%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	Acetone
<b>Solvent control (final concentration):</b>	1%
<b>Positive control:</b>	Absence of S9 mix: Ethylmethanesulphonate (EMS), 200 µg/mL

### Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media: DMEM</b>			
Properly maintained?	X	Yes	No
Periodically checked for Mycoplasma contamination?		Yes	No
Periodically checked for karyotype stability?		Yes	No
Periodically “cleansed” against high spontaneous background?	X	Yes	No

X indicates those that apply

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na <sup>+</sup> /K <sup>+</sup> ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

#### Test compound concentrations used:

Absence of S9 mix                      12.5, 25, 50, 100 µg/mL

#### Study Design and Methods:

**In-life dates:** Not indicated

#### Test performance:

##### Mutation assay

The forward mutation assay on 6-thioguanine resistance in V79 cells was based on the replating method described previously (McMillan and Fox, 1979).  $1 \times 10^6$  cells were plated in 100 mm dishes in 9 mL of medium and cultured for 24 h. The test chemical was then added to the dishes in 1 mL of medium to give the desired final concentrations. For each concentration, two dishes were used. Each experiment included a solvent control and a positive control of EMS at 200 µg/mL. The maximum solvent concentration was 1%. This level had no effect on cell growth or spontaneous mutant frequency. After 24 h of treatment, cells were dissociated with a trypsin-EDTA solution, and cell survival was determined by plating 200 cells in 60 mm dishes (4 dishes/dose) in 4 mL of medium and staining the dishes with Giemsa 6 days later. Colonies of at least 50 cells were counted and survival was calculated relative to the solvent control. At the time cells were plated for survival,  $1-2 \times 10^6$  cells of each treatment condition were plated in 100 mm dishes in 10 mL of medium to allow phenotypic expression. Cells were subcultured every 48 h, maintaining  $1 \times 10^6$  cells at each subculture.

After a 6 day expression time,  $1 \times 10^3$  cells were plated in 100 mm dishes (10 dishes/dose) in 9 mL of medium, and 6-thioguanine was added in 1 mL of medium 4 h later to give a final concentration of 10 µg/mL. The number of mutant colonies was determined 10 days later, after Giemsa staining. To determine cell viability at the time of mutant selection, 200 cells were plated in 60-mm dishes (4 dishes/dose) in 4 mL of medium, and the colonies were stained with Giemsa and counted 6 days later. Mutant frequencies were expressed as the numbers of mutants per  $10^6$  viable cells.

#### Statistical Methods and Evaluation Criteria:

A chemical was considered to be mutagenic if the mutant frequency exceeded the 99% upper confidence limit of the historical background mutant frequency and a dose-related increase in the mutagenicity was observed. The historical background mutant frequency (the solvent control frequencies in 12 independent experiments including those reported here) was  $7.0 \times 10^{-6} \pm 4.5 \times 10^{-6}$  (mean  $\pm$  standard deviation). The 99% confidence interval calculated by multiplying the standard deviation by the appropriate *t* value of the Student's *t* distribution (one sided) requiring 99% confidence was  $12.2 \times 10^{-6}$ , and the 99% upper confidence limit was  $19.2 \times 10^{-6}$ .

## RESULTS

Over the concentration range tested, 2,4,6-TCP (12.5-100 µg/mL) reduced the plating efficiency (% cell survival) in a dose-dependent manner, indicating cytotoxicity to the V79 cell line.

At the concentrations tested, 2,4,6-TCP did not produce significant increases in the frequency of 6-thioguanine-resistant mutants.

Evidence that the cells in these experiments were responsive was shown by EMS treatment, which induced marked dose related increases in 6-thioguanine resistance.

**INDUCTION OF MUTATION TO 6-THIOGUANINE (TG) RESISTANCE IN V79 CHINESE HAMSTER CELLS BY TREATMENT WITH CHLOROPHENOLS FOR 24 h**

Chemical	Dose ( $\mu\text{g/ml}$ )	Survival (%) <sup>a</sup>	Number of TG-resistant mutants/ $10^6$ viable cells
EMS	200	64	1 262
2,4,6-Trichloro-phenol	0	100	2
	12.5	85	3
	25	79	4
	50	72	2
	100	53	0
EMS	0	100	8
	50	74	180
	100	73	259
	200	53	613
	400	13	1 367

<sup>a</sup> The average absolute plating efficiency of control cells was 75%, range 62–89%.

<sup>b</sup> Positive control, ethyl methanesulfonate.

## CONCLUSION:

In a publication from 1986, in a mammalian cell gene mutation HGPRT assay, V79 cells cultured *in vitro* were exposed to 2,4,6-TCP dissolved in acetone at concentrations of 12.5–100  $\mu\text{g/mL}$ , in the absence of metabolic activation only. Marked concentration-related cytotoxicity was observed, as indicated by the relative survival (survival reduced to 53% of control at 100  $\mu\text{g/mL}$ ). Over the concentration range tested, no increase in the number of 6-thioguanine resistant mutants was observed. Relevant vehicle and positive controls responded as expected.

Overall, 2,4,6-TCP did not increase the number of 6-thioguanine resistant mutants in V79 cells *in vitro*, in the absence of metabolic activation. 2,4,6-TCP was therefore non mutagenic in this study. HSE notes that the study was broadly comparable to OECD TG 476, but the result was inconsistent with that of the previous study.

## REFERENCES:

McMillan S and Fox M (1979). Failure of caffeine to influence induced mutation frequencies and the independence of cell killing and mutation induction in V79 Chinese hamster cells. *Mutation Res.*, 60:91-107.

(Jansson K and Jansson V, 1986)

**Report:** K-CA 5.8.1/31 Jansson K. and Jansson V. (1992). Genotoxicity of 2,4,6-Trichlorophenol in V79 Chinese Hamster Cells. Department of Cell Biology, University of Jyväskylä, SF-40100 Jyväskylä Finland. Published: Jansson K and Jansson V (1992). Genotoxicity of 2,4,6-trichlorophenol in V79 Chinese hamster cells. *Mutation Research* 280:175-179. Syngenta File No. NA\_13758.



**STUDY TYPE:** Assessment of multiple genotoxic endpoints in mammalian cells *in vitro*.

**TEST MATERIAL (PURITY):** 2,4,6- TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The potential for 2,4,6-trichlorophenol (2,4,6-TCP) to induce a range of genotoxic endpoints (6-thioguanine resistant mutants, chromosome aberrations, hyperdiploidy and micronuclei) was investigated over a range of concentrations in V79 cells *in vitro*, in the absence of metabolic activation only.

In the mammalian cell gene mutation assay, using hypoxanthine-guanine-phosphoribosyl transferase (HGPRT) as the target gene, V79 cells were exposed to 2,4,6-TCP dissolved in 0.5% DMSO at concentrations of 10-180 µg/mL. No increase in 6-thioguanine (6-TG) mutant colonies was observed.

In the chromosome aberration, hyperdiploidy and micronuclei assays, V79 cells were exposed to 2,4,6-TCP dissolved in 0.5% DMSO at concentrations of 10-90 µg/mL. A statistically significant, dose dependant increase in the number of hyperdiploid cells, and those with micronuclei was observed. No increase in the number of cells with chromosome aberrations was observed.

The positive control Ethylmethanesulphonate (EMS) gave an appropriate response for all endpoints tested.

**Under the conditions of the assay described, 2,4,6-TCP caused an increase in hyperdiploidy and micronuclei, but not an increase in 6-thioguanine resistant mutants or chromosome aberrations in V79 cells, in the absence of metabolic activation.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	99.7%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	DMSO
<b>Solvent control (final concentration):</b>	0.5%
<b>Positive control:</b>	Ethylmethanesulphonate, 500 µg/mL

**Test cells: mammalian cells in culture**

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media: DMEM</b>			
Properly maintained?		X	Yes
Periodically checked for Mycoplasma contamination?		X	Yes
Periodically checked for karyotype stability?			Yes
Periodically “cleansed” against high spontaneous background?		X	Yes

X indicates those that apply

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na <sup>+</sup> /K <sup>+</sup> ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

**Test concentrations**

Assay		Concentration
Hprt mutation assay	Absence of S9 mix	10, 30, 60, 90, 120, 150 and 180 µg/mL
Chromosome aberration assay and Hyperdiploidy/Micronuclei assay	Absence of S9 mix	10, 30, 60 and 90 µg/mL

**Study Design and Methods:****Test performance:****Cell survival and mutation**

The cells were plated at  $3 \times 10^5$  cells per 100 mm dish (3 dishes/dose) in 10 mL of medium and allowed to grow for 24 h. The medium was then replaced with 10 mL of fresh medium containing the test chemical. After 24 h of treatment, the cells were washed twice with Dulbecco's phosphate buffered saline (PBS) and harvested with 0.05% trypsin and 0.02% EDTA in PBS. To determine cell survival, 200 cells were plated in 4 mL of medium per 60 mm dish (4 dishes/dose), and after 6 days, the colonies were fixed in ethanol and stained with Giemsa. For phenotypic expression,  $2 \times 10^6$  cells were plated in a 80 cm<sup>2</sup> flask in 20 mL of medium. The cells were subcultured every 2 days, maintaining  $2 \times 10^6$  cells at each subculture. After a 6 day expression period, the cells were plated at  $1 \times 10^5$  cells per 100 mm dish (20 dishes/dose) in 10 mL of medium containing 30 µM 6-TG. The mutant colonies were fixed and stained 10 days later. To determine cloning efficiency at the time of mutant selection, 200 cells were plated in 4 mL of medium per 60 mm dish (4 dishes/dose), and after 6 days, the colonies were fixed and stained. The mutant frequency was expressed as mutants per  $10^6$  clonable cells.

**Chromosome aberrations, hyperdiploidy, and micronuclei**

The cells were plated at  $3 \times 10^5$  cells per 100 mm dish (2 dishes/dose) in 10 ml of medium and allowed to grow for 24 h. The medium was then replaced with 10 mL of fresh medium containing the test chemical. After 24 h of treatment, the cells were washed twice with PBS, and harvested as above either immediately or after an additional 24 h incubation in fresh medium. Colcemid was added to a final concentration of 0.1 µg/mL 2 h before harvest. The cells were suspended in a hypotonic solution of 75 mM KCl at 37°C for 10 min, fixed 3 times in ice-cold methanol: acetic acid (3:1), dropped onto wet glass slides, air-dried, and stained with 5% Giemsa in 10 mM phosphate buffer (pH 6.8). The slides were mounted in DPX and coded. For chromosome aberrations, 100 metaphase cells per dose (50 cells/slide) with 21-23 chromosomes were scored. The aberrations excluding gaps and endoreduplications were grouped into categories of 'simple' (breaks and terminal deletions), 'complex' (exchanges and rearrangements), 'other' (including pulverized chromosomes), and 'total' as described

(Galloway et al. 1987). For hyperdiploidy, 200 metaphase cells per dose (100 cells/slide) were scored. Only diploid cells and hyperdiploid cells with 23-28 chromosomes were registered. For micronuclei, 2000 interphase cells per dose (1000 cells/slide) were scored using the criteria previously described (Countryman and Heddle, 1976).

### Statistical Analysis and Data Evaluation

The variance of the mutant frequency was calculated as described by Leong *et al.* (1985), and a one-tailed normal deviate ( $z$ ) test was used to examine increases over control levels at each dose. For chromosome aberrations ('total' category), hyperdiploidy, and micronuclei, the one-tailed  $z$  test for binomial proportions with the continuity correction was used.

## RESULTS

### Gene mutation

2,4,6-TCP was unable to induce 6-TG-resistant mutants at doses up to 180 µg/ml. At this dose, the relative survival was reduced to 14%. A qualitative reduction in the size of surviving colonies occurred at 90 µg/ml and higher.

**Table 6.8.1-77a: Induction of 6-thioguanidine resistant mutants in V79 chinese hamster cells by treatment with 2,4,6 TCP for 24h**

Treatment	Relative survival (%) <sup>a</sup>		Mutants per 10 <sup>6</sup> clonable cells <sup>b</sup>	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
DMSO (0.5%)	100	100	13 (19)	8 (13)
TCP (10 µg/ml)	109	NT	7 (9)	NT
TCP (30 µg/ml)	101	92	10 (13)	12 (18)
TCP (60 µg/ml)	76	83	7 (11)	13 (21)
TCP (90 µg/ml)	65	52	14 (18)	9 (14)
TCP (120 µg/ml)	50	51	11 (17)	9 (15)
TCP (150 µg/ml)	38	33	9 (13)	5 (7)
TCP (180 µg/ml)	NT	14	NT	7 (10)
EMS (200 µg/ml)	83	71	982 * (1097)	785 * (950)

<sup>a</sup> Relative survival was calculated as compared to the solvent control cloning efficiency (75% for Expt. 1, 69% for Expt. 2).  
<sup>b</sup> Mutant frequency was calculated by dividing the number of mutant colonies (numbers in parentheses) by the number of cells plated for selection ( $2 \times 10^6$ ) corrected by the cloning efficiency at the time of selection.  
 NT, not tested.  
 \* Significantly greater than the solvent control ( $P < 0.05$ ).

### Chromosome aberration

2,4,6-TCP did not induce chromosome aberrations in cells fixed immediately after treatment. The highest dose scored was 60 µg/mL, where the mitotic index was reduced by about 78%. At this dose, 2,4,6-TCP did not induce chromosome aberrations in cells fixed 24 h after treatment, either.

**Table 6.8.1-77b: Induction of chromosome aberrations in V79 chinese hamster cells by treatment with 2,4,6 TCP for 24h**

Treatment		Mitoses per 1000 cells scored	Cells with aberrations per 100 cells scored		
			Simple	Complex	Total
Expt. 1	DMSO (0.5%)	43	1	0	1
	TCP (10 µg/ml)	37	1	0	1
	TCP (30 µg/ml)	14	1	0	1
	TCP (60 µg/ml)	8	2	0	2
	TCP (90 µg/ml)	1	ND	ND	ND
	EMS (200 µg/ml)	37	6	3	9 *
Expt. 2	DMSO (0.5%)	47	2	0	2
	TCP (10 µg/ml)	41	1	1	2
	TCP (30 µg/ml)	19	3	0	3
	TCP (60 µg/ml)	12	2	1	3
	TCP (60 µg/ml)	52 <sup>a</sup>	1 <sup>a</sup>	0 <sup>a</sup>	1 <sup>a</sup>
	EMS (200 µg/ml)	31	7	5	12 *

<sup>a</sup> Cells were harvested 24 h after treatment.

ND, not determined.

\* Significantly greater than the solvent control ( $P < 0.05$ ).

### Hyperdiploidy and micronuclei

2,4,6-TCP produced significant, dose-related increases in hyperdiploidy and micronuclei in cells fixed 24 h after treatment. For both responses, the lowest observed effective dose was 30 µg/mL. At doses up to 60 µg/mL, there was no reduction in the mitotic index.

**Table 6.8.1-77c: Overview of gene mutation, chromosome aberration, hyperploidy and micronuclei following exposure of V79 cells to 2,4,6-TCP**

Treatment		Mitoses/1000 cells scored	Hyperdiploid cells/200 cells scored	Cells with micronuclei/2000 cells scored
Expt. 1	DMSO (0.5%)	46	11	32
	2,4,6- TCP (10 µg/mL)	51	18	44
	2,4,6-TCP (30 µg/mL)	59	31*	56*
	2,4,6-TCP (60 µg/mL)	68	34*	88*
	2,4,6-TCP (90 µg/mL)	24	42*	116*
	EMS (200 µg/mL)	34	17	126*
Expt. 2	DMSO (0.5%)	53	8	36
	2,4,6-TCP (10 µg/mL)	55	14	46
	2,4,6-TCP (30 µg/mL)	50	32*	69*
	2,4,6-TCP (60 µg/mL)	62	32*	77*
	2,4,6-TCP (90 µg/mL)	30	36*	114*
	EMS (200 µg/mL)	39	12	112*

\* Significantly greater than the solvent control ( $P < 0.05$ ).

It appears that 2,4,6-TCP inhibits cell proliferation by a reversible mechanism rather than by causing cell killing. The positive results for hyperdiploidy indicate that 2,4,6-TCP produces chromosome malsegregation leading to aneuploidy in V79 cells.

Evidence that the cells were responsive to DNA damage was shown using the positive control, which gave appropriate responses across the endpoints investigated.

### CONCLUSION:

In a publication from 1992, the potential for 2,4,6-TCP to induce a range of genotoxic effects (6-thioguanine resistant mutants, chromosome aberrations, hyperdiploidy and micronuclei) was investigated over a range of concentrations in V79 cells in vitro, in the absence of metabolic activation only. In the mammalian cell gene mutation assay, using hypoxanthine-guanine-phosphoribosyl

transferase (HGPRT) as the target gene, V79 cells were exposed to 2,4,6-TCP at concentrations of 10-180 µg/mL. No increase in 6-thioguanine (6-TG) mutant colonies was observed.

In the chromosome aberration, hyperdiploidy and micronuclei assays, V79 cells were exposed to 2,4,6-TCP at concentrations of 10-90 µg/mL. A statistically significant, concentration-dependant increase in the number of hyperdiploid cells, and cells with micronuclei was observed. No increase in the number of cells with chromosome aberrations was reported. The positive control Ethylmethanesulphonate (EMS) gave an appropriate response for all endpoints tested.

Overall, 2,4,6-TCP caused an increase in hyperdiploidy and micronuclei, but not an increase in 6-thioguanine resistant mutants or chromosome aberrations in V79 cells, in the absence of metabolic activation.

#### REFERENCES:

Countryman P, and Heddle J (1976). *The production of micronuclei from chromosome aberrations in irradiated cultures of human lymphocytes. Mutation Res. 41:321- 332*

Galloway S, Armstrong M, Reuben C, Colman S, Brown B, Cannon C, Bloom A, Nakamura F, Ahmed M, Duk S, Rimpo J, Margolin B, Resnick M, Anderson B and Zeiger E (1987). *Chromosome aberrations and sister chromatid exchanges in Chinese hamster ovary cells: evaluations of 108 chemicals, Environ. Mol. Mutagen., 10 (Suppl. 10), 1-175.*

Leong P, Thilly W and Morgenthaler S (1985). *Variance estimation in single-cell mutation assays: comparison to experimental observations in human lymphoblasts at 4 gene loci. Mutation Res. 150:403-410.*

(Jansson K and Jansson V, 1992)

<b>Report:</b>	K-CA 5.8.1/32 McGregor D. <i>et al.</i> , (1988). Responses of the L5178Y tk <sup>+</sup> /tk <sup>-</sup> Mouse Lymphoma Cell Forward Mutation Assay: III. 72 Coded Chemicals. Inveresk Research International, Limited, Musselburgh, United Kingdom and Cellular and Genetic Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina. Accepted: 12 March 1988. Published: McGregor D, Brown A, Cattanaach P, Edwards I, McBride D, Riach C, Caspary W (1988). Responses of the L5178Y tk <sup>+</sup> /tk <sup>-</sup> Mouse Lymphoma Cell Forward Mutation Assay: III. 72 Coded Chemicals. Environmental and Molecular Mutagenesis 12:85-154. Syngenta File No. NA_13800.
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**STUDY TYPE:** *In Vitro* Mammalian Cell Gene Mutation Test

**TEST MATERIAL (PURITY):** 2,4,6-TCP (purity not stated)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

The study was performed to investigate the potential of 2,4,6-trichlorophenol (2,4,6-TCP) to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y. The assay was performed in two independent trials, using two parallel cultures each. Trials I and II were performed without liver microsomal activation only, with a treatment period of 4 hours. A treatment in the presence of metabolic activation was not conducted.

In both trials, the maximum concentration was limited by test item induced cytotoxicity, as reflected by decreases in the relative total growth (RTG). These concentrations were determined by a preliminary cytotoxicity test. The main experiments were conducted at the following concentrations:

Trial I	
without S9 mix:	0, 6.25, 12.5, 25, 50, 100 and 200* µg/mL
Trial II	
without S9 mix:	0, 10, 20, 40, 80, 120 and 160* µg/mL

\*Cultures not evaluated due to excessive cytotoxicity

A dose related increase in cytotoxicity, as measured by a decrease in RTG, was observed in both trials. Optimum toxicity in the RTG range of 10-20% was achieved, at concentrations of 100 and 120 µg/mL in trials I and II respectively.

Significant mutagenic responses were obtained from both trials in the absence of S9 mix. The lowest effect dose (LOED) was 80 µg/mL, at which the RTG was about 23%.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

**2,4,6-TCP induced mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence of metabolic activation. Therefore, 2,4,6-TCP is considered to be mutagenic in this mouse lymphoma assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Molecular weight:</b>	197.45 g/mol
<b>Purity</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated
<b>Control Materials:</b>	
<b>Negative:</b>	-
<b>Solvent control (final concentration):</b>	DMSO
<b>Positive control:</b>	Methylmethanesulphonate 15.0 µg/mL Ethylmethanesulphonate 250.0 µg/mL

**Test cells: mammalian cells in culture**

X	Mouse lymphoma L5178Y cells		V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others
<b>Media:</b> Fischer's medium			
Properly maintained?	X	Yes	No
Periodically checked for Mycoplasma contamination?	X	Yes	No
Periodically checked for karyotype stability?		Yes	X No
Periodically "cleansed" against high spontaneous background?	X	Yes	No

X indicates those that apply

Locus Examined:		Thymidine kinase (TK)		Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)		Na <sup>+</sup> /K <sup>+</sup> ATPase
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)		6-thioguanine (6-TG)		
	X	Trifluorothymidine (TFT)				

X indicates those that apply

**Test compound concentrations used:**

Trial I (absence of metabolic activation) 0, 6.25, 12.5, 25, 50, 100 and 200 µg/mL

Trial II (absence of metabolic activation) 0, 10, 20, 40, 80, 120 and 160 µg/mL

**Study Design and Methods:**

**In-life dates:** Not stated

**Test performance:**

**Cell treatment:** Each exposed culture consisted of  $6 \times 10^6$  cells in a final volume of 10 mL F<sub>5p</sub> in a 30-mL screw-cap plastic tube. This tube was incubated for 4 h on a horizontal axis roller drum rotating at 10 rpm. At the end of the incubation time, the cells were sedimented by centrifugation at 500 g.av. for 10 min, washed, and finally resuspended in 20 mL F<sub>10p</sub>. These cell suspensions ( $3 \times 10^5$  cells/mL) were incubated for a 2 day expression period, the cell population density being adjusted back to 20 ml of  $3 \times 10^5$  cells/mL after 24 h. After 48 h, the cell population densities were estimated and culture volumes containing  $3 \times 10^6$  cells adjusted to 15 mL with F<sub>10p</sub>, giving a cell population density of  $2 \times 10^5$  cells/mL.

**Cloning efficiency**

A 0.1 mL sample of the cell suspension was withdrawn and diluted 1:100. Three 0.1 mL samples (200 cells) of the diluted cultures were transferred to 30 mL tubes, mixed with 25 mL cloning medium (Fischer's medium containing 20% heat inactivated horse serum, i.e., F<sub>20p</sub>) containing 0.35% Noble agar and poured into 90 mm Petri plates.

**Mutant selection**

Three aliquots (each containing  $10^6$  cells) of the remaining culture were distributed to 30 mL tubes, mixed with 20 mL cloning medium to give final concentrations of 0.35% Noble agar and 3 µg trifluorothymidine/mL, then poured into 90 mm Petri plates. The agar was gelled at 4°C for 5-10 min, then the plates were incubated for 11-14 days in 5% CO<sub>2</sub>:95% air at 37°C.

Colonies were counted using an Artek 880 Automated Colony Counter, with the colony size discriminator control in the "off" position. Toxicity was expressed as either a reduction of cell population growth in suspension during the expression period or a reduction in cloning efficiency.

Any pH shifts observed from phenol red color changes to yellow or purple were noted.

**Statistical Methods:**

The statistical analysis was based upon the mathematical model proposed for this system (Lee and Caspary, 1983) and consisted of a dose-trend test and a variance analysis of pair-wise comparisons of each dose against the vehicle control. Significant differences from concurrent vehicle control values at the 5% level were indicative of a mutagenic response. Where a statistically significant response occurred, the lowest observed effective dose (LOED) was noted.

**Evaluation Criteria:** Four response categories were defined. Primary judgments were made at the level of individual experiments, but judgment on the mutagenic potential of a chemical was made on a basis of consensus of all valid experimental results

**Positive response ( + )**

The dose-related trend and the response at one of the three highest acceptable doses were statistically significant.

**Negative response (-)**

Two categories were used. In both there was

- a) no dose-related trend,
- b) no statistically significant response at any dose,
- c) an acceptable positive control response.

**Nontoxic, negative response (=)**

There was an RTG among the acceptable doses of >30% (approximately), higher toxicities being unattainable due to intrinsic properties of either the compound or the system.

**Toxic, negative response (-)**

There was either an RTG of <30% (approximately) at the maximum acceptable dose, or the lethal concentration was no greater than 1.5 X a lower concentration at which the RTG was >30%.

**Inconclusive (i)**

There was

- a) no dose-related trend and a statistically significant dose was any other than one of the highest three doses,
- b) a response which would have been negative, but the lowest RTG acceptable doses was >35%,
- c) a response which would have been negative, but there were no acceptable positive controls.

**Questionable (?)**

There was either

- a) no dose-related trend, but a statistically significant response occurred at one of the highest three doses, or significant on its own.
- b) a statistically significant dose-related trend, but none of the acceptable doses was statistically

**RESULTS****Preliminary toxicity assay:**

The first experiment was a toxicity test in which cell population expansion was measured. Ten-fold differences in test compound concentrations were used, the highest being 5 mg/mL unless a much lower concentration was indicated by the poor solubility of a compound. Data from the preliminary toxicity trial was not presented.



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The concentrations of the main experiments were selected based on evident test item induced cytotoxicity. The concentrations used were as follows:

Trial I (absence of metabolic activation) 0, 6.25, 12.5, 25, 50, 100 and 200 µg/mL

Based on the observed cytotoxicity, a modified concentration range was used for trial II:

Trial II (absence of metabolic activation) 0, 10, 20, 40, 80, 120 and 160 µg/mL

**Mutation assay:**

The study was performed to investigate the potential of 2,4,6-TCP to induce mutations at the mouse lymphoma thymidine kinase locus of the cell line L5178Y.

The assay was performed with two independent experiments, using two parallel cultures each. The main experiments were performed without liver microsomal activation and a treatment period of 4 hours.

Relevant cytotoxic effects occurred in both trials, as indicated by RTG of less than 50% in both cultures, at 100 and 80 µg/mL in trials I and II respectively. Cultures at 200 (trial I) and 160 µg/mL (trial II) were not evaluated due to excessive cytotoxicity.

Statistical evaluation of the test item induced mutant frequency values, compared to concurrent control values, demonstrated significant increases in the mutant frequency at 100 µg/mL (trial I) and 80 and 120 µg/mL (trial II). The LOED was 80 µg/mL.

MMS (15 µg/mL) and EMS (250 µg/mL) were used as positive controls, both showed a distinct increase in induced total mutant colonies at acceptable levels of toxicity.

**2,4,6,-Trichlorophenol**

WITHOUT S9 TRIAL 1 [+]						WITHOUT S9 TRIAL 2 [+]					
CONC. ug/ml	CE	RTG	MC	MF	AVG MF	CONC. ug/ml	CE	RTG	MC	MF	AVG MF
DMSO	82	115	70	28		DMSO	109	115	126	39	
0.	89	101	55	21		0.	92	100	106	38	
	74	92	106	48			114	90	141	41	
	78	92	60	26	31		97	96	129	45	41
6.25	86	94	68	27		10.	104	89	159	51	
	97	89	130	45	36		92	106	141	51	51
12.5	69	81	41	20		20.	80	87	155	65	
	67	78	70	35	27		76	88	114	50	58
25.	83	73	90	36		40.	84	68	174	69	
	66	79	94	48	42		99	74	154	52	60
50.	73	70	91	42		80.	91	19	212	78	
	78	66	98	42	42		79	26	199	85	81
100.	60	10	119	66		120.	71	9	496	233	
	48	11	122	85	75		93	11	385	138	185
200.	LETHAL					160.	LETHAL				
	LETHAL						LETHAL				
EMS	64	75	255	134		EMS	56	71	403	238	
250.	63	99	280	149	182	250.	64	70	455	236	237
ug/ml						ug/ml					
MMS	43	40	116	91		MMS	45	31	219	162	
15.	48	40	163	114	102	15.	38	25	207	183	173
ug/ml						ug/ml					

### CONCLUSION:

In a publication from 1988, the potential of 2,4,6-TCP to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y was investigated. The assay was performed in two independent trials, using two parallel cultures each. Trials I and II were performed without liver microsomal activation only, with a treatment period of 4 hours. A treatment in the presence of metabolic activation was not conducted. In both trials, the maximum concentration was limited by cytotoxicity, as reflected by decreases in the relative total growth (RTG). The main experiments were conducted up to concentrations of 160-200 µg/mL.

A concentration-related increase in cytotoxicity, as measured by a decrease in RTG, was observed in both trials. Optimum toxicity in the RTG range of 10-20% was achieved, at concentrations of 100 and 120 µg/mL in trials I and II respectively. Significant mutagenic responses were obtained from both trials in the absence of S9 mix. The lowest effect concentration was 80 µg/mL, at which the RTG was about 23%. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

Overall, 2,4,6-TCP induced mutations in the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence of metabolic activation. HSE notes that the study was broadly comparable to OECD TG 490.

### REFERENCES:

Lee YJ, Caspary WJ (1983). *Mathematical model of L5178Y mouse lymphoma forward mutation assay. Mutat Res* 113:417-430.

(McGregor D. *et al*, 1988)

<b>Report:</b>	K-CA 5.8.1/33 Armstrong M.J. <i>et al.</i> , (1993). 2,4,6-Trichlorophenol (TCP) Induces Chromosome Breakage and Aneuploidy <i>In Vitro</i> . Merck Research Laboratories, WP45-305, West Point, PA 19486, USA. Accepted 7 July 1993. Published: Armstrong MJ, Galloway SM, Ashby J (1993). 2,4,6-Trichlorophenol (TCP) induces chromosome breakage and aneuploidy <i>in vitro</i> . Mutation Research 303:101-108. Syngenta File No. NA_13759.
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**STUDY TYPE:** *In Vitro* Mammalian Chromosome Aberration Test.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (>99.7 and >99.95%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 2**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The potential for 2,4,6-trichlorophenol (2,4,6-TCP) to induce structural and numerical chromosome aberrations was investigated using CHO and V79 cells *in vitro*. A range of treatment/exposure periods was used, utilising concentration ranges inducing cytotoxicity as indicated by a decrease in mitotic index.

In the absence of S9 metabolic activation, 2,4,6-TCP induced structural chromosome aberrations in CHO cells and in V79 cells using a 3 h treatment and 20 h sampling time (17 h recovery). There was no increase in aberrations observed when a 24 h treatment with sampling either immediately, or with a 24-h recovery period was used. However, positive results were obtained when a recovery time of 4-12 h was allowed after the 24-h treatment with 2,4,6-TCP. All these results were obtained without S9 metabolic activation.

2,4,6-TCP was also positive for chromosome aberration induction in CHO cells when tested in the presence of S9.

2,4,6-TCP also induced hyperdiploidy in V79 cells harvested 24 h after the end of a 24 h treatment. An increase in the frequency of roughly triploid and tetraploid cells was also observed.

**Under the conditions of the study, 2,4,6-TCP induces both structural and numerical chromosome aberrations *in vitro*.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	>99.7 and >99.95%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	DMSO
<b>Solvent control (final concentration):</b>	1.0%
<b>Positive control:</b>	No positive control used

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbital		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

S9 activation system contained serum-free McCoy's 5A medium and 0.8 mg/ml NADP (1.0 mM /3-nicotinamide- adenosine dinucleotide phosphate, sodium salt), 1.5 mg/mL trisodium isocitrate (5.8 mM) and 15 µL/ml rat liver S9.

The S9 was prepared from male Sprague-Dawley rats induced with phenobarbital and / β - naphthoflavone, and the protein concentration of the homogenate was about 40 mg/mL.

### Test cells: mammalian cells in culture

X	V79 cells (Chinese hamster lung fibroblasts)
	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 donors (female for Experiment 1 and male for Experiment 2) were pooled together for each experiment. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
X	Chinese hamster ovary (CHO) cells

#### Media:

**CHO cells:** McCoy's 5A medium with 10% foetal bovine serum, 2 mM L-glutamine, 100units/ml penicillin and 100 µg/mL streptomycin

**V79 cells:** Minimal Essential Medium Eagle with 5% foetal bovine serum, 1 mM L-glutamine, 0.1 mM MEM non-essential amino acids, 0.11 mg/mL sodium pyruvate and 0.05 mg/ml gentamycin

Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

**Test compound concentrations used:**

<b>Chromosome aberration</b>		
CHO cells	Treatment / Recovery time (h)	Concentrations
Absence of S9 mix	3 / 17	0, 300, 400 and 500 µg/mL
Presence of S9 mix	3 / 17	0, 400, 500 and 600 µg/mL
Absence of S9	24 / 0, 4, 8, 12, 24	0, 50, 100, 150 and 200 µg/mL
<b>Hyperdiploidy</b>		
V79 cells	Treatment / Recovery time (h)	Concentrations
Absence of S9 mix	24 / 0	0, 25, 50, 100, 150, 200 and 250 µg/mL

**Study Design and Methods:****In-life dates:** Not indicated**TEST PERFORMANCE****Preliminary Cytotoxicity Assay:**

Not performed.

**Cytogenetic Assay:**

Exponentially growing cells were exposed to 2,4,6-TCP for a range of exposure and recovery periods, as indicated below. Cells were harvested for aberration analysis at the end of the recovery period.

Cell type	+/-S9	Exposure period	Recovery period
CHO	-	3	17
	+	3	17
V79	-	3	17
	+	3	17
	-	24	0
	+	24	4
	+	24	8
	-	24	12
	-	24	24

**Toxicity assessment:**

Cell samples from each treatment were counted twice using a Coulter counter and the mean was expressed as a percentage of controls. For metaphase preparations, cells were treated with 75 mM KCl for 1-3 min at room temperature, washed twice with fixative (methanol:glacial acetic acid, 3:1 v/v), dropped onto slides, air-dried, stained with 5% Giemsa in water then mounted. The mitotic index (MI) was the percentage of metaphase figures among 1000 cells. The doses tested were selected to give a range of toxicity, aiming to include a dose giving about a 50% reduction in cell counts at harvest.

<b>Spindle inhibition:</b>	
Inhibitor used/ concentration:	Colcemid 0.1 µg/mL
Administration time:	2-3 hours (before cell harvest)

### Metaphase analysis

No. of cells examined per dose: 200				
Scored for structural?	X	Yes		No
Scored for numerical?	X	Yes (polyploidy noted if observed)		No
Coded prior to analysis?	X	Yes		No

Slides were scored under code by one observer. For aberration analysis, 200 metaphase cells containing 19-23 chromosomes were scored for each treatment unless aberration frequencies were high or few cells were available for analysis due to mitotic suppression. Gaps (achromatic region less than or equal to the width of a chromatid), polyploidy and endoreduplicated cells, and pulverized chromosomes were noted but not included in structural aberration totals. Results are shown as the percentage of cells with aberrations (aberrant cells).

200 cells per treatment were scored for hyperdiploidy according to the criteria of Jansson (Jansson *et al.*, 1992), where metaphase cells with 23-28 chromosomes were scored as hyperdiploid. Cells with roughly 3n and 4n chromosome numbers were also scored. Results are shown as the number of hyperdiploid cells per 200 metaphases.

### Statistical analysis

For selected points, the percentage of aberrant cells in treated cultures was compared to concurrent controls by a chi-squared test based on a standard normal approximation.

## RESULTS

**Preliminary cytotoxicity assay:** Not performed.

### Cytogenetic assay:

Aberration induction by 2,4,6-TCP (>99.7 and >99.95% purity) was measured in CHO and V79 cells at various intervals after 3 or 24 h treatments in the absence of metabolic activation.

A clearly positive result was found in CHO cells treated for 3 h and harvested 20 h from the beginning of treatment. Cell numbers over the dose range 300-600 µg/ml were reduced from 80 to 34% of controls after treatment with repurified 2,4,6-TCP, and from 75 to 27% of controls with commercial 2,4,6-TCP.

The same test in V79 cells (3 h treatment, 17 h recovery –S9) was conducted, again a positive increase in chromosome aberrations was observed.

2,4,6-TCP also induced an increase in chromosome aberrations in CHO cells when tested in the presence of metabolic activation (3 h treatment, 17 h recovery).

**Structural chromosome aberrations in CHO cells 17h after 3-h treatment with 2,4,6-TCP with and without S9 activation:**

2,4,6-TCP ( $\mu\text{g/ml}$ )	Cell counts <sup>a</sup> (% controls)	% Aberrant cells	Frequencies of aberrations per 100 cells					
			Total	Chromatid		Chromosome		SD
				Del	Exc	Del	Exc	
<i>Without S9</i>								
0 <sup>b</sup>		1.8	1.8	0.5	0.0	0.5	0.8	0.0
300	75	1.5	1.5	0.5	0.0	0.5	0.5	0.0
400	63	13.5	22.0	9.5	11.0	1.5	0.0	0.0
500	47	33.0 <sup>c</sup>	59.0	19.0	34.0	4.0	2.0	0.0
<i>With S9</i>								
0 <sup>b</sup>		2.8	3.8	0.3	0.5	2.3	0.8	0.0
400	83	0.0	0.0	0.0	0.0	0.0	0.0	0.0
500	81	2.5	2.5	0.5	0.5	0.5	1.0	0.0
600	42	66.0 <sup>d</sup>	372.0	42.0	36.0	14.0	0.0	28.0

<sup>a</sup> Cell counts at time of harvest.  
<sup>b</sup> Combined DMSO and medium controls.  
Del, deletions; Exc, exchanges; SD, severely damaged ( $\geq 10$  aberrations/cell): each counted as one in % aberrant cells and as 10 in total frequency of aberrations/cell.  
200 cells scored/point except where noted: <sup>c</sup> 100 or <sup>d</sup> 50 cells scored.

### Influence of recovery period length

V79 cells were also treated for 24 h then harvested immediately or following recovery periods of 4, 8, 12 or 24 h. No significant increases in aberrations were seen in cells harvested immediately after treatment (i.e. at 24 h) up to toxic doses (e.g. cell counts reduced to 20% of controls). After a 24 h recovery period, there were 5% cells with aberrations, a small but statistically significant increase over the concurrent control value of 0.25%, a result that is questionable at best. In contrast, there was a clear increase in aberrations after a 12 h recovery period, with up to 21% aberrant cells. At 36 and 48 h the mitotic indices at lower doses were markedly higher than the control values suggesting that there was a cell-cycle block followed by some degree of synchrony in the recovering cells. There were considerable reductions in cell growth at the doses that induced aberrations.

The results were confirmed in a second test with additional sampling times: aberration induction (albeit weak) was seen after a 4, 8 and 12 h recovery period but not when sampled immediately post 24 h of treatment.

### Numerical aberration induction

2,4,6-TCP induced hyperdiploidy in V79 cells harvested 24 h after the end of the 24 h treatment (12/200 at 100  $\mu\text{g/mL}$ ). An increase in the frequency of roughly triploid and tetraploid cells at 150-250  $\mu\text{g/mL}$  2,4,6-TCP was also observed.

### Hyperdiploidy in V79 cells 24h after a 24-h treatment with 2,4,6-TCP:

2,4,6-TCP ( $\mu\text{g/ml}$ )	Hyperdiploid cells per per 200 cells scored (23–28)	Polyploid cells per 200 cells scored			
		(29–36)	(37–46)	(49)	(51)
0 <sup>a</sup>	6		1		
25	3		1		
50	16	1			
100	12	1	3		
150	21	5	9		
200	18	9	16		1
250	30	2	28	1	

<sup>a</sup> Combined DMSO and medium controls.  
( ) number of chromosomes per metaphase.

### CONCLUSION:

In a publication from 1993, the potential for 2,4,6-TCP to induce chromosome aberrations was investigated using CHO and V79 cells *in vitro*. A range of treatment/exposure periods was used, utilising concentration ranges inducing cytotoxicity as indicated by a decrease in mitotic index. In the absence of metabolic activation, 2,4,6-TCP induced structural chromosome aberrations in CHO cells and in V79 cells using a 3 h treatment and 20 h sampling time (17 h recovery). There was no increase in aberrations observed when a 24 h treatment with sampling either immediately, or with a 24-h recovery period was used. However, positive results were obtained when a recovery time of 4-12 h was allowed after the 24-h treatment with 2,4,6-TCP. All these results were obtained without metabolic activation. 2,4,6-TCP was also positive for chromosome aberration induction in CHO cells when tested in the presence of S9. 2,4,6-TCP also induced hyperdiploidy in V79 cells harvested 24 h after the end of a 24 h treatment. An increase in the frequency of roughly triploid and tetraploid cells was also observed.

Overall, 2,4,6-TCP induced chromosome aberrations *in vitro* in the presence and absence of metabolic activation. HSE notes that the study used a non-standard protocol.

#### REFERENCES:

Jansson K., and Jansson V (1992). *Genotoxicity of 2,4,6-trichlorophenol in V79 Chinese hamster cells*. *Mutation Res.* 280, 175-179.

(Armstrong M. J. *et al*, 1993)

<b>Report:</b>	K-CA 5.8.1/34 Galloway S. <i>et al.</i> , (1987). Chromosome Aberrations and Sister Chromatid Exchanges in Chinese Hamster Ovary Cells: Evaluations of 108 Chemicals. Department of Molecular Toxicology, Litton Bionetics, Inc., Kensington, Maryland. Published: Galloway S, Armstrong M, Reuben C, Colman S, Brown B, Cannon C, Bloom A, Nakamura F, Ahmed M, Duk S, Rimpo J, Margolin B, Resnick M, Anderson B, Zeiger E (1987). Chromosome Aberrations and Sister Chromatid Exchanges in Chinese Hamster Ovary Cells: Evaluations of 108 Chemicals. <i>Environmental and Molecular Mutagenesis</i> 10:1-175. Syngenta File no. NA_13783.
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**STUDY TYPE:** *In Vitro* Mammalian Chromosome Aberration and sister chromatid exchange Test.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (Not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 2**

**HSE comment:** supportive study

#### EXECUTIVE SUMMARY

The potential for 2,4,6-trichlorophenol (2,4,6-TCP) to induce cytogenetic damage, in the form of sister chromatid exchanges (SCE) or chromosome aberrations (CA), was investigated in CHO cells *in vitro*, both in the presence and absence of metabolic activation.

Over a concentration range of 50-500 µg/mL both in the presence and absence of metabolic activation, no increase in the number of cells with chromosome aberrations was observed.



Similarly, over a concentration range of 5-50 µg/mL (–S9) and 16-500 µg/mL (+S9), no increase in the number of cells with sister chromatid exchange was observed.

No discussion of test item induced toxicity or test item precipitation in the culture was included.

**Under the conditions of the study, 2,4,6-TCP did not induce sister chromatid exchange or chromosome aberrations in CHO cells *in vitro*. 2,4,6-TCP therefore did not induce cytogenetic damage *in vitro* in the assays described.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	Untreated
<b>Solvent control (final concentration):</b>	Not indicated (Either water, dimethyl sulfoxide (DMSO), ethanol, or acetone, selected in that order of preference)
<b>Positive control:</b>	Absence of S9 mix: Mitomycin C (Both assays) Triethylenemelamine (Aberration assay only)
	Presence of S9 mix: Cyclophosphamide (Both assays)

### Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other β-naphthoflavone		Other		

The S9 mix consisted of 15 µL/mL liver homogenate (from male Sprague-Dawley rats, induced with Aroclor 1254), 2.4 mg/mL NADP, and 4.5 mg/mL isocitric acid in serum-free medium

### Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 donors (female for Experiment 1 and male for Experiment 2) were pooled together for each experiment. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
X	Chinese hamster ovary (CHO) cells

Media: McCoy's 5A medium				
Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

**Study Design and Methods:****In-life dates:** Not indicated**TEST PERFORMANCE****Preliminary Cytotoxicity Assay:**

Initially, dose selection was based on a preliminary growth inhibition test in which cells that excluded trypan blue were counted 24 h after treatment. The top doses selected for the cytogenetics assays were those estimated to reduce growth by 50%. This approach was subsequently modified such that toxicity estimates were made from observations of cell monolayer confluence and mitotic activity in the same cultures used for analysis of SCEs or aberrations. A maximum dose of 5-10 mg/mL, the limit of solubility in culture medium or cytotoxicity were selected as criteria for limiting top doses in the assay.

**Test compound concentrations used:**

Assay type	+/- metabolic activation	Concentrations 2,4,6-TCP (µg/mL)
Chromosome aberration test	Absence of S9 mix	50-500
	Presence of S9 mix	50-500
Sister chromatid exchange test	Absence of S9 mix	5-50
	Presence of S9 mix	16-500

**Treatment times:**

Assay	+/- S9	Test Material (h)	Solvent Control (h)	Positive Control (h)
Sister chromatid exchange assay	-	25	25	25
	+	2	2	2
Chromosome aberration assay	-	25	25	25
	+	2	2	2

**Sister Chromatid Exchange Test**

5-Bromodeoxyuridine (BrdUrd; 10 µM) was added 2 h after addition of the test chemical (without S9) or immediately after the S9 mix plus chemical had been removed. The chemical treatment periods were approximately 25 h without S9 and 2 h with S9. The total incubation time with BrdUrd was 25-26 h, with colcemid (0.1 µg/mL) present during the final 2-3 h. Immediately before the cells were harvested, the cell monolayers were examined, and the degree of confluence and availability of mitotic cells were noted. Cells were collected by mitotic shake-off at doses up to the maximum considered likely to yield sufficient metaphase cells for analysis. After 1-3 min treatment with hypotonic solution (75 mM KCl), cells were fixed in 3:1 methanol:glacial acetic acid (V/V). For a preliminary assessment of cell cycle delay, test slides were prepared from cells treated at the highest dose levels to see if later harvests were necessary. These test slides were stained with “dilute” Hoechst 33258 (0.5 µg/ml in Sorensen’s buffer, pH 6.8) and examined by fluorescence microscopy to assess cell cycle kinetics.

For scoring SCEs, slides were stained by a modification of the method of Perry and Wolff (Perry and Wolff, 1974) as adapted by Goto et al (Goto *et al.*, 1978). After staining for 10 min in “concentrated” Hoechst 33258 (5 µg/mL in pH 6.8 buffer) and exposure to “black light” at 55 to 60°C for about 5 min, slides were stained in Giemsa. All slides were coded, and 50 cells per dose were scored from the three highest doses at which sufficient M2 cells were available, from a solvent control, and from a “weak positive” control (in later experiments) treated with a low dose of mitomycin C (without S9) or cyclophosphamide (with S9). This weak-positive control was designed to give a small (20-40 %) increase in SCEs and was included to assess the ability of the system to detect small increases in SCEs

(Margolin and Resnick, 1985). After the introduction of the weak-positive control into the protocol, only five to ten cells were scored from “strong-positive” controls (higher doses of mitomycin C or cyclophosphamide) that were not coded. When cell cycle delay was noted, cell kinetics were recorded, by classifying each of 100 metaphases as M1, M1+, or M2, ie, having completed one (M1), two (M2), or between one and two (M1 +) cell cycles in BrdUrd.

<b>Spindle inhibition:</b>	
Inhibitor used/ concentration:	Colcemid 0.1 µg/mL
Administration time:	2-3 h (before cell harvest)

### Chromosome aberration test

Cells were exposed to the test chemical for 2 h in the presence of S9 or throughout the incubation period without S9. Cells were collected by mitotic shake-off. Slides were stained with Giemsa and coded and 100 cells were scored from each of the three highest dose groups having sufficient metaphases for analysis and from positive (triethylenemelamine, mitomycin C, or cyclophosphamide) and solvent controls. All types of aberrations were recorded separately, but for data analysis they were grouped into categories of “simple” (breaks and terminal deletions), “complex” (exchanges and rearrangements), “other” (includes pulverized chromosomes), and “total”. Gaps and endoreduplications were recorded but were not included in the totals. Aberrations were not scored in polyploidy cells but metaphases with 19-23 chromosomes were used (the modal number being 21).

### Evaluation criteria and Statistical analysis:

The statistical procedures for evaluation of the test data and the assessment of the results have been modified from those described in detail (Galloway et al., 1985 and Margolin *et al.*, 1986). Briefly, the analyses examined the evidence for a dose relation and the absolute increase over the solvent control at each dose.

### Sister Chromatid Exchanges

For SCE data, a linear regression test (trend test) of SCEs per chromosome *vs* the log of the dose was used (Armitage, 1955). For individual doses, absolute increases in SCEs per chromosome of 20% or more over the solvent control were considered significant (Galloway *et al.*, 1985).

### Aberrations

For chromosome aberrations, linear regression analysis of the percentage of cells with aberrations *vs.* the log-dose was used as the test for trend. To examine absolute increases over control levels at each dose, a binomial sampling assumption (as opposed to Poisson) was used, and the test was that described by Margolin (Margolin *et al.* 1983). The P values were adjusted by Dunnett’s method to take into account the multiple dose comparisons. For data analysis, the “total” aberration category was used, and the criterion for a positive response was that the adjusted P value be < 0.05.

## RESULTS

**Preliminary cytotoxicity assay:** Performed, but not reported in detail. The top doses selected for the cytogenetics assays were those estimated to reduce growth by 50%.

### Main assay:

2,4,6-TCP was tested over a range of concentrations, both in the presence and absence of S9-mix for the induction of chromosome aberrations and sister chromatid exchange.

### Chromosome aberrations:

Over a concentration range of 50-500 µg/mL, 2,4,6-TCP did not cause an increase in the number of cells with chromosome aberrations (data not presented).

**Sister chromatid exchange:**

Over a concentration range of 5-50 µg/mL (-S9) and 16-500 µg/mL (+S9), 2,4,6-TCP did not cause an increase in sister chromatid exchange (data not presented).

In the study detailed, no mention of cytotoxicity induction or compound precipitation in the cultures was discussed.

The sensitivity of the test system, and the metabolic activity of the S9-mix employed, were clearly demonstrated by the increases in the frequencies of cells with aberrations or SCE induced by the positive control agents, triethylenemelamine, mitomycin C and cyclophosphamide.

**CONCLUSION:**

In a publication from 1987, the potential for 2,4,6-TCP to induce cytogenetic damage, in the form of sister chromatid exchanges (SCE) or chromosome aberrations (CA), was investigated in CHO cells in vitro, both in the presence and absence of metabolic activation. Over a concentration range of 50-500 µg/mL both in the presence and absence of metabolic activation, no increase in the number of cells with chromosome aberrations was observed. Similarly, over a concentration range of 5-50 µg/mL (-S9) and 16-500 µg/mL (+S9), no increase in the number of cells with sister chromatid exchange was observed. No discussion of test item induced toxicity or test item precipitation in the culture was included.

Overall, 2,4,6-TCP did not induce SCEs or CAs in CHO cells in vitro. HSE notes that there are several reporting deficiencies in this study.

**REFERENCES:**

Perry P and Wolff S (1974). *New Giemsa method for the differential staining of sister chromatids. Nature* 251:156-158.

Margolin B and Resnick M (1985). *A simple experimental method to probe the resolving power of an in vitro toxicological assay. Environ Mutagen* 7(suppl 3):48.

Margolin B, Resnick M, Rimpo J, Archer P, Galloway S, Bloom A, Zeiger E (1986). *Statistical analyses for in vitro cytogenetic assays using Chinese hamster ovary cells. Environ Mutagen* 8:183-204.

Margolin B, Collings B, Mason J (1983). *Statistical analysis and sample-size determinations for mutagenicity experiments with binomial responses. Environ Mutagen* 5:705-715.

Goto K, Maeda S, Kano Y, Sugiyama T (1978). *Factors involved in differential Giemsa-staining of sister chromatids. Chromosoma* 66:351-359.

Galloway S, Bloom A, Resnick M, Margolin B, Nakamura F, Archer P, Zeiger E (1985). *Development of a standard protocol for in vitro cytogenetic testing with Chinese hamster ovary cells: Comparison of results for 22 compounds in two laboratories. Environ Mutagen* 7:1-51.

(Galloway S, et al., 1987)

<b>Report:</b>	K-CA 5.8.1/35 Matsuoka A. et al., (1998). <i>In vitro</i> clastogenicity of 19 organic chemicals found in contaminated water and 7 structurally related chemicals. National Institute of Health Sciences, Japan. Accepted 31 July 1998. Published: Matsuoka A, Hayashi M, Sofuni T (1998). <i>In vitro</i> clastogenicity of 19 organic chemicals found in contaminated water and 7 structurally related chemicals. <i>Environ. Mutagen Res.</i> , 20:159-165. Syngenta File No. NA_13789.
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**STUDY TYPE:** Chromosome Aberration Test in Chinese Hamster Lung Fibroblasts *In Vitro*.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (99.9%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 2**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

This *in vitro* assay was performed to assess the potential of 2,4,6-TCP to induce structural chromosomal aberrations in cultured Chinese hamster lung fibroblasts in the absence and presence of an exogenous metabolic activation system.

100 metaphases were evaluated for structural chromosomal aberrations at each test item concentration.

The highest applied concentration in this study was 1.5 mg/mL (inferred from graphical representation of the data), no justification for the top concentration of 2,4,6-TCP was provided.

2,4,6-TCP induced structural aberrations, that exceeded the criteria for a positive clastogenic response, in the presence of S9 mix. Criteria for a positive response were based on laboratory historical control data (Matsuoka, 1991).

**In conclusion, it can be stated that under the experimental conditions reported, 2,4,6-TCP induced structural chromosomal aberrations in chinese hamster lung fibroblasts *in vitro*. Therefore, 2,4,6-TCP is considered to be clastogenic in this chromosome aberration test.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	99.9 %
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	DMSO
<b>Solvent control (final concentration):</b>	DMSO
<b>Positive control:</b>	Not described.

### Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254		Rat	X	Liver
	Non-induced		Phenobarbital	X	Mouse		Lung
			None		Hamster		Other

		X	Other Polychlorinated biphenyl		Other		
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X indicates those that apply

The S9 fraction was prepared from the liver of male BALB/c mice pretreated with polychlorinated biphenyl. 10 mL of S9 mix consisted of 2 mL of 20 mM HEPES buffer (pH 7.2), 1 mL each of 50 mM MgCl<sub>2</sub>, 330 mM KCl, 50 mM glucose-6-phosphate, 40 mM NADP, and distilled water, all mixed and filter sterilised and 3ml of the S9 fraction.

#### Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells
X	CHL, Chinese hamster lung fibroblasts

X indicates those that apply

Media: Eagle's minimum essential medium supplemented with 10% foetal calf serum				
Properly maintained?	X	Yes		No
Periodically checked for <i>Mycoplasma</i> contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

#### Test compound concentrations used:

Absence of S9 mix	0, 0.5, 1 and 1.5 mg/mL *
Presence of S9 mix	0, 0.5, 1 and 1.5 mg/mL *

\* Concentrations inferred from graphical representation of the data.

#### TEST PERFORMANCE

**Preliminary cytotoxicity assay:** Not performed.

#### Cytogenetic assay:

Cell exposure time:	Test Material	Solvent Control	Positive Control
- S9 mix	6h	6h	Not conducted
+ S9 mix	6h	6h	

Spindle inhibition:	
Inhibitor used/ concentration:	Colcemid 0.2 µg/mL
Administration time:	2 hours (before cell harvest)

Cell harvest time after termination of treatment:	Test Material	Solvent Control	Positive Control
- S9 mix	18h	18h	Not conducted
+ S9 mix	18h	18h	

#### Details of slide preparation

**Exposure time 6 hours:** Cells were seeded at a density of  $2 \times 10^4/5$  mL of medium in a glass culture bottle. On the third day they were treated for 6 h with 0.5 mL of S9-mix or medium, 2.5 mL of medium, and 0.015 mL of a DMSO solution containing one dose of 2,4,6-TCP.

The reaction mixture was replaced with fresh medium, and the cells were incubated for 18 hours. Chemicals were tested up to the dose showing clear cytotoxicity or practically preparable (homogeneously suspended in medium or the solubility limit in the solvent).

### Metaphase analysis

No. of cells examined per dose: 100				
Scored for structural?	X	Yes		No
Scored for numerical?	X	Yes (polyploidy noted if observed)		No
Coded prior to analysis?	X	Yes		No

X indicates those that apply

Colcemid (final concentration 0.2 µg/mL) was added to the culture 2 h before cell harvesting. The cells were trypsinised and incubated in 75 mM KCl hypotonic solution for 15 min at 37°C. They were fixed with ice cold fixative (methanol:glacial acetic acid, 3:1) three times. Two drops of the cell suspension were spread on a clean glass slide and stained with Giemsa solution. Slides were coded before observation. The number of cells with chromosomal aberrations was counted on 100 well spread metaphases. The incidence of polyploidy was also recorded. Solvent controls were run in parallel.

**Evaluation criteria:** The percentage of aberrant metaphases was calculated for each treatment scored, both including and excluding cells with only gap-type aberrations.

Classification of a treatment as clastogenic was based on laboratory historical control data (Matsuoka, 1991).

Positive – 10.0% or more cells were aberrant

Equivocal – 5.0 – 9.9% of cells were aberrant

Negative – Less than 4.0% of cells were aberrant

**Statistical analysis:** No statistical analysis was conducted

## RESULTS

**Preliminary cytotoxicity assay:** Not performed.

### Cytogenetic assay:

2,4,6-TCP induced structural aberrations in the presence of S9 mix. Detailed description of the results is not given. Data is shown as a graph only.

## CONCLUSION:

In a publication from 1998, the potential of 2,4,6-TCP to induce chromosome aberrations in cultured Chinese hamster lung fibroblasts in the absence and presence of an exogenous metabolic activation system was assessed. 100 metaphases were evaluated at each test item concentration. The highest applied concentration in this study was 1.5 mg/mL (inferred from graphical representation of the data); no justification for the top concentration of 2,4,6-TCP was provided. 2,4,6-TCP induced structural aberrations in the presence of S9. Criteria for a positive response were based on laboratory historical control data (Matsuoka, 1991).

Overall, 2,4,6-TCP induced chromosomal aberrations in chinese hamster lung fibroblasts in vitro. HSE notes that there are several reporting deficiencies in this study.

#### REFERENCES:

Matsuoka A, Sofuni T, Miyata N, Ishidate M (1991). *Clastogenicity of 1-nitropyrene, dinitropyrenes, fluorene and mononitrofluorenes in cultured Chinese hamster cells. Mutat. Res., 259, 103-110.*

(Matsuoka A *et al*, 1998)

<b>Report:</b>	K-CA 5.8.1/36 Juhl U. (1989). The <i>In Vitro</i> Metabolites of 2,4,6-Trichlorophenol and their DNA Strand Breaking Properties. Fachbereich Biologie, Universität Oldenburg, 2900 Oldenburg (F.R.G.). Published: Juhl U, Blum K, Witte I (1989). The in vitro metabolites of 2,4,6-trichlorophenol and their DNA strand breaking properties. Chem-Biol Interactions 69:333-344. Syngenta File No. NA_13757.
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**STUDY TYPE:** *In vitro* metabolism and DNA strand breakage assay.

**TEST MATERIAL (PURITY):** 2,4,6-TCP, (purchased at 98%, recrystallized purity not stated)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

#### EXECUTIVE SUMMARY

After incubation of 2,4,6-trichlorophenol (2,4,6-TCP) with a rat liver S9 fraction, three metabolites were identified: 2,6-dichloro-1,4- hydroquinone (DHQ), and two isomers of hydroxypentachlorodiphenyl ether. The 2,6-dichloro-1,4-semiquinoneDHQ free radical was identified by electron spin resonance (ESR) spectroscopy.

Incubation of a mixture of metabolites with PM2 DNA at pH 7.2 resulted in single strand breaks. Addition of catalase and dimethylsulfoxide (DMSO) inhibited this DNA strand scission.

**After *in vitro* metabolism using rat liver S9 fractions, 2,4,6-TCP induced single strand breaks in PM2 DNA. Mechanistic studies concluded that reactive oxygen species, produced during the formation of a semiquinone radical, were responsible for the observed DNA damage.**

#### MATERIALS AND METHODS

##### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Purchased at 98%, recrystallized purity not stated
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated



<b>Control Materials:</b>	
<b>Negative:</b>	DMSO/Cremophore (1:1)
<b>Positive control:</b>	No positive control used

**Mammalian metabolic system: S9 derived**

	Induced	X <sup>a</sup>	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
X	Induced and non-induced		None		Hamster		Other
			Other β-naphthoflavone		Other		

X indicates those that apply

X<sup>a</sup> induced fractions

Aroclor-1254-induced rat liver fractions were purchased from Organon Technika (Freiburg, F.R.G.). For non-induced liver S9 fractions 8-10-week old male Wistar rats were used. The homogenates were prepared by the method of Ames (Ames et al, 1975). The S9 mix contained 10% S9 fraction and cofactors (8 mmol/l MgCl<sub>2</sub>, 33 mmol/L KCl, 5 mmol/l glucose-6-phosphate, 4 mmol/l NADP, 0.1 mol/L sodium phosphate buffer pH 7.2). The final protein concentration was 2.0-3.0 mg/mL.

**Study Design and Methods:**

**In-life dates:** Not indicated

**Test performance:****Metabolism with S9 fraction**

2,4,6-TCP in phosphate buffer was added to the S9 fraction at a final concentration of 1 mmol/L. Control experiments either did not contain the NADPH-generating system, or contained no 2,4,6-TCP.

Incubations proceeded at 37 °C. At various time intervals from 0 to 24 h, 0.5-mL aliquots were mixed with 2 vols. of ice-cold acetone, and the precipitate was removed by centrifugation. The supernatant was dried in a rotary evaporator, and the residue re-dissolved in 0.5 mL ethyl acetate. For gas chromatographic (GC) analysis the samples were mixed with one quarter vol. of 0.02 mol/L triethylsulfoniumhydroxide (TESH) in methanol/water (70:30) for pyrolytic ethylation of hydroxyl groups. GC analysis was performed on a Varian 3700 GC instrument, equipped with an electron capture detector, and a 50 m x 0.25 mm SE54 column. Separation was obtained with a temperature program of 170-270°C over a 30-min period (5°C per min, hold 10 min). 2,4,6-Tribromophenol was used as an external standard. The relative concentrations of metabolites were calculated from the ratio of the peak area of metabolite to the peak area of external standard. For identification of metabolites by GC-MS the residue was directly dissolved in a small volume of TESH after evaporation. The sample was analysed on a Varian 3700 GC, coupled to a Finnigan MAT 212 MS equipped with a SS 300 data system.

**Measurement of DNA strand breakage**

Aliquots (0.5 mL) of supernatant from microsomal incubations were dried, dissolved in 1 mL ethyl acetate and transferred to a new bottle. After a second evaporation the residue was carefully redissolved in 0.2 mL 0.1 mol/L sodium phosphate buffer (pH 7.2). Quantitative GC analysis showed that this solution contained 50, 60 and 65% of metabolites I, II and III, respectively, relative to the amounts in the supernatant. Aliquots of 30 µL of the metabolite solution were immediately incubated for 4 h at 37°C with PM2 DNA (0.2 µg DNA per 35-µL sample) from Boehringer, Mannheim (F.R.G.). The reaction was stopped by placing the samples on ice and by addition of 0.25% bromophenol blue and 10% Ficoll in water. Superhelical DNA was separated from the nicked form on 0.8% agarose slab gels by electrophoresis for 7 h at 80 V. The gels were stained with ethidiumbromide (1 mg/L) for 1 h, destained for 15 min in water, and photographed during illumination with long-wave UV light using a Polaroid instant pack film. The DNA bands were scanned with a Joyce Loeble Chromoscan, and the

peak areas were determined with a Kontron Digiplan semiautomatic integrator. The strand breaks N per PM2 molecule were calculated from  $N = -\ln \alpha$ , where  $\alpha$  is the fraction of superhelical DNA molecules. In specified experiments, DMSO or catalase (from beef liver, Boehringer/Mannheim, spec. act.: 65,000 U/mg) were added to the incubation solution as inhibitors of ROS.

## RESULTS

### Gas chromatographic analyses

During the in vitro activation of 2,4,6-TCP with rat liver fractions three metabolites were gas chromatographically observed. Metabolites I, II, and III had retention times of 5.6, 17.7 and 18.9 min, respectively. Metabolite I and III production was enhanced with induced fractions, and metabolite II was not.

### Identification of metabolites

Identification of the metabolic products was performed by GC-MS. Metabolite I was identified as a dichlorodihydroxy-benzene on the basis of the molecular ion peak cluster (234/236/238), the fragment ion cluster (206/ 208/210), and the base ion cluster (178/180/182). The position of dechlorination and subsequent hydroxylation could not be determined from the mass spectrum; this was done by ESR spectroscopy. The metabolite was identified as 2,6-dichloro-1,4-hydroquinone. The corresponding semiquinone radical gave a three-line ESR spectrum (relative intensities 1:2:1) with broadened hyperfine lines. Metabolites II and III were isomeric compounds. They eluted with different retention times, but their mass spectra were very similar. The compounds were identified as isomers of hydroxypentachlorodiphenyl ether on the basis of the molecular ion cluster (384/386/388) and the fragment ion cluster of 356/358/360. Both show ratios of abundances of five chlorine atoms in the molecule. Subsequent losses of two chlorine atoms are also visible in the fragmentation pattern (from 358 to 323 and from 323 to 288).

### DNA strand scission

The average single strand break per PM2 molecule for samples with non-induced S9 was 0.2 and with Aroclor-induced fractions, 0.3. When Aroclor induced S9 fraction was used, the number of breaks/PM2 molecules increased with the time of metabolic activation up to 24 h and was much higher than with non-induced S9. The metabolite-containing solutions, which were incubated with DNA, were gas chromatographically examined to determine the concentrations of each metabolite. The number of strand breaks/PM2 DNA molecule and the concentrations of every metabolite of 14 different time periods (three experiments) were correlated with each other. The dihydroquinone (metabolite I) showed excellent correlation ( $r = 0.97$ ), while metabolites III and II had medium ( $r = 0.55$ ) to little correlation ( $r = 0.21$ ), respectively. This indicated that the hydroquinone metabolite was involved in the formation of DNA strand breaks.

### Scavenging of oxygen radicals

The addition of catalase, a specific  $H_2O_2$  scavenging enzyme, and DMSO, which reacts with  $\bullet OH$  radicals, resulted in the total inhibition of strand breaks. These data show that ROS are responsible for the DNA single strand breakage.

## CONCLUSION:

In a publication from 1989, 2,4,6-TCP was incubated with a rat liver S9 fraction. Three metabolites were identified: 2,6-dichloro-1,4- hydroquinone (DHQ), and two isomers of hydroxypentachlorodiphenyl ether. These metabolites were then incubated with PM2 DNA at pH 7.2 and produced single strand breaks.

Overall, after in vitro metabolism using rat liver S9 fractions, 2,4,6-TCP induced single strand breaks in PM2 DNA. HSE notes that this test is not a standard genotoxicity test.

## REFERENCES:

Ames B, McCann J and Yamasaki E (1975). *Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. Mutat. Res., 31:347-364.*

(Juhl U *et al.*, 1989)

<b>Report:</b>	K-CA 5.8.1/37 Kitchin K. and Brown J. (1988). Biochemical Effects of Three Chlorinated Phenols in Rat Liver. Developmental and Cell Toxicology Division, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA. Published: Kitchin K and Brown J (1988). Biochemical Effects of Three Chlorinated Phenols in Rat Liver. Toxicological and Environmental Chemistry 16:165-172. Syngenta File No. NA_13781.
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**STUDY TYPE:** Measurement of Biochemical changes in the liver after acute exposure in the rat.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (98%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1 (3 missing positive control)**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

2,4,6-trichlorophenol (2,4,6-TCP) was dosed to female rats at 164 and 500 mg/kg p.o., 21 and 4 hours prior to sacrifice, following which, ornithine decarboxylase (ODC) activity, serum alanine aminotransferase (SGPT) activity, alanine aminotransferase levels, glutathione content and cytochrome P-450 content were measured. DNA damage in liver and white blood cells was investigated in the animals using an alkaline elution test.

No evidence of DNA damage in either blood or liver was found after administration of 2,4,6-TCP.

2,4,6-TCP did not alter either hepatic glutathione content, cytochrome P-450 content or SGPT activity.

At a higher dose of 500mg/kg (data not presented) 2,4,6-TCP, hepatic ODC activity was significantly increased from  $2.44 \pm 0.70$  to  $5.03 \pm 0.60$  nmol CO<sub>2</sub>/g liver/hour ( $P < 0.05$ , data not presented). Cytochrome P-450 content was also significantly increased from  $2.31 \pm 0.30$  to  $3.26 \pm 0.30$  nmol/g liver ( $P < 0.05$ , data not presented). No alterations were observed in SGPT activity, glutathione content or alkaline elution of DNA from rats given 500 mg/kg of 2,4,6-TCP.

**Under the conditions of the study reported, the test substance 2,4,6-TCP had no effect on hepatic glutathione content and SGPT activity, or alkaline elution of DNA in both white blood cells and hepatocytes. At a higher dose of 500 mg/kg, hepatic ODC activity and cytochrome P-450 content were both significantly increased.**

## MATERIALS AND METHODS

### Materials:

<b>Non-Radiolabelled Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	98%
<b>Contaminants:</b>	Not indicated
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

Vehicle: 16% Acetone/84% Corn oil

**Preparation of dosing solutions:** 2,4,6-TCP was administered by oral gavage, after formulation in 16% acetone/84% corn oil.

<b>Test Animals:</b>	
<b>Species:</b>	Rat
<b>Strain:</b>	Sprague-Dawley
<b>Age/weight at dosing:</b>	90 day old
<b>Source:</b>	
<b>Housing:</b>	Not indicated
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	Not indicated
<b>Water:</b>	Not indicated
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Not indicated

### Study Design and Methods:

**In-life dates:** Not indicated

#### Animal treatment

Adult female rats were orally given 2,4,6-TCP, 21 and 4 hours before sacrifice. Doses of 1/5 LD50 values were selected, 164 mg/kg was selected for the main study. Treatment and control groups consisted of 9 and 11 rats respectively.

Results from animals dosed at 500 mg/kg are mentioned in the results section, but this dose group is not discussed in materials and methods (e.g. dose rationale/preparation), and data is not presented.

#### Liver tissue preparation.

To prepare subcellular tissue fractions, liver tissue (1.5 g) was homogenized in 6 ml of ice-cold pH 7.5 buffer containing NaCl (136 mM), KCl (5.4 mM), HEPES (20 mM), dithioerythritol (5mM), EDTA (4 mM) and pyridoxal-5'-phosphate (0.08 mM). Liver samples were homogenized with six strokes with a size C Potter-Elvehjem homogenizer (clearance 0.15-0.23 mm) operated at 300 rpm. After a 10-min settling period at 4°C, 75 µL of the whole liver homogenate were used for alkaline elution.

### White cell preparation

White cells were separated from reticulocytes by layering 4 ml of a 50% solution of blood in physiological saline on top of LSM Lymphocyte Separation Medium and centrifuging for 10 minutes at 500g.

### DNA elution

The Stout and Becker (Stout and Becker, 1982) modification of the Kohn (Kohn *et al.*, 1981) basic alkaline elution procedure for minimizing protein adsorption was employed. A 48-hour delay in the SDS-lysis step (Nicolini *et al.* 1985) addition of 0.6 % Sarkosyl to the first EDTA wash and addition of 5mM phosphate buffer to increase the buffering capacity of the pH 12.10 eluting solution were the additional modifications of the alkaline elution technique. Alkaline elution data are expressed as the fraction of DNA eluted during the 14-hour period. A fraction of 1.00 DNA eluted means 100% of the DNA was eluted from the filter.

### Liver biochemistry

Ornithine decarboxylase activity (Nebert *et al.*, 1982), glutathione content (Cohn *et al.*, 1966, Hissin *et al.*, 1976), cytochrome P-450 content (Omura *et al.*, 1964) and serum alanine aminotransferase activity assays (Plaa *et al.*, 1982) were performed by standard methods. The highspeed supernatant, whole homogenate, microsomes and serum, respectively, were utilized for these four assays. The post-mitochondrial supernatant (20 min, 20000g) was spun at 193 000 g for 40 min to produce the microsomal fraction and the high-speed supernatant. The subcellular fractionation method used in this study gives a low yield of microsomal protein per gram liver. Thus the values of cytochrome P-450 content per gram liver reported here are lower than many published values.

### Statistical analysis

Statistical analysis employed analysis of variance and Dunnet's test. Where statistically significant differences were found, they were then evaluated with a Student's ttest.

## RESULTS

No evidence of DNA damage in either blood or liver was found after administration of 2,4,6-TCP.

2,4,6-TCP did not alter either hepatic glutathione content, cytochrome P-450 content or SGPT activity.

**Table 6.8.1-78: Results of 2,4,6-TCP exposure on DNA damage in blood and liver and markers of hepatotoxicity**

Treatment	Blood alkaline elution (fraction of DNA eluted)	Liver alkaline elution (fraction of DNA eluted)	Ornithine decarboxylase (nmol CO <sub>2</sub> /g liver/h)	Glutathione (umol/g)	Cytochrome P-450 (nmol/g)	Alanine aminotransferase (1U/L)
Vehicle	0.082 ± 0.013	0.150 ± 0.024	2.02 ± 0.30	4.71 ± 0.26	4.34 ± 0.45	10.6 ± 1.6
2,4,6-TCP 164 mg/kg	0.057 ± 0.009	0.117 ± 0.009	2.28 ± 0.39	4.39 ± 0.40	4.71 ± 0.43	11.0 ± 1.1

At a higher dose of 500 mg/kg (data not presented) 2,4,6-TCP, hepatic ODC activity was significantly increased from 2.44 ± 0.70 to 5.03 ± 0.60 nmol CO<sub>2</sub>/g liver/h ( $P < 0.05$ , data not presented). Cytochrome P-450 content was also significantly increased from 2.31 ± 0.30 to 3.26 ± 0.30 nmol/g liver ( $P < 0.05$ , data not presented). No alterations were observed in SGPT activity, glutathione content or alkaline elution of DNA from rats given 500 mg/kg of 2,4,6-TCP.

## CONCLUSION:

In a publication from 1988, 2,4,6-TCP was dosed to female rats at 164 and 500 mg/kg bw orally, 21 and 4 hours prior to sacrifice, following which, ornithine decarboxylase (ODC) activity, serum alanine aminotransferase (SGPT) activity, alanine aminotransferase levels, glutathione content and cytochrome P-450 content were measured. DNA damage in liver and white blood cells was investigated in the animals using an alkaline elution test.

No evidence of DNA damage in either blood or liver was found after administration of 2,4,6-TCP. 2,4,6-TCP did not alter either hepatic glutathione content, cytochrome P-450 content or SGPT activity. At a higher dose of 500 mg/kg bw, hepatic ODC activity and cytochrome P-450 content were both significantly increased.

Overall, 2,4,6-TCP caused no DNA damage in liver and blood in vivo in rats. HSE notes that this test is not a standard in vivo genotoxicity test.

#### REFERENCES:

Kohn K, Ewig R, Erickson L and Zwelling L. *DNA Repair, A Laboratory Manual of Research Procedures* (P. C. Hanawalt and E. C. Friedberg, eds.) (Marcel-Dekker, New York, 1981), pp. 379-401.

Stout D and Becker F (1982). *Fluorometric quantitation of single stranded DNA: A method applicable to the technique of alkaline elution. Analytical Biochemistry* 127:302-307.

Nicolini C, Robbiano L, Pino A, Maura A, Finollo R and Brambilla G (1985). *Higher sensitivity for the detection of chemically-induced DNA damage: role of DNA unfolding in determining alkaline elution rate. Carcinogenesis* 6: 385-389.

Nebert D, Jensen N, Perry J and Oka T (1980). *Association between ornithine decarboxylase induction and the Ah locus in mice treated with polycyclic aromatic compounds. Biol. Chem.* 255:6836-6842.

Cohn V and Lyle J (1966). *A fluorometric assay for glutathione. Analytical Biochemistry* 14:434-440.

Hissin P and Hilf R (1976). *A fluorometric method for determination of oxidized and reduced glutathione in tissues. Analytical Biochemistry* 74:214-226.

Omura T and Sato R (1964). *The Carbon Monoxide-binding Pigment of Liver Microsomes. I. evidence for its hemoprotein nature. Biological Chemistry* 239:2370-2378.

Plaa G and Hewitt W. In: *Principles and Methods of Toxicology* (A. W. Hayes, ed.) (Raven Press, New York, 1982), pp. 407-445.

(Kitchin K and Brown J, 1988)

<b>Report:</b>	K-CA 5.8.1/38 Miyagawa M. <i>et al.</i> , (1995). The <i>in vivo-in vitro</i> Replicative DNA Synthesis (RDS) Test with Hepatocytes Prepared from Male B6C3F1 Mice as an Early Prediction Assay for Putative Nongenotoxic (Ames-negative) Mouse Hepatocarcinogens. Yokohama Laboratory, Mitsubishi Chemical Safety Institute Ltd., 1000, and Kamoshida-cho, Aoba-ku, Yokohama 227, Japan. Published: Miyagawa M, Takasawa H, Sugiyama A, Inoue Y, Murata T, Uno Y, Yoshikawa K, (1995). The <i>in vivo-in vitro</i> replicative DNA synthesis (RDS) test with hepatocytes prepared from male B6C3F1 mice as an early prediction assay for putative nongenotoxic (Ames-negative) mouse hepatocarcinogens. <i>Mutation Research</i> 343:157-183. Syngenta File No. NA_13787.
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**STUDY TYPE:** *In vivo-in vitro* Replicative DNA synthesis test with hepatocytes prepared from mice.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 2**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The potential for 2,4,6-trichlorophenol (2,4,6-TCP) to induce replicative DNA synthesis in hepatocytes isolated from the liver of B6C3F1 mice was investigated, after the compound was administered by oral gavage up to 2000 mg/kg.

At the sampling times of 24, 39 and 48 h, 2,4,6-TCP did not cause an increase in replicative DNA synthesis in the hepatocytes at any dose tested.

A decrease in hepatocyte viability was observed after mice were dosed with 2000 mg/kg and sampled at 24 h ( $74\% \pm 7.6$  vs.  $56 \pm 0.1$ ). Although still decreased at later time points, the cell viabilities were no statistically significant compared to control.

**Under the experimental conditions reported, 2,4,6-TCP did not cause an increase in replicative DNA synthesis in the liver of B6C3F1 mice after oral gavage dosing. 2,4,6-TCP was therefore negative in the assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>			
<b>Negative control (if not vehicle) :</b>	N/A	<b>Final Volume:</b> N/A	<b>Route:</b> N/A
<b>Vehicle:</b>	<b>Corn oil</b>	<b>Final Volume:</b> Not indicated	<b>Route:</b> oral
<b>Positive control :</b>	N/A	<b>Final Doses:</b> N/A	<b>Route:</b> oral

<b>Test Animals:</b>	
<b>Species</b>	Mice
<b>Strain</b>	B6C3F1
<b>Age/weight at dosing</b>	8 weeks
<b>Source</b>	
<b>Housing</b>	4/5 per cage
<b>Acclimatisation period</b>	Not specified
<b>Diet</b>	<i>ad libitum</i>
<b>Water</b>	Mains water <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 20-24°C Humidity: 40-70% Air changes: Not specified Photoperiod: 12h dark/12 h light

**Test compound administration:**

	<b>Dose Levels (mg/kg)</b>	<b>Final Volume</b>	<b>Route</b>
<b>Preliminary:</b>	Not specified	Not specified	Oral
<b>Main Study:</b>	1000, 2000	Not specified	Oral

**Study Design and Methods:**

**In-life dates:** Not indicated

**TEST PERFORMANCE****Preliminary Toxicity Assay:**

Simple acute toxicity testing was first conducted using 4 or 5 mice, and the MTD was set at about half the LD50, with a limit of 2000 mg/kg. Subsequently, 4 or 5 mice per group were exposed to MTD and ½ MTD dosages. After 24, 39 and 48 h, hepatocytes were prepared at each set time as described below.

**Experimental design**

<b>Treatment</b>	<b>Dose</b>	<b>Number of Animals /Time of kill</b>		
		<b>24 h</b>	<b>39 h</b>	<b>48 h</b>
Vehicle control	0 mg/kg	4 or 5	4 or 5	4 or 5
2,4,6-TCP	1000 mg/kg	4 or 5	4 or 5	4 or 5
2,4,6-trichlorophenol TCP	2000 mg/kg	4 or 5	4 or 5	4 or 5

**Hepatocyte isolation and measurement of RDS induction**

The procedures were principally conducted according to our methods for rat-hepatocyte preparation (Uno *et al.*, 1992a,b), essentially in line with those reported by other researchers (Williams *et al.*, 1977; Mirsalis *et al.*, 1985; Doolittle *et al.*, 1987a). After anesthetizing with 5% pentobarbital sodium, the portal veins were cannulated with 25G needles and the livers perfused in situ with Hanks' balanced salt solution without Ca<sup>2+</sup> and Mg<sup>2+</sup>, containing 0.5 mM EGTA and 10 mM Hepes, pH 7.2 to 7.3, at 37°C and a flow rate of 6 mL/min for a total of 6 mL. Subsequently, 0.05% collagenase type IV solution containing 10 mg/mL of BSA, 50 µg/mL of trypsin inhibitor 560 µg/mL of calcium chloride and 10 mM Hepes, pH 7.5, maintained at 37°C, was perfused for 6 min at 6 mL/min. Perfused livers were minced and filtered, and hepatocytes were suspended in Hanks' balanced salt solution. Hepatocyte viability was determined using the conventional trypan blue exclusion test. Aliquots of 5 × 10<sup>4</sup>/mL cells obtained were seeded into each well (8.9 × 8.9 mm) of a Lab-Tek chamber, and incubated for 4 h at 37°C 5% CO<sub>2</sub> in Williams' medium E containing 370 kBq/mL of [methyl-<sup>3</sup>H]thymidine. Lab-Tek chamber slides were dipped in photographic NR-M2 emulsion, exposed at 4°C for 7 to 10 days and stained with hematoxylin solution. RDS incidences were calculated as the percentage of [methyl-<sup>3</sup>H]thymidine-



incorporating cells relative to 2000 hepatocytes counted per animal. For binuclear mouse hepatocytes, each labelled nucleus was counted individually, since it was difficult to clearly distinguish binuclear hepatocytes in observing slide samples.

#### Statistical analysis:

The statistical significance of differences in hepatocyte viability was checked with Student's t-test or the Welch test ( $\alpha = 0.05$ ).

#### Evaluation criteria:

When the RDS incidence value was 0.4% or more at 24, 39 or 48 h after compound exposure, the compound was judged as positive. An incidence of less than 0.4% was judged to be negative.

## RESULTS

#### Preliminary toxicity test

To examine the LD<sub>50</sub> for 2,4,6-TCP, simple acute toxicity experiments were performed with groups of 4 or 5 male B6C3F1 mice. The MTD dose of 2,4,6-TCP was set at the limit dose of the assay; 2000 mg/kg, and the ½ MTD dose of 1000 mg/kg.

#### Distribution of spontaneous RDS incidences in hepatocytes isolated from 8-week-old mice

Spontaneous RDS incidence data, in hepatocytes isolated from 8-week-old mice from numerous studies was presented. Spontaneous RDS incidences in hepatocytes isolated from 8-week-old mice were determined under density conditions of  $5 \times 10^4$  viable hepatocytes/mL from animals dosed with corn oil or distilled water.

The average mean RDS across the studies was  $0.15 \pm 0.08\%$  (mean + SE), and the hepatocyte viability being in order of  $77 \pm 5.3\%$  (mean + SE). The data for the mean spontaneous RDS incidences in 8 week-old mice fell within the range of 0 to 0.39% (mean +  $3 \times$  SE) with a 99.7% probability. It was considered therefore that the maximum spontaneous RDS incidence is below the 0.4% level in hepatocytes from untreated 8-week-old male B6C3F1 mice.

#### Induction of RDS in mouse hepatocytes by putative non-genotoxic mouse hepatocarcinogens and non-carcinogens

When administered 2,4,6-TCP at doses of 1000 and 2000 mg/kg, the limit dose of the assay, no increase in replicative DNA synthesis was observed at any time point sampled.

A statistically significant decrease in cell viability was observed at the 24 hour sampling time when mice were dosed with 2000 mg/kg 2,4,6-TCP, as measured by the trypan blue exclusion test.

**Table 6.8.1-79: Induction of RDS in mouse hepatocytes following exposure to 2,4,6-TCP by oral gavage**

Test substance	Sampling time (h)	Dose (mg/kg)	RDS incidence (%; Mean $\pm$ SD)	Cell viability (%; Mean $\pm$ SD)
Corn oil	-	0	$0.05 \pm 0.07$	$74 \pm 7.6$
2,4,6-TCP	24	1000	$0.08 \pm 0.07$	$79 \pm 2.1$
	39		$0.35 \pm 0.30$	$76 \pm 1.8$
	48		$0.13 \pm 0.06$	$71 \pm 5.0$
	24	2000	$0.02 \pm 0.03$	$56 \pm 0.1^a$
	39		$0.03 \pm 0.05$	$56 \pm 14.8$
	48		$0.23 \pm 0.28$	$58 \pm 8.2$

<sup>a</sup> Significant decrease compared to control ( $p < 0.01$ )

## CONCLUSION:

In a publication from 1995, the potential for 2,4,6-TCP to induce replicative DNA synthesis in hepatocytes isolated from the liver of B6C3F1 mice was investigated, after the compound was administered by oral gavage up to 2000 mg/kg bw. At the sampling times of 24, 39 and 48 h, 2,4,6-TCP did not cause an increase in replicative DNA synthesis in the hepatocytes at any dose tested. A decrease in hepatocyte viability was observed after mice were dosed with 2000 mg/kg and sampled at 24 h ( $74\% \pm 7.6$  vs.  $56 \pm 0.1$ ). Although still decreased at later time points, the cell viabilities were no statistically significant compared to control.

Overall, 2,4,6-TCP did not cause an increase in replicative DNA synthesis in the liver of B6C3F1 mice after oral gavage dosing. HSE notes that this test is not a standard in vivo genotoxicity test.

#### REFERENCES:

Uno Y, Takasawa H, Miyagawa M, Inoue Y, Murata T, Ogawa M and Yoshikawa K (1992). *In vivo-in vitro* replicative DNA synthesis (RDS) test using perfused rat livers as an early prediction assay for nongenotoxic hepatocarcinogens (I): establishment of a standard protocol, *Toxicol. Lett.* 63:191-199.

Williams G, Bermudez M and Scaramuzzino D (1977). Rat hepatocyte primary cell cultures. III. Improved dissociation and attachment techniques and enhancement of survival by culture medium, *in vitro*. *J. Tissue Cult. Assoc.* 13:809-817.

Mirsalis J, Tyson C, Loh E, Steinmetz K, Bakke J, Hamilton C, Spak D and Spalding J (1985). Induction of hepatic cell proliferation and unscheduled DNA synthesis in mouse hepatocytes following in vivo treatment. *Carcinogenesis* 6:1521-1524.

Doolittle D, Muller G and Scribner H (1987). The in vivo-in vitro hepatocyte assay for assessing DNA repair and DNA replication: studies in the CD-1 mouse. *Food Chem. Toxicol.* 25:399-405.

(Miyagawa M *et al.*, 1995)

<b>Report:</b>	K-CA 5.8.1/39 Sasaki Y.F. <i>et al.</i> , (2000). The Comet Assay with Multiple Mouse Organs: Comparison of Comet Assay Results and Carcinogenicity with 208 Chemicals Selected from the IARC Monographs and U.S. NTP Carcinogenicity Database. Hachinohe National College of Technology, Hachinohe, Japan. Published: Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. (2000). The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. <i>Crit Rev Toxicol.</i> 2000 Nov; 30(6):629-799. Syngenta File No. NA_13763.
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**STUDY TYPE:** *In vivo* mammalian alkaline comet assay.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

#### EXECUTIVE SUMMARY

208 chemicals selected from International Agency for Research on Cancer (IARC) Groups 1, 2A, 2B, 3, and 4, and from the U.S. National Toxicology Program (NTP) Carcinogenicity Database were tested for their ability to induce DNA strandbreaks *in vivo* using the comet (alkaline single cell gel electrophoresis) assay. 2,4,6-Trichlorophenol (2,4,6-TCP) was one of the compounds tested.

ddY mice were dosed with 500 mg/kg/day 2,4,6-TCP by oral gavage at the maximum tolerated dose (MTD). Organs of interest were isolated after 3 and 8h and the induction of DNA strandbreaks was analysed.

2,4,6-TCP caused a statistical significant increase in DNA strandbreaks in the stomach, colon, liver, kidney, urinary bladder, lung and brain, but not bone marrow, after a 3h sampling time, when compared to control animals. No DNA damage was observed after 8 h sampling time.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

**In conclusion, it can be stated that there were DNA strandbreaks observed in stomach, colon, liver, kidney, urinary bladder, lung and brain following oral administration of 2,4,6-TCP.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated.
<b>Lot/Batch number:</b>	Not indicated.
<b>Purity:</b>	Not indicated.
<b>Stability of test compound:</b>	Not indicated.

<b>Control Materials:</b>			
<b>Negative control (if not vehicle) :</b>	Physiological saline, Phosphate buffer, olive oil,	<b>Final Volume:</b> N/A	<b>Route:</b> oral and i.p.
<b>Vehicle:</b>	Olive oil	<b>Final Volume:</b> N/A	<b>Route:</b> oral
<b>Positive control :</b>	Methyl methansulfonate (MMS)	<b>Final Doses:</b> 80 mg/kg bw.	<b>Route:</b> i.p.

<b>Test Animals:</b>	
<b>Species</b>	Mice, male
<b>Strain</b>	ddY
<b>Age/weight at dosing</b>	7 – 10 weeks (at start of experiment) / Not indicated.
<b>Source</b>	N/A
<b>Housing</b>	N/A
<b>Acclimatisation period</b>	Performed, but no details given.
<b>Diet</b>	Pelleted standard diet, <i>ad libitum</i>
<b>Water</b>	Tap water, <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: N/A Humidity: N/A Photoperiod: N/A

**Test compound administration:**

	Dose Levels	Final Volume	Route
<b>Preliminary:</b>	Dose-sighting phase: 1000, 2000 mg/kg/day	N/A	oral
<b>Main Study:</b>	500 mg/kg/day males only	N/A	oral

**Study Design and Methods:**

**Preliminary Toxicity Assay:** Toxicity was assessed by acute toxicity testing. 2,4,6-TCP tested up to 2000 mg/kg/day by oral (gavage). The MTD was calculated as  $\frac{1}{2}$  LD<sub>50</sub>.

**In vivo comet assay:** 2,4,6-TCP was administered once by oral gavage at a dose of 500 mg/kg/day, which corresponds to the calculated MTD. The dose group consisted of 4 animals. Sampling times were 0, 3, and 8 h, and the analysed organs were stomach, colon, liver, kidney, urinary bladder, lung, brain, and bone marrow. From shortly after treatment until just before the animals were humanely killed, they were carefully observed for pharmacotoxic signs.

**Slide preparation:** Nuclei were prepared by homogenization and placed in a chilled lysing solution (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Trizma, 1% sarkosyl, 10% DMSO, and 1% Triton X-100, pH 10) and kept at 0 °C in the dark for at least 60 min. Subsequently, the slides incubated in chilled alkaline solution (300 mM NaOH and 1mM Na<sub>2</sub>EDTA, pH 13) for 10 min in the dark at 0 °C.

**Electrophoresis:** Electrophoresis was conducted at 0 °C in the dark for 15 min at 25 V (0.96 V/cm) and approximately 250 mA. All slides from mice treated with the same chemical (including the corresponding 0 time control) were electrophoresed at the same time. After being neutralized with 400 mM Trizma (pH 7.5), the slides were stained with 50 µL of 20 µg/mL ethidium bromide. 50 nuclei on one slide per organ were examined at 200 x magnification, and the length of the whole comet and the diameter measured. Migration, the difference between length and diameter were determined for each comet and the average length of DNA migration for each organ was calculated.

**Statistical analysis:** The average length of DNA migration per treatment group was compared by one way analysis of variance. When significance was obtained by this analysis, differences between treatment and control groups were compared using Dunnet's test. A p-value < 0.05 was considered statistically significant.

**Histopathology:** Necropsies were performed, and the organs were examined for changes in size, colour, and texture. A small portion of each organ was fixed in 10% formaldehyde, dehydrated, and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin and analysed by light microscopy to identify necrotic/apoptotic cells and degenerated cells. Histopathological examination was conducted when positive results were obtained in the comet assay.

**RESULTS**

**Preliminary toxicity assay:** The LD<sub>50</sub> was established at 1000 mg/kg/day. The MTD was defined as  $\frac{1}{2}$  LD<sub>50</sub>. 500 mg/kg/day was set as the concentration to be tested in the main study.

**In vivo comet assay:** 2,4,6-TCP caused a statistical significant increase in DNA strandbreaks in the stomach, colon, liver, kidney, urinary bladder, lung and brain, but not bone marrow, after a 3h sampling time, when compared to control animals. No increase in DNA strandbreaks was observed after 8 h sampling time.

**Table 6.8.1-80: Results for the *in vivo* comet assay for 2,4,6-TCP**

Dose	Tissue
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500 mg/kg 2,4,6-TCP (n=4)		S	C	L	K	Ub	Lu	Br	BM
		Migration (µM, group mean)							
Sampling time	3h	17.5	35.9	11.7	9.54	17.5	6.98	11.3	-
	8h	-	-	-	-	-	-	-	-
0-time control (n=380)		Mean migration ± SD (µM)							
		6.0 ± 1.41	4.91 ± 1.57	2.17 ± 1.01	1.97 ± 1.11	4.64 ± 1.47	2.71 ± 0.88	1.38 ± 1.01	1.18 ± 1.05

- = negative for DNA damage

S (stomach), C (colon), L (Liver), K (Kidney), Ub (Urinary bladder), Lu (Lung), Br (Brain), BM (Bone marrow)

Histopathological examination was conducted on tissues with positive results in the comet assay to exclude that the DNA damage was caused by secondary cytotoxicity. No cytotoxicity was present in the organs analysed after treatment with 2,4,6-TCP.

Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

## CONCLUSION:

In a publication from 2000, 208 chemicals selected from International Agency for Research on Cancer (IARC) Groups 1, 2A, 2B, 3, and 4, and from the U.S. National Toxicology Program (NTP) Carcinogenicity Database were tested for their ability to induce DNA strandbreaks *in vivo* using the comet (alkaline single cell gel electrophoresis) assay. 2,4,6-TCP was one of the compounds tested.

ddY mice were dosed with 500 mg/kg bw/d 2,4,6-TCP by oral gavage at the maximum tolerated dose (MTD). Organs of interest were isolated after 3 and 8 h and the induction of DNA strandbreaks was analysed. 2,4,6-TCP caused a statistical significant increase in DNA strandbreaks in the stomach, colon, liver, kidney, urinary bladder, lung and brain, but not bone marrow, after a 3 h sampling time, when compared to control animals. No DNA damage was observed after 8 h. Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced mutant colonies, indicating that the tests were sensitive and valid.

Overall, 2,4,6-TCP caused DNA strandbreaks in stomach, colon, liver, kidney, urinary bladder, lung and brain of mice following oral administration. HSE notes that there are significant deviations between this protocol and OECD TG 489.

(Sasaki Y.F *et al*, 2000)

<b>Report:</b>	K-CA 5.8.1/40 Yin D. <i>et al.</i> , (2009). Genotoxic Effect of 2,4,6-Trichlorophenol on p53 Gene in Zebrafish Liver. School of the Environment, State Key Laboratory of Pollution Control and Resource Reuse, Nanjing University 22 Hankou Road, Nanjing 210093, China. Accepted: 17 September 2008. Published: Yin D, Zhu H, Hu P, Zhao Q (2009). Genotoxic Effect of 2,4,6-trichlorophenol on P53 Gene in Zebrafish Liver. Environmental Toxicology and Chemistry 28:603-608. Syngenta File No. NA_13752.
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**STUDY TYPE:** Denaturing High-Performance Liquid Chromatography and DNA Sequencing Analyses of the p53 Gene in Zebrafish Liver.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (>98%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

No globally or regionally accepted regulatory guidelines were followed.

**KLIMISCH SCORE: 2**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

Zebrafish were treated in water with 2,4,6-Trichlorophenol (2,4,6-TCP) at doses up to 50 µg/L, to assess induction of mutations in the p53 gene. Livers were harvested after 1, 3, 6 or 10 days of treatment. An increased mutation frequency was observed at the 10 d exposure at an intermediate dose of 5 µg/L only. No increase in mutant frequency was observed after shorter time points (1, 3, and 6 d). No obvious dose response was seen.

**In conclusion, it can be stated that under the experimental conditions reported the test substance 2,4,6-TCP increases the number of mutated p53 molecules in zebrafish liver. Therefore, 2,4,6-TCP is considered to be mutagenic in the assay described.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP		
<b>Description:</b>	Not indicated		
<b>Lot/Batch number:</b>	Not indicated		
<b>Purity:</b>	>98%		
<b>CAS#:</b>	88-06-2		
<b>Stability of test compound:</b>	Not indicated		

<b>Control Materials:</b>			
<b>Negative control (if not vehicle) :</b>	Acetone	Route: Water	
<b>Vehicle:</b>	Acetone 0.05%	Route: Water	
<b>Positive control :</b>	None used	Route: N/A	

<b>Test Animals:</b>	
<b>Species</b>	Zebrafish ( <i>Danio rerio</i> )
<b>Strain</b>	Not specified
<b>Age/weight at dosing</b>	Adult
<b>Source</b>	Not indicated
<b>Housing</b>	10/tank (preliminary toxicity assay) 3/tank (genotoxicity assay)
<b>Acclimatisation period</b>	Not indicated
<b>Diet</b>	Not indicated
<b>Environmental conditions</b>	Not indicated

Test compound administration:		
	Dose Levels	Route
Preliminary:	0.82, 0.91, 1.00, 1.10, 1.21, 1.33, or 1.46 mg/L	Water
Main Study:	0.5, 5, and 50 µg/L	Water

## Study Design and Methods:

### Preliminary Toxicity Assay:

Adult zebrafish were exposed to 0.82, 0.91, 1.00, 1.10, 1.21, 1.33, or 1.46 mg/L 2,4,6-TCP for acute toxicity testing. Water containing 0.05% acetone was set as the control. For each test, 10 animals were placed in 2 L of test solution for 24, 48, 72, or 96 h in three replicates. The median lethal concentration value (LC<sub>50</sub>) was calculated according to the Trimmed Spearman–Kärber method (Hamilton, 1978).

### DHPLC Genotoxicity assay

Three fish in each group were exposed to 2,4,6-TCP at 0 (control), 0.5, 5, and 50 µg/L, respectively, for 1, 3, 6, or 10 d with normal feeding. The final vehicle (acetone) concentration was always 0.05% for all experiments. One-third volume of test solution was changed daily.

Zebrafish genomic DNA was isolated from zebrafish livers. The DNA was then used as a template to amplify the 132-bp fragment containing exon 7 of the zebrafish p53 gene via polymerase chain reaction (PCR) with the primers 5'-GTTTAACAGTCACATTTTCCT-3' (forward) and 5'-ACAAGAGGAGGAATCAAATA-3' (reverse) and the use of high-fidelity DNA polymerase. The PCR products were subcloned into the pGEM-T vector. Each positive recombinant containing the target p53 fragment was used as a template in PCR amplification, and the amplified products were subjected to denaturing high-performance liquid chromatography (DHPLC) analysis with the use of the WAVE DNA Fragment Analysis System. Every four PCR products were grouped and then denatured at 95°C for 5 min and 94.5°C for 20s and cooled down to 25°C with a temperature ramp of 0.5°C /20 s to form heteroduplexes. Eight microliters of the reannealed DNA was injected into a reversed-phase column. The melting temperature for the tested fragment was 60°C. DNA was eluted at a flow rate of 0.9 ml/min within a linear acetonitrile gradient consisting of buffer A (0.1 M triethylammonium acetate) and buffer B (0.1 M triethylammonium acetate with 25% acetonitrile) from 44% buffer B to 49% buffer B in 0.5 min, then 49% B to 54% B in 3 min. If DHPLC elution profiles indicated heteroduplexes present in a group, each PCR product was then mixed with a wild-type product, and DHPLC was performed again to finally determine which recombinant had the potential mutation. The positive recombinant was then directly sequenced from both directions to confirm the mutations. The base mutation frequency of the p53 genomic region in zebrafish was calculated as the (number of mutated bases)/(132 x number of molecules examined).

### Statistical analysis

The experimental data were subjected to the analysis of variance statistical test with a least significant difference method or  $\chi^2$  test.

## RESULTS

### Preliminary toxicity assay:

Adult zebrafish (n = 10) were treated with 0.82, 0.91, 1.00, 1.10, 1.21, 1.33, and 1.46 mg/L 2,4,6-TCP in 2 L of test solution for 96 h. The results showed that all exposed fish were dead at 1.21, 1.33, and 1.46 mg/L 2,4,6-TCP within 24 h and alive at 0.82 mg/L within 96 h, whereas some of fish survived at 0.91, 1.00, and 1.10 mg/L 2,4,6-TCP within 96 h. On the basis of the trimmed Spearman–Kärber method, the LC<sub>50</sub> values of zebrafish exposed to 2,4,6-TCP at 24, 48, 72, and 96 h were determined to be 0.98, 0.96, 0.96, and 0.96 mg/L, respectively.

**DHPLC Genotoxicity Assay:**

Adult zebrafish were treated with 0, 5 and 50 µg/L 2,4,6-TCP for 1, 3, 6 and 10d. DHPLC analysis was carried out to assess the mutation rates of the p53 gene.

Overall, among 3,126 amplified molecules examined, DHPLC analyses suggested that 96 had altered sequences. Sequencing confirmed that 81 molecules had point mutations.

The general frequencies of mutated molecules and mutated bases of the control group were 1.89% (7 of 371) and  $1.84 \times 10^{-4}$  [9 of (371· 132)], which are consistent ( $p > 0.05$ ,  $\chi^2$  test) with the error rates [3 of 394 = 0.76% or 3 of (394· 132) =  $0.58 \times 10^{-4}$ ] incurred by the PCR process, and similar to previous laboratory data (Yin, 2006)

On average, the mutation frequencies (mean  $\pm$  standard deviation) of the amplified molecules from the 10 d exposures of groups with 0, 0.5, 5, and 50 µg/L 2,4,6-TCP were  $2.21 \pm 1.47\%$  (n = 3),  $1.42 \pm 1.77\%$  (n = 3),  $5.43 \pm 1.22\%$  (n = 3), and  $3.66 \pm 4.32\%$  (n = 4), respectively. Base mutation rates were  $2.17 \pm 1.55 \times 10^{-4}$ ,  $1.41 \pm 1.31 \times 10^{-4}$ ,  $6.05 \pm 1.18 \times 10^{-4}$  and  $3.94 \pm 5.07 \times 10^{-4}$ , respectively. Analyzed by analysis of variance, the results demonstrated that the rates for groups at 5 µg/L 2,4,6-TCP were significantly higher than those of the control and 0.5 µg/L 2,4,6-TCP group ( $p < 0.05$ ); the rates for groups at 0.5 or 50 µg/L 2,4,6-TCP were similar to those of the control group ( $p > 0.05$ ).

Of the 90 mutated bases found in 2,4,6-TCP treated groups, 24 are in the introns, whereas the other 66 mutations are in exon 7, of which 13 mutations are same-sense and 53 mutations are missense, including 16 mutations that cause a premature stop codon.

In the 10 d exposure group with 5 µg/L 2,4,6-TCP, a total of 37 mutation sites are in nucleotides (NT) 23 (n = 5), 27 (n = 6), 41 (n = 1), 43 (n = 2), 44 (n = 2), 47 (n = 6), 58 (n = 1), 64 (n = 1), 66 (n = 1), 81 (n = 1), 94 (n = 1), 106 (n = 2), 116 (n = 1), 120 (n = 1), 124 (n = 2), 135 (n = 2), 136 (n = 1), and 148 (n = 1). Statistical tests reveal that the rates of mutations occurring at NT23, NT27, and NT47 are significantly higher than those at other sites ( $p < 0.05$ ,  $\chi^2$  test). The results suggest that NT23 (intron 6), NT27 (intron 6), and NT47 (exon 7) are hot-spot mutation sites caused by 2,4,6-TCP exposure.

To test whether it is time-dependent for 2,4,6-TCP to induce point mutation, zebrafish were treated with 5 µg/L 2,4,6-TCP for 1, 3, and 6 d. Results showed that the mutation frequencies of the amplified molecules were  $1.18 \pm 0.55$  (n = 3) at 1 d,  $0.59 \pm 1.02$  (n = 3) at 3 d, and  $1.47 \pm 1.00$  (n = 3) at 6 d. Also, the base mutation rates at 1, 3, and 6 d were  $0.90 \pm 0.42 \times 10^{-4}$ ,  $0.45 \pm 0.78 \times 10^{-4}$  and  $1.12 \pm 0.76 \times 10^{-4}$ , respectively. Statistical analysis revealed that the rates were all similar to those of the control group without 2,4,6-TCP exposure ( $p > 0.05$ ) but significantly less than ( $p < 0.05$ ) those of the 5 µg/L group with 2,4,6-TCP exposure for 10 d.

**Number of mutated molecules detected by denaturing high-performance liquid chromatography (DHPLC), sequencing or both:**



TCP exposure (µg/L) <sup>a</sup>	Exposure (d)	Zebrafish examined	No. of molecules examined	Mutated molecules				
				DHPLC	Sequencing		Mutated nucleotide	
					Total	Mutation rate (%)	Total	Mutation rate (%)
0	10	C1	149	3	2	1.34	3	1.53
		C2	145	2	2	1.38	2	1.04
		C3	77	3	3	3.90	4	3.94
		Subtotal	371	8	7	2.21 ± 1.47 <sup>b</sup>	9	2.17 ± 1.55 <sup>b</sup>
0.5	10	D1	114	0	0	0.00	0	0.00
		D2	117	4	4	3.41	4	2.59
		D3	116	1	1	0.86	1	0.65
		Subtotal	346	5	5	1.42 ± 1.77 <sup>b</sup>	5	1.41 ± 1.31 <sup>b</sup>
5	10	B1	113	11	7	6.19	11	7.37
		B2	199	11	8	4.02	15	5.71
		B3	164	11	10	6.09	11	5.08
		Subtotal	476	33	25	5.43 ± 1.22 <sup>b</sup>	37	6.05 ± 1.18 <sup>b</sup>
	6	B61	111	1	1	0.90	1	0.68
		B62	112	2	1	0.89	1	0.68
		B63	114	3	3	2.63	3	1.99
		Subtotal	347	6	5	1.47 ± 1.00 <sup>b</sup>	5	1.12 ± 0.76 <sup>b</sup>
	3	B31	112	0	0	0.00	0	0.00
		B32	120	0	0	0.00	0	0.00
		B33	112	2	2	1.78	2	1.35
		Subtotal	344	2	2	0.59 ± 1.02 <sup>b</sup>	2	0.45 ± 0.78 <sup>b</sup>
	1	B11	110	3	2	1.82	2	1.38
		B12	114	1	1	0.88	1	0.66
		B13	117	1	1	0.85	1	0.65
		Subtotal	341	5	4	1.18 ± 0.55 <sup>b</sup>	4	0.90 ± 0.42 <sup>b</sup>
50	10	A1	163	15	13	7.98	14	6.51
		A2	102	3	2	1.96	2	1.48
		A3	126	14	13	10.32	20	12.02
		A4	116	2	2	1.72	2	1.31
		Subtotal	507	34	30	3.66 ± 4.32 <sup>b</sup>	38	3.94 ± 5.07 <sup>b</sup>
Plasmid <sup>c</sup>	HF2		394	3	3	0.76	3	0.58
Total			3,126	96	81		102	

<sup>a</sup> TCP = 2,4,6-trichlorophenol.  
<sup>b</sup> Mean ± standard deviation.  
<sup>c</sup> Plasmid and HF2 indicate that the molecules used as controls for DHPLC analysis or DNA sequencing are amplified from the plasmid holding the 173-base pair fragment of the *p53* gene with high-fidelity DNA polymerase (HF2).

## CONCLUSION:

In a publication from 2009, zebrafish were treated in water with 2,4,6-TCP at concentrations doses up to 50 µg/L, to assess induction of mutations in the *p53* gene. Livers were harvested after 1, 3, 6 or 10 days of treatment. An increased mutation frequency was observed at the 10 d exposure at an intermediate concentration of 5 µg/L only. No increase in mutant frequency was observed after shorter time points (1, 3, and 6 d). No obvious dose response was seen.

Overall, 2,4,6-TCP increased the number of mutations in *p53* gene in zebrafish liver. HSE notes that this is not a standard in vivo genotoxicity assay.

## REFERENCES:

Hamilton MA, Russo RC, Thurston RV (1977). *Trimmed Spearman–Kärber method for estimating median lethal concentrations in toxicity bioassays. Environ Sci Technol* 11:714–719; Correction 12:417 (1978).

Yin D, Gu Y, Li Y, Wang X, Zhao Q (2006). *Pentachlorophenol treatment in vivo elevates point mutation rate in zebrafish p53 gene. Mutat Res* 609:92–101.

(Yin D et al, 2009)

Following the EU peer-review process, it was concluded that the genotoxic potential of 2,4,6-TCP was inconclusive, based on positive results observed in vitro and inconsistent results observed in vivo, and needed to be clarified. On this basis, Syngenta recently submitted a modern package of three in vitro tests (Ames, micronucleus and mammalian cell gene mutation tests) and an in vivo TGR (Transgenic rodent) assay in rats.

These new studies have been fully evaluated by HSE and are presented below.

## Ames test

Report reference	██████████, 2020
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Report year	2020
Report title	CA6519 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay.
Report No	2113900
Document No	VV-877637
Guidelines followed in study	OECD 471 (1997)
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
Test substance and purity	2,4,6-TCP, batch MES663/2, 99% w/w
GLP	Yes
Acceptability/Reliability	Yes

## Methods

In a GLP and OECD guideline study, 2,4,6-TCP was assessed for its potential to induce gene mutations in the plate incorporation test (Experiment I) and the pre-incubation test (Experiment II) using *S. typhimurium* strains TA1535, TA1537, TA98, and TA100, and the *E. coli* strains WP2 (pKM101) and WP2 uvrA (pKM101).

In the pre-experiment, the concentration range of the test substance was 3 - 5000 µg/plate. The pre-experiment is reported as Experiment I. Depending on the cytotoxic effects observed in experiment I, seven or eight concentrations were tested in Experiment II. The chosen maximal concentration in Experiment II was 2500 µg/plate for the *S. typhimurium* strains and 1000 µg/plate for the *E. coli* strains due to the levels of cytotoxicity observed in Experiment I in all strains.

The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

## Results

In Experiment I, cytotoxic effects were seen, depending on strain, from 333 µg/plate with and without S9. In Experiment II, cytotoxicity was seen from around 1000 µg/plate in the presence and absence of S9.

Precipitation of the test item in the overlay agar on the incubated agar plates was observed in Experiment I at 5000 µg/plate in the absence of S9 and from 2500 to 5000 µg/plate in the presence of S9 and in Experiment II at 2500 µg/plate in strains TA1535, TA1537, TA98, and TA100 in the presence of S9. The undissolved particles had no influence on the data recording. Based on a lower top concentration, no precipitation of the test substance was observed in Experiment II in any strains without S9, and no precipitation was observed in strains WP2 uvrA (pKM101) and WP2 (pKM101) in the presence of S9.

No relevant increase in revertant colony numbers of any of the six tester strains was observed following treatment with 2,4,6-TCP at any concentration level, neither in the presence nor absence of metabolic activation. There was also no observed tendency of higher mutation rates with increasing concentrations.

**Table 6.8.1-81: Summary of Results Pre-Experiment/Experiment I**

Metabolic Activation	Test Group	Concentration (per plate)	Revertant Colony Counts (Mean ±SD)

			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		10 ± 3	12 ± 2	29 ± 10	101 ± 5	224 ± 12	311 ± 30
	Untreated		14 ± 2	15 ± 4	29 ± 3	110 ± 9	257 ± 38	337 ± 26
	CA6519	3 µg	10 ± 1	14 ± 4	27 ± 5	107 ± 8	256 ± 15	310 ± 22
		10 µg	11 ± 4	13 ± 3	32 ± 1	117 ± 9	245 ± 5	297 ± 4
		33 µg	8 ± 1	17 ± 3	25 ± 3	105 ± 9	195 ± 13	287 ± 8
		100 µg	10 ± 3	11 ± 3	16 ± 4	107 ± 9	128 ± 12	214 ± 11
		333 µg	11 ± 1	7 ± 3	14 ± 4	102 ± 10	25 ± 3	60 ± 4
		1000 µg	9 ± 4	4 ± 1	3 ± 1	10 ± 4	1 ± 1	9 ± 2
		2500 µg	1 ± 1	0 ± 0	0 ± 1	1 ± 1	0 ± 1	1 ± 1
		5000 µg	0 ± 0 P	0 ± 0 P	0 ± 1 P	0 ± 1 P	0 ± 0 P	0 ± 0 P
	NaN3	10 µg	1130 ± 78			1671 ± 9		
	4-NOPD	10 µg			686 ± 46			
	4-NOPD	50 µg		92 ± 8				
	MMS	2.0 µL					3473 ± 154	3488 ± 127
With Activation	DMSO		14 ± 2	14 ± 4	36 ± 1	96 ± 6	238 ± 36	347 ± 9
	Untreated		12 ± 3	16 ± 3	39 ± 9	108 ± 15	286 ± 6	367 ± 13
	CA6519	3 µg	12 ± 1	12 ± 3	29 ± 9	97 ± 8	263 ± 32	363 ± 22
		10 µg	11 ± 2	14 ± 2	37 ± 5	110 ± 17	237 ± 8	304 ± 22
		33 µg	10 ± 2	13 ± 2	33 ± 4	102 ± 11	201 ± 12	314 ± 19
		100 µg	11 ± 1	8 ± 2	29 ± 9	112 ± 14	160 ± 19	277 ± 43
		333 µg	13 ± 3	2 ± 1	25 ± 4	121 ± 13	42 ± 9	92 ± 18
		1000 µg	14 ± 3	1 ± 1	3 ± 1	17 ± 3	6 ± 1	29 ± 7
		2500 µg	5 ± 1 P	0 ± 1 P	0 ± 0 P	5 ± 1 P	0 ± 1 P	0 ± 1 P
		5000 µg	0 ± 1 P	0 ± 0 P	0 ± 0 P	0 ± 0 P	0 ± 0 P	0 ± 0 P
	2-AA	2.5 µg	276 ± 40	318 ± 29	2591 ± 84	3398 ± 174		
							970 ± 18	1730 ± 197
	2-AA	10.0 µg						
NaN3	sodium azide			P	Precipitate			
2-AA	2-aminoanthracene							
4-NOPD	4-nitro-o-phenylene-diamine							
MMS	methyl methane sulfonate							

Table 6.8.1-82: Summary of Results Experiment II

Metabolic Activation	Test Group	Concentration (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		9 ± 3	10 ± 3	32 ± 7	110 ± 21	289 ± 15	385 ± 7
	Untreated		7 ± 0	10 ± 1	36 ± 6	142 ± 7	315 ± 32	401 ± 3
	CA6519	1 µg		13 ± 2	38 ± 11		300 ± 16	393 ± 15
		3 µg	10 ± 2	8 ± 3	28 ± 8	112 ± 23	297 ± 42	383 ± 9
		10 µg	9 ± 3	9 ± 2	30 ± 6	118 ± 12	279 ± 22	377 ± 28
		33 µg	7 ± 3	10 ± 3	30 ± 7	109 ± 12	262 ± 26	372 ± 22
		100 µg	8 ± 2	10 ± 1	39 ± 3	105 ± 7	248 ± 5	343 ± 14
		333 µg	11 ± 1	8 ± 3	24 ± 4	140 ± 18	150 ± 13	257 ± 10
		1000 µg	6 ± 2	1 ± 1 R	1 ± 1 R M	21 ± 7 R	16 ± 4	82 ± 15
		2500 µg	0 ± 0 R	0 ± 0 R	0 ± 0 R	0 ± 0 R		
	NaN3	10 µg	784 ± 49			1850 ± 49		
	4-NOPD	10 µg			1099 ± 84			
	4-NOPD	50 µg		92 ± 18				

	MMS	2.0 µL					4219 ± 267	±	3903 ± 91
With	DMSO		10 ± 1	11 ± 3	48 ± 14	98 ± 9	293 ± 16		392 ± 7
Activation	Untreated		8 ± 3	10 ± 3	49 ± 11	114 ± 5	340 ± 5		472 ± 10
	CA6519	1 µg		13 ± 3	34 ± 2		326 ± 8		404 ± 10
		3 µg	11 ± 1	13 ± 3	42 ± 13	133 ± 31	321 ± 24		396 ± 7
		10 µg	10 ± 3	11 ± 1	35 ± 4	141 ± 14	297 ± 16		399 ± 7
		33 µg	9 ± 3	14 ± 2	37 ± 10	126 ± 24	296 ± 38		386 ± 34
		100 µg	8 ± 2	7 ± 3	40 ± 4	129 ± 10	268 ± 59		391 ± 4
		333 µg	7 ± 1	8 ± 1	20 ± 6	123 ± 15	187 ± 24		309 ± 41
		1000 µg	7 ± 3	4 ± 2 R	2 ± 1 R M	25 ± 7 R	14 ± 4		74 ± 10
		2500 µg	0 ± 0 R P	0 ± 0 R P	0 ± 0 R P	0 ± 0 R P			
	2-AA	2.5 µg	194 ± 11	275 ± 11	4387 ± 267	± 3639 ± 350			
	2-AA	10.0 µg					1024 ± 30	±	2284 ± 149

NaN <sub>3</sub>	sodium azide	R	Reduced background growth
2-AA	2-aminoanthracene	P	Precipitate
4-NOPD	4-nitro-o-phenylene-diamine	M	Manual count
MMS	methyl methane sulfonate		

Appropriate reference mutagens were used as positive controls, which showed a distinct increase of induced revertant colonies consistent with the laboratory's historical control data demonstrating the sensitivity of the test system and the efficacy of the S9 mix. Each batch of S9 was also tested with 2 pro-mutagens, benzo(a)pyrene and 2-aminoanthracene.

## Conclusion

In conclusion, in this GLP and guideline Ames test, 2,4,6-TCP did not induce gene mutations with and without metabolic activation up to concentrations causing cytotoxicity and/or precipitation.

(██████, 2020)

## Cell Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT)

Report reference	██████, 2020
Report year	2020
Report title	CA6519 - Cell Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT).
Report No	2114300
Document No	VV-877648
Guidelines followed in study	OECD 476 (2016)
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
GLP	Yes
Test substance and purity	2,4,6-TCP, batch MES663/2, 99% w/w
Acceptability/Reliability	Yes

## Methods

In a GLP and guideline gene mutation assay at the HPRT locus in V79 cells, 2,4,6-TCP concentrations between 15.4 µg/mL and 1975.0 µg/mL were used in the pre-experiment with and without metabolic activation, following 4 hr treatment. The highest concentration was equal to a molar concentration of approximately 10 mM. Precipitation occurred at 987.5 µg/mL and above without metabolic activation, and at 493.8 µg/mL and above with metabolic activation. A relevant cytotoxic effect, indicated by a relative cloning efficiency of 50% or below was observed at 987.5 µg/mL and above with and without metabolic activation.

Based on the results of the pre-experiment, the following concentrations were applied in the main experiment with and without metabolic activation: 12.5; 25.0; 50.0; 100.0; 200.0; 400.0; 600.0; 800.0; and 1000.0 µg/mL for 4 hours. Duplicate cultures were employed.

## Results

In the absence of metabolic activation, precipitation occurred at 1000.0 µg/mL. Relevant cytotoxic effects indicated by a relative adjusted cloning efficiency I (survival rate) below 50% were noted at 200.0 µg/mL and above. The maximum recommended cytotoxicity level of 10-20% relative survival was achieved at 200 µg/ml. Consequently, concentrations of 25.0 to 200.0 µg/mL were evaluated for mutagenicity in the absence of metabolic activation. The observed mean mutant frequency (MF) of the solvent control and all evaluated test substance concentrations were within the 95% control limits of the solvent historical control data. In addition, linear regression analysis showed no statistically significant trend. A t-test was performed at 100.0 and 200.0 µg/mL without metabolic activation (treatment concentrations showing an MF greater than the concurrent solvent control MF). The t-test shows no statistical significance at these concentrations.

In the presence of metabolic activation, precipitation occurred at 800.0 µg/mL. Relevant cytotoxic effects indicated by a relative adjusted cloning efficiency I (survival rate) below 50% (mean value of both parallel cultures) were noted at 200.0 µg/mL and above. The maximum recommended cytotoxicity level of 10-20% relative survival was achieved at 200 µg/ml. Consequently, concentrations of 25.0 to 200.0 µg/mL were evaluated for mutagenicity. The observed MF of the solvent control and all evaluated test substance concentrations were within the 95% control limits of the solvent historical control data. Linear regression analysis showed a statistically significant trend, which was reciprocal and therefore without relevance. A t-test was performed at 25.0 and 50.0 µg/mL (treatment concentrations showing an MF greater than the concurrent solvent control MF). The t-test showed no significance.

**Table 6.8.1-83: Summary of Results, Main Experiment**

	conc. µg/mL	P	S9 mix	relative cloning efficiency I %	relative cell density %	rel. adjusted cloning efficiency I %	(MF) mutant colonies/ 106 cells	95% control limit	statistical analysis**	
Column	1	2	3	4	5	6	7	8	t-test	linear regression
Main Experiment / 4 h treatment				mean values of culture I and II						
Solvent control with DMSO*			-	100.0	100.0	100.0	10.2	3.5 - 31.0 53.5-	0.000 S	
Positive control (EMS)	300.0		-	92.3	96.9	89.3	244.3	443.6		
Test item	12.5		-	96.9	102.1	96.4	culture not continued#			
Test item	25.0		-	92.4	94.2	87.0	8.0	3.5 - 31.0	n.c.	
Test item	50.0		-	70.8	90.8	64.3	8.2	3.5 - 31.0	n.c.	0.123
Test item	100.0		-	60.1	87.8	52.6	11.4	3.5 - 31.0	0.535	

Test item	200.0	-	20.0	60.6	11.3	12.6	3.5 31.0	-	0.073		
Test item	400.0	-	0.0	30.8	0.0	culture not continued##					
Test item	600.0	-	##	4.2	culture not continued##						
Test item	800.0	-	##	7.0	culture not continued##						
Test item	1000.0	-	##	8.1	culture not continued##						
Solvent control with DMSO*		+	100.0	100.0	100.0	13.3	4.2 30.7	-	0.000	0.035***	
Positive control (DMBA)	2.3	+	85.6	115.4	98.6	89.2	38.1- 265.2	-	S		
Test item	12.5	+	101.5	105.3	105.2	culture not continued#					
Test item	25.0	+	111.2	101.6	110.5	13.3	4.2 30.7	-	0.937		
Test item	50.0	+	75.1	103.2	79.5	14.0	4.2 30.7	-	0.759		
Test item	100.0	+	63.4	104.6	65.9	10.9	4.2 30.7	-	n.c.		
Test item	200.0	+	23.7	84.2	19.9	9.7	4.2 30.7	-	n.c.		
Test item	400.0	+	0.4	10.6	0.1	culture not continued##					
Test item	600.0	+	0.0	8.4	0.0	culture not continued##					
Test item	800.0	P +	##	1.9	culture not continued##						
Test item	1000.0	P +	culture not continued##								
Test item	0	P +	culture not continued##								

\* mean value calculation based on the data of two solvent controls in each culture

\*\* statistical analysis based on the mean values of culture I and II

\*\*\* inverse trend without biological relevance

n.c. not calculated / S = significant trend (< 0.05) / P = Precipitation observed at the end of treatment

MF Mutant frequency

# culture not continued as a minimum of only four analyzable concentrations are required

## culture not continued due to strong toxic effects

The positive controls induced distinct increases in mutant frequencies, consistent with the laboratory's historical positive control data, thus demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

## Conclusion

Overall, in a GLP and guideline study, 2,4,6-TCP did not induce gene mutations at the HPRT locus in V79 cells with and without metabolic activation up to concentrations causing precipitation and/or cytotoxicity.

(██████████, 2020)

## In vitro micronucleus test

<b>Report reference</b>	██████████, 2020
<b>Report year</b>	2020
<b>Report title</b>	CA6519 - Micronucleus Test in Human Lymphocytes In vitro.
<b>Report No</b>	2114000
<b>Document No</b>	VV-877675
<b>Guidelines followed in study</b>	OECD 487 (2016)
<b>Deviations from current test guideline</b>	None
<b>Previous evaluation</b>	No, not previously submitted
<b>Test substance and purity</b>	2,4,6-TCP, batch MES663/2, 99% w/w

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GLP	Yes
Acceptability/Reliability	Yes

### Methods

In a GLP and guideline study, 2,4,6-TCP, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes in vitro in two independent experiments. In each experimental group, two parallel cultures were analysed. Per culture, 1000 binucleated cells were evaluated for micronucleus formation. The highest applied concentration was 1975 µg/mL, approx. 10 mM, the limit concentration for this assay. The test substance was applied for 4 hours with and without metabolic (Experiment I) and for 20 hours without metabolic activation (Experiment II). With S9, the highest concentration assessed for micronucleus formation was 263 µg/mL. Without S9, the highest concentration evaluated was 60.2 and 81.6 µg/mL for 4 and 20 hour respectively.

### Results

In Experiment I in the absence and presence of S9, no cytotoxicity was observed up to the highest evaluated concentration, which however showed precipitation. In Experiment II (20 hr exposure), performed without S9, clear cytotoxicity (50.2% cytostasis) was observed at 81.6 µg/mL which was the highest evaluated concentration.

In the absence and presence of S9, no relevant increases in the numbers of micronucleated cells were observed after treatment with the test substance. The mean percentage of micronuclei in all treated conditions was within the 95% control limits of the laboratory's historical control data. None of the values were statistically significantly increased, when compared to the solvent control and none of the treatment conditions showed a statistically significant concentration related increase in micronuclei formation. The statistically significant trend in Experiment II (20 h exposure without S9) is not biologically relevant as it is caused by an inverse trend (concentration related decrease in micronuclei).

Appropriate mutagens were used as positive controls. They induced statistically significant increases in binucleated cells with micronuclei demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

**Table 6.8.1-84: Summary of Results of the Micronucleus Assay**

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**	95% Ctrl limit in %
Exposure period 4 h without S9 mix						
I	40 h	Solvent control1	1.88		0.35	0.00 – 1.04
		Positive control2	1.67	23.0	10.95S	3.57 – 16.79
		11.2	1.80	8.8	0.25	
		19.7	1.76	13.1	0.00	
		34.4	1.79	9.9	0.20	
		60.2P	1.81	7.4	0.15	
Trend test: p-value 0.475						
Exposure period 20 h without S9 mix						
II	40 h	Solvent control1	1.81		0.60	0.00 – 0.86
		Positive control3	1.49	38.9	3.25 S	2.09 - 7.03
		15.2	1.73	9.8	0.55	
		26.7	1.65	18.9	0.50	
		46.6	1.72	10.8	0.50	
		81.6	1.40	50.2	0.30	
Trend test: p-value 0.009I						
Exposure period 4 h with S9 mix						
I	40 h	Solvent control1	1.69		0.45	0.00 – 1.03
		Positive control4	1.38	45.3	2.85S	1.30 - 6.05
		34.4	1.67	3.2	0.25	
		60.2	1.61	11.5	0.15	
		105	1.54	22.1	0.15	
		263P	1.43	37.2	0.15	
Trend test: p-value 0.258						
*	For the positive control groups and the test item treatment groups the values are related to the solvent controls					
**	The number of micronucleated cells was determined in a sample of 2000 binucleated cells					
P	Precipitation occurred at the end of treatment					
S	The number of micronucleated cells is statistically significantly higher than corresponding control values					
I	inverse trend without biological relevance					
n.c.	Not calculated as the CBPI is equal or higher than the solvent control value					
1	DMSO	0.5 % (v/v)				
2	MMC	0.8 µg/mL				
3	Demecolcine	50 ng/mL				
4	CPA	17.5 µg/mL				

## Conclusion

Overall, in a GLP and guideline study, 2,4,6-TCP did not induce micronuclei in vitro in human lymphocytes with and without metabolic activation up to concentrations causing precipitation and/or cytotoxicity.

(████████, 2020)

## Dose-range finding study for subsequent TGR assay in rats

Report reference	████████, 2021a
Report year	2021
Report title	CA6519 – 14-Day In Vivo Dose Range Finder Assay in Rats.
Report No	AG23LM.DRF000R.BTL
Document No	VV-898118
Guidelines followed in study	None – range-finding study
Deviations from current test guideline	Not Applicable
Previous evaluation	No, not previously submitted
Test substance and purity	2,4,6-TCP, batch MES663/2, 99% w/w
GLP	Yes
Acceptability/Reliability	Yes



## Methods

The purpose of this study was to assess the toxicity of 2,4,6-TCP and determine the maximum tolerated dose (MTD) or maximum feasible dose (MFD) suitable for use in a 28 day Big Blue® Transgenic rat model assay. Groups of 5 male and female F344 rats were given 2,4,6-TCP in corn oil by gavage at 0, 500, 750 or 1000 mg/kg bw/d for 14 days.

## Results and Conclusions

Mortality was observed in two males and two females at 1000 mg/kg bw/d and in one male at 750 mg/kg/day. The cause of death for the male at 750 mg/kg bw/d was undetermined. During cage side observations, ruffled fur, hunched posture, thin appearance, and decreased motor activity were observed from the lowest dose tested.

Based on these results, 2,4,6-TCP was well tolerated in rats at doses up to 500 mg/kg bw/d for 14 days, and therefore 500 mg/kg bw/d may be used as the high dose level for the 28 day Big Blue® Transgenic assay.

(██████, 2021a)

## Big Blue® Transgenic rat model assay

Report reference	██████, 2021b
Report year	2021
Report title	<i>In vivo</i> mutation assay of CA6519 at the <i>cII</i> locus in Big Blue® transgenic F344 Rats
Report No	AG23LM.171.BTL
Document No	VV-898121
Guidelines followed in study	OECD 488 (2020)
Deviations from current test guideline	None
Previous evaluation	No, not previously submitted
Test substance and purity	2,4,6-TCP, batch MES663/2, 99% w/w
GLP	Yes
Method of analysis	Acceptable method : Refer to Vol 3CA B5.1.2.3
Acceptability/Reliability	Yes

## Methods

The purpose of this GLP and guideline study was to determine the effect of 2,4,6-TCP following gavage administration, on mutant frequency at the *cII* gene in liver, bone marrow and duodenum from male transgenic Fischer 344 Big Blue® rats. 2,4,6-TCP was administered at 0, 100, 250 and 500 mg/kg bw/d (Groups 1-4, respectively), to six male rats per group in Groups 1-3 and seven male rats in Group 4 (500 mg/kg bw/d) for 28 consecutive days. Corn oil was used as the vehicle. Animals were sacrificed on Day 31. At necropsy, the liver (medial lobe), bone marrow (femurs) and duodenum were collected for mutant analysis, weighed (except for bone marrow), flash frozen and stored in a freezer set to -80°C. These tissues from the first five surviving animals/group were processed for DNA isolation and analysis of *cII* mutants, along with the DNA from the packaging positive controls. DNA isolated from previous positive control animals (F344 transgenic male rats exposed to N-ethyl-N-nitrosourea (ENU) by oral gavage, at 20 mg/kg bw/d on Study Days 1, 2, 3, 10, 17 and 24 and necropsied on Study Day 31) was used as a

positive control. These positive control animals were as Group 5. ENU is a potent mutagen, demonstrated to be mutagenic in the target tissues selected in this study.

## Results

No mortality or moribundity was observed during the course of the study. There were no treatment related effects on bodyweight or bodyweight gain. Clinical signs of crusty eyes (vehicle), hypersalivation (250 and 500 mg/kg bw/d) and ruffled fur (500 mg/kg bw/d) were observed during the study. Food consumption was reduced in animals at 500 mg/kg bw/d compared to the other doses. Overall, some toxicity started to appear at 250 mg/kg bw/d, with the MTD clearly reached at the top dose.

Treatment with 2,4,6-TCP in corn oil did not cause a statistically elevated mutant frequency at the *cH* gene in the liver, bone marrow or duodenum of Big Blue® rats. Individual animal and group mean mutant frequencies of 2,4,6-TCP treated animals remained within the 95% control limit of the vehicle historical control data. The ENU treatment produced a statistically significant increase in mutant frequencies for all tissues tested, demonstrating the utility of the test system to detect and quantify induced mutants, following exposure to a known direct acting mutagen.

**Table 6.8.1-85: Mutant frequencies (Liver)**

2,4,6-TCP [mg/kg bw/d]	Mean number of plaque forming units	Number of packagings	Mean number of mutants	Mean mutant frequency ± Standard deviation x 10 <sup>-6</sup>
0 (control)	399,550	2-4	13	32.8 ± 3.8
100	400,975	2-3	14	37.4 ± 21.0
250	358,019	2-4	16	41.2 ± 14.8
500	373,545	2-5	13	37.1 ± 10.1
Positive control	357,549	2-4	63	181.9 ± 45.3*
Historical negative controls (based on group means from 21 studies (2014-2019):				Mean ± SD 43.6 ± 13.0
				Acceptable range 25.2 – 76.2
				95% control limits 17.6 – 69.6

\* = Statistically significant (1-Way ANOVA, p < 0.001)

**Table 6.8.1-86: Mutant frequencies (Bone Marrow)**

2,4,6-TCP [mg/kg bw/d]	Mean number of plaque forming units	Number of packagings	Mean number of mutants	Mean mutant frequency ± Standard deviation x 10 <sup>-6</sup>
0 (control)	258,827	2-3	6	24.2 ± 14.5
100	453,052	2-4	14	28.6 ± 6.8
250	290,215	2-3	7	24.0 ± 16.1
500	328,258	2-3	9	26.0 ± 14.7
Positive control	320,076	2-4	129	388.9 ± 95.1*
Historical negative controls (based on group means from 11 studies (2014-2019):				Mean ± SD 30.0 ± 5.5
				Acceptable range 19.3 – 38.0
				95% control limits 19.0 – 41.0

\* = Statistically significant (1-Way ANOVA, p < 0.001)

**Table 6.8.1-87: Mutant frequencies (Duodenum)**

2,4,6-TCP [mg/kg bw/d]	Mean number of plaque forming units	Number of packagings	Mean number of mutants	Mean mutant frequency ± Standard deviation x 10 <sup>-6</sup>
0 (control)	371,985	2-3	15	42.2 ± 4.4 [43.2]

100	302,538	3	10	<b>32.9 ± 13.7</b>
250	345,813	3	11	<b>33.2 ± 9.2</b>
500	358,924	3	10	<b>33.6 ± 18.6</b>
Positive control	372,773	3-5	345	<b>956.2 ± 192.7*</b> <b>[983.6]</b>
Historical negative controls (based on group means from 9 studies (2016-2019):				Mean ± SD 41.0 ± 11.5
				Acceptable range 27.4 – 61.9
				95% control limits 18.0 – 64.0

\* = Statistically significant (Kruskal-Wallis test, p = 0.009)

[ ] = median mutant frequency

## Conclusion

Overall, in this GLP and guideline TGR study in rats, the oral administration of 2,4,6-TCP at doses up to and including 500 mg/kg bw/d (MTD) was negative for the induction of *cH* mutants in liver, bone marrow and duodenum.

(██████, 2021b)

## LONG-TERM/CARCINOGENICITY STUDIES

<b>Report:</b>	K-CA 5.8.1/41 National Cancer Institute. (1979). Bioassay of 2,4,6-Trichlorophenol for Possible Carcinogenicity. National Cancer Institute Technical Report Series No. 155, 1979. National Institutes of Health, Bethesda, Maryland 20014, USA. Syngenta File No. NA_13753.
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**STUDY TYPE:** Dietary Carcinogenicity Studies in B6C3F1 Mice and Fischer 344 Rats.

**Guidelines:** Not conducted to a regulatory guideline, although some of the principles of Carcinogenicity studies OECD 451 (1981) have been applied.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (96-97%)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**KLIMISCH SCORE: 2**

Where discrepancies exist between data presented in tables and text, data from tables has been used.

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

Bioassays of 2,4,6-trichlorophenol (2,4,6-TCP) for possible carcinogenicity were conducted by administering the test chemical in feed to F344 rats and B6C3F1 mice for 2 years.

Over the course of the studies, group mean body weights of all 2,4,6-TCP treated groups (both sexes of rats and mice) were decreased compared to controls.

Survival of 2,4,6-TCP treated groups (both sexes of rats and mice) was not reduced compared to concurrent controls. No clinical signs were observed in 2,4,6-TCP treated groups that were not observed at similar incidences in controls.

2,4,6-TCP was carcinogenic in male F344 rats, inducing lymphomas or leukemias and also carcinogenic in both sexes of B6C3F1 mice, inducing liver hepatocellular carcinomas and/or adenomas.

**It is concluded that under the conditions of this bioassay, 2,4,6-TCP was carcinogenic in male Fischer 344 rats, inducing lymphomas or leukemias<sup>13</sup>. The test chemical was also carcinogenic in both sexes of B6C3F1 mice, inducing liver hepatocellular carcinomas and/or adenomas.**

## MATERIALS AND METHODS

The study was conducted by NCI Frederick Cancer Research Centre (FCRC).

### Materials:

<b>Test Material:</b>	2,4,6-TCP (Omal®, Dowicide®2S)
<b>Description:</b>	Light, pinkish-orange solid
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	96-97%
<b>CAS#:</b>	88-06-2
<b>Impurity analysis:</b>	Up to 17 'minor' impurities detected by gas-liquid chromatography, chlorinated dibenzo-p-dioxin content not determined.
<b>Stability of test compound:</b>	Not indicated
<b>Source:</b>	DOW Chemical Company

**Vehicle and/or positive control:** The test substance was administered via Wayne® Sterilizable Lab Meal containing 4% fat (Allied Mills, Inc., Chicago, Ill).

<b>Test Animals:</b>	
<b>Species</b>	Mouse / Rat
<b>Strain</b>	B6C3F1 mice, Fischer 344 Rats
<b>Age/weight at dosing</b>	4 week old on arrival, 6 weeks at dose initiation Rats: 90 to 105 g (Males) and 80 to 95 g (Females) Mice: 18 to 22 g (Males) and 17 to 21 g (Females)
<b>Source</b>	
<b>Housing</b>	Polycarbonate cages Rats: 4/cage (same sex) Mice: 5/cage (same sex)
<b>Acclimatisation period</b>	2 weeks
<b>Diet</b>	Wayne Sterilizable Lab Meal containing 4% fat <i>ad libitum</i>
<b>Water</b>	Acidified to pH 2.5 <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 22-24°C Humidity: 45-55% Air changes: 15 times/h Photoperiod: 12 h light/dark cycle

### Study Design and Methods:

**In-life dates:** Not indicated

<sup>13</sup> Large granular lymphocyte leukemia (LGLL) is a common fatal disease in aging F344 rats. Therefore, interpretations of bioassays and declaring treatment-related increases in LGLL should involve a weight of evidence approach. Thomas J. *et al.*, (2007). Published in Thomas J; Haseman J.K; Goodman J.I; Ward J.M; Loughran T.P; Spencer P.J. (2007). A review of large granular lymphocytic leukemia in Fischer 344 rats as an initial step toward evaluating the implication of the endpoint to human cancer risk assessment. Toxicological Science. 99(1): 3-19. Syngenta File No. NA13801.

### Preliminary toxicity studies

Subchronic feeding studies were conducted to estimate the maximum tolerated doses (MTD's) of 2,4,6-TCP, on the basis of which two concentrations (referred to in this report as "low" and "high" doses) were selected for administration in the chronic studies. Groups of rats or mice consisting of five males or five females were fed diets *ad libitum* which contained 10,000 to 46,000 ppm 2,4,6-TCP for rats and 6,800 to 31,500 for mice for a period of 7 weeks, followed by 1 week of additional observation. Each animal was weighed twice per week. At the end of the subchronic studies, all animals were killed using CO<sub>2</sub> and necropsied.

The low and high doses for chronic studies were set at 5,000 and 10,000 ppm for male and female rats; 5,000 and 10,000 ppm for male mice; and 10,000 and 20,000 ppm for female mice.

### Chronic/carcinogenicity studies

#### Mice

##### Study design in mice

Test group	Dietary concentration (ppm) Males/Females	Dose to animal	# male	# female
Control	0	Food consumption not recorded	20	20
Low	5000 / 5214 <sup>a</sup>		50	50
High	10000 / 10428 <sup>a</sup>		50	50

<sup>a</sup>Female mice were offered 2,4,6-TCP in feed at 0, 10,000, or 20,000 ppm. After 38 weeks because of excessive lowered body weights, dietary concentrations for female mice were reduced to 2500 and 5000 ppm for 67 weeks (total = 105 weeks); time-weighted averages for female mice given above.

Time-weighted average dose =  $\frac{\sum (\text{dose in ppm} \times \text{no. of weeks at that dose})}{\sum (\text{no. of weeks receiving each dose})}$

#### Rats

##### Study design in rats

Test group	Dietary concentration (ppm)	Dose to animal	# male	# female
Control	0	Food consumption not recorded	20	20
Low	5000		50	50
High	10000		50	50

### Diet preparation and analysis:

2,4,6-TCP test diets were prepared by mixing the appropriate amount of the chemical with autoclaved Wayne® Sterilizable Lab Meal containing 4% fat. The weighed chemical was first mixed with an equal amount of the lab meal using a mortar and pestle. The mixing was continued with second and third additions of feed, and final mixing was performed with the remaining quantity of feed for a minimum of 15 minutes in a Patterson-Kelly twin-shell blender with an intensifier bar. The material was then stored in sealed 3 kg plastic bags at 7°C until used. No details are provided with regard to analysis of prepared diets for achieved concentration, homogeneity or stability. The duration of storage of prepared diets at 7°C are not reported.

### Observations:

Animals were observed twice daily. Clinical examination and palpation for masses were performed each month.

**Bodyweight:**

Animals were weighed at least once per month

**Food consumption:**

Food consumption was not recorded. It is not possible to derive achieved intake on 2,4,6-TCP by the animals.

**Investigations *post mortem*:**

Moribund and those animals surviving to termination were killed by CO<sub>2</sub> and necropsied.

**Macroscopic examination:**

The pathologic evaluation consisted of gross and microscopic examination of major tissues, major organs, and all gross lesions.

**Tissue submission:**

The following tissues were preserved in neutral buffered 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, followed by microscopic examination

adrenal	pituitary
bone marrow (femur)	prostate
brain (cerebrum and cerebellum)	salivary glands (parotid, sublingual, and submaxillary)
bronchi	skin
esophagus	small and large intestines
heart	spleen
kidney	stomach (glandular and nonglandular),
liver	testis
lungs	thymus
lymph nodes (mesenteric and submandibular)	thyroid
mammary gland	trachea
ovary	urinary bladder
pancreas	uterus
parathyroid	all tissue masses

Peripheral blood smears also were made for all animals, whenever possible.

Necropsies were performed on all animals found dead unless precluded in whole or in part by autolysis or cannibalization.

**Microscopic examination:**

All sectioned tissues were examined by light microscopy

**Statistical analysis**

Probabilities of survival were estimated by the product-limit procedure of Kaplan and Meier (1958). Animals were statistically censored as of the time that they died of other than natural causes or were found to be missing; animals dying from natural causes were not statistically censored. Statistical analyses for a possible dose-related effect on survival used the method of Cox (1972) for testing two groups for equality and Tarone's (1975) extensions of Cox methods for testing for a dose-related trend.

One-tailed P values have been reported for all tests except the departure from linearity test, which is only reported when its two-tailed P value is less than 0.05.

The purpose of the statistical analyses of tumour incidence is to determine whether animals receiving the test chemical developed a significantly higher proportion of tumours than did the control animals. As a part of these analyses, the one-tailed Fisher exact test (Cox, 1970) was used to compare the tumour incidence of a control group with that of a group of dosed animals at each dose level. When results for a number of dosed groups (k) are compared simultaneously with those for a control group, a correction to ensure an overall significance level of 0.05 may be made. The Bonferroni inequality (Miller, 1966) requires that the P value for any comparison be less than or equal to  $0.05/k$ . In cases where this correction was used, it is discussed in the narrative section. It is not, however, presented in the tables, where the Fisher exact P values are shown.

The Cochran-Armitage test for linear trend in proportions, with continuity correction (Armitage, 1971), was also used. Under the assumption of a linear trend, this test determines if the slope of the dose-response curve is different from zero at the one-tailed 0.05 level of significance. Unless otherwise noted, the direction of the significant trend is a positive dose relationship. This method also provides a two-tailed test of departure from linear trend.

A time-adjusted analysis was applied when numerous early deaths resulted from causes that were not associated with the formation of tumours. In this analysis, deaths that occurred before the first tumour was observed were excluded by basing the statistical tests on animals that survived at least 52 weeks, unless a tumour was found at the anatomic site of interest before week 52. When such an early tumour was found, comparisons were based exclusively on animals that survived at least as long as the animal in which the first tumour was found. Once this reduced set of data was obtained, the standard procedures for analyses of the incidence of tumours (Fisher exact tests, Cochran-Armitage tests, etc.) were followed.

When appropriate, life-table methods were used to analyze the incidence of tumours. Curves of the proportions surviving without an observed tumour were computed as in Saffiotti et al. (1972). The week during which an animal died naturally or was sacrificed was entered as the time point of tumour observation. Cox's methods of comparing these curves were used for two groups; Tarone's extension to testing for linear trend was used for three groups. The statistical tests for the incidence of tumours which used life-table methods were one-tailed and, unless otherwise noted, in the direction of a positive dose relationship. Significant departures from linearity (P less than 0.05, two-tailed test) were also noted.

The approximate 95 percent confidence interval for the relative risk of each dosed group compared with its control was calculated from the exact interval on the odds ratio (Gart, 1971). The relative risk is defined as  $p/p_0$  where  $p$  is the true binomial probability of the incidence of a specific type of tumour in a dosed group of animals and  $p_0$  is the true probability of the spontaneous incidence of the same type of tumour in a control group. The hypothesis of equality between the true proportion of a specific tumour in a dosed group and the proportion in a control group corresponds to a relative risk of unity. Values in excess of unity represent the condition of a larger proportion in the dosed group than in the control.

The lower and upper limits of the confidence interval of the relative risk have been included in the tables of statistical analyses. The interpretation of the limits is that in approximately 95% of a large number of identical experiments, the true ratio of the risk in a dosed group of animals to that in a control group would be within the interval calculated from the experiment. When the lower limit of the confidence interval is greater than one, it can be inferred that a statistically significant result (P less than 0.025 one-tailed test when the control incidence is not zero, P less than 0.050 when the control incidence is zero) has occurred. When the lower limit is less than unity, but the upper limit is a greater than unity, the lower limit indicates the absence of a significant result while the upper limit indicates that there is a theoretical possibility of the induction of tumours by the test chemical, which could not be detected under the conditions of this test.

## RESULTS

### Preliminary toxicity studies

A summary of the survival and group mean body weight at week 7 data from the preliminary toxicity studies are shown in Table 6.8.1-88.

**Table 6.8.1-88: 2,4,6-TCP preliminary toxicity studies – survival and group mean body weight data**

Dose ppm	Male		Female	
	Survival	Mean weight at Week 7 as % of control	Survival	Mean weight at Week 7 as % of control
<b>Rats</b>				
0	5/5	100	5/5	100
10000	5/5	96	5/5	92
14700	5/5	89	5/5	84
21500	4/5	73	5/5	73
31500	4/5	47	4/5	67
46000	3/5	39	2/5	42
<b>Mice</b>				
0	5/5	100	4/5	100
6800	5/5	99	5/5	110
10000	5/5	99	5/5	110
14700	5/5	83	5/5	101
21500	5/5	79	5/5	93
31500	3/5	57	3/5	68

The lowest dose at which histopathologic findings were observed in the rats was 46,000 ppm; at this dose moderate to marked increase in splenic hematopoiesis was seen in male and female rats and midzonal vacuolation of hepatocytes was seen in two males. In male and female mice dosed at 21,500 ppm, all tissues were essentially normal.

A ten percent depression in body weight was a major criterion for the estimation of MTD's. The doses that were required to produce this response were determined by the following procedure: first, least squares regressions of mean body weights versus days on study were used to estimate mean body weights of each of the dosed groups at day 49. Next, probits of the percent weights of each of the dosed groups at day 49 relative to weights of corresponding control groups were plotted against logarithms of the doses, and least squares regressions fitted to the data were used to estimate the doses required to induce 10% depression in weight.

For the chronic studies, doses were set at 5,000 and 10,000 ppm for male and female rats; 5,000 and 10,000 ppm for male mice; and 10,000 and 20,000 ppm for female mice. No histopathologic lesions were observed at the doses selected.

### Chronic studies

#### Mortality:

Survival across the studies was unaffected (male rats: controls 18/20, 90% vs. 35/50, 34/50; female rats: 14/20, 70% vs. 39/50, 39/50; male mice: 16/20, 80% vs. 44/50, 45/50; female mice 17/20, 85% vs. 44/50, 40/50); male rat controls were numerically greater [90% vs. 70% & 68%] but not statistically different. There were no statistically different dose-related trends in mortality.

#### 2,4,6 –TCP Survival- Fisher rat study: overall mortality

Dietary concentration M/F (ppm)	Dose M/F (mg/kg/day)	Sex	
		Males	Females



0	0	2/20 (10)	6/20 (30)
5000	258	15/50 (30)	11/50 (22)
10000	544	16/50 (32)	11/50 (22)

( ) percent

#### 2,4,6–TCP Survival- B6C2F1 mouse study: overall mortality

Dietary concentration M/F (ppm)	Dose M/F (mg/kg/day)	Sex	
		Males	Females
0	0	4/20 (20)	3/20 (15)
5000	650	6/50 (12)	6/50 (12)
10000	1300	5/50 (10)	10/50 (20)

( ) percent

#### Clinical observations:

Clinical signs observed in rats and mice of both sexes were common to both 2,4,6-TCP and control treated groups.

#### Bodyweight and weight gain:

Mean body weights were dose related and considerably lower in rats and mice throughout much of the bioassay duration, and even in female mice after reducing dietary levels to one-fourth at week 39. Averaged mean body weights were decreased compared to controls: Male rats ~9% & 15%; Female rats ~18% & 24%; Male mice ~11% & 21%; Female mice ~20% & 33%.

**Food consumption:** Data were not available.

#### Sacrifice and pathology

#### Macroscopic findings:

A small number of lesions were observed in rats and mice, none of which were related to treatment.

**Microscopic findings:** Treatment-related findings for rats and mice, both neoplastic and non-neoplastic, are summarised in Table 6.8.1-89

**Table 6.8.1-89: Lesions/Tumours induced by 2,4,6-TCP in two-year dietary exposure bioassays in Fischer 344 rats and B6C3F1 mice**

Dose group (ppm)	Male rats			Female rats		
	Control	5000	10000	Control	5000	10000
No. necropsied	20	50	50	20	50	50
Location Lesion: number of animals with lesion (%)						
Peripheral blood Leukocytosis	0 <sup>b</sup>	13 (26) <sup>b</sup>	11 (22) <sup>b</sup>	0	6 (12)	3 (6)
Bone Marrow Hyperplasia	0	26 (52) <sup>b</sup>	15 (30) <sup>b</sup>	0	16 (32)	2 (4)
Hematopoietic system						

Dose group (ppm)	Male rats			Female rats		
	Control	5000	10000	Control	5000	10000
Lymphoma	1 (5)	2 (4)	0	0	0	2 (4)
Leukemia	3 (15) <sup>b</sup>	23 (46) <sup>b</sup>	28 (56) <sup>b</sup>	3 (15)	11 (22)	10 (20)
Combined	4 (20) <sup>b</sup>	25 (50) <sup>a</sup>	29 (58) <sup>b</sup>	3 (15)	11 (22)	13 (26)
Historical controls: Male rats 11/255, 4% (Monocytic leukemia); female rats 42/420, 10% (Combined leukemia or lymphomas)						
Dose group (ppm)	Male mice			Female mice <sup>c</sup>		
	0	5000	10000	0	5214	10428
No. necropsied	20	49	47	20	50	48
Liver						
Hyperplasia	2 (10)	12 (24)	6 (13)	1 (5)	1 (2)	6 (13)
Adenoma	3 (15) <sup>b</sup>	22 (45)	32 (68)	1 (5) <sup>b</sup>	12 (24) <sup>a</sup>	17 (35) <sup>b</sup>
Carcinoma	1 (5)	10 (20)	7 (15)	0 <sup>b</sup>	0	7 (15)
Combined	4 (20) <sup>b</sup>	32 (65) <sup>b</sup>	39 (83) <sup>b</sup>	1 (5) <sup>b</sup>	12 (24) <sup>a</sup>	24 (50) <sup>b</sup>
Historical controls (adenoma or carcinoma): Male mice 99/323 30.7%; female mice 14/324, 4.3%						
Hematopoietic system						
Lymphoma	2 (10)	3 (6)	1 (2)	2 (10)	12 (24)	7 (15)

<sup>a</sup> P values: P < 0.05

<sup>b</sup> P values: P < 0.01 d = decrease; P at control column = dose response trend; at dose groups = compared to controls.

<sup>c</sup> Average dietary levels for female mice: 38 weeks at 10000 and 20000 ppm, then 67 weeks at 2500 and 5000 ppm resulting in time-weighted averages 5214 and 10428 ppm.

<sup>d</sup> Decrease

### Non-neoplastic:

#### Rats

Adverse effects at two years for rats were leukocytosis (another 4 males and 1 female had monocytosis) of peripheral blood and hyperplasia of bone marrow. As these were seen only in 2,4,6-TCP dosed rats, and despite lack of dose responses, these are considered chemical related.

#### Mice

No microscopic, non-neoplastic findings were observed in mice.

### Neoplastic:

#### Rats

In male rats, leukemias/lymphomas were increased and dose related, and likewise numerically increased with dose in female rats. Incidences for both sexes increased with dose but differences between dose groups were not particularly robust (50% vs. 58% for males and 22% vs. 26% for females), although increased over male (20%) and female (15%) controls. Both control groups exceeded averages for historic controls.

Note: The HCD is presented as a cumulative incidence and not presented on a per study basis. The HCD from the performing laboratory were provided on select primary tumors for studies conducted on or before 1979. The number of studies used to compile the HCD was not provided nor any information on those studies other than the combined tumor incidence.

#### Mice

In mice, liver toxicity, including individual liver cell abnormalities, focal areas of cellular alteration, and focal and nodular areas of hyperplasia were commonly present in 2,4,6-TCP groups of mice. In both sexes of mice, hepatocellular carcinomas/adenomas were increased, dose related, significantly higher in all four 2,4,6-TCP groups, and exceeded contemporary historic control rates.

Additionally female but not male mice showed numerical non-dose related increases in lymphoma.

**CONCLUSION:**

Bioassays of 2,4,6-TCP for possible carcinogenicity were conducted by administering the test chemical in feed at 0, 5000 and 10,000 ppm to groups of 50 (20 in controls) male and 50 (20 in controls) female F344 rats and B6C3F1 mice for 2 years. Equivalent doses were 258 and 544 mg/kg bw/d in rats and 650 and 1300 mg/kg bw/d in mice.

Survival of 2,4,6-TCP treated groups (both sexes of rats and mice) was not reduced compared to concurrent controls. No clinical signs were observed in 2,4,6-TCP treated groups that were not observed at similar incidences in controls. Group mean body weights of all 2,4,6-TCP treated groups (both sexes of rats and mice) were decreased > 10% compared to controls.

2,4,6-TCP was carcinogenic in male F344 rats, inducing lymphomas or leukemias from the lowest dose of 258 mg/kg bw/d. It was also carcinogenic in both sexes of B6C3F1 mice, inducing liver hepatocellular carcinomas and/or adenomas from the lowest dose of 650 mg/kg bw/d.

(National Cancer Institute, 1979)

<b>Report:</b>	K-CA 5.8.1/42 Huff, J. (2012). Long-term Toxicology and Carcinogenicity of 2,4,6-Trichlorophenol. National Institute of Environmental Health Sciences. Research Triangle Park, NC, US. Published: Huff, J. (2012). Long-term toxicology and carcinogenicity of 2,4,6-Trichlorophenol. Chemosphere 89, 521-525. Syngenta File No. NA_13807.
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**STUDY TYPE:** Dietary Carcinogenicity Studies in B6C3F1 Mice and Fischer 344 Rats – further review of data conducted by NCI, (1979).

**Guidelines:** Review article, not applicable.

**TEST MATERIAL (PURITY):** Not applicable

**GLP:** Not applicable.

**KLIMISCH SCORE:** Not applicable, re-evaluation of existing data.

**EXECUTIVE SUMMARY/CONCLUSIONS**

Carcinogenesis bioassays were conducted by giving (2,4,6-TCP) in feed to groups of 50 male and female Fischer rats and male B6C3F1 mice for two years (NCI, 1979). Dietary concentrations were 0 [20/group], 5000 [0.5%], or 10,000 [1%] ppm. Female mice began with 10,000 and 20,000 ppm but after 38 weeks were lowered due to reduced body weights to 2500 and 5000 ppm for 67 weeks; exposures averaged 5200 and 10,400 ppm. Adverse effects at two years were leukocytosis and monocytosis of peripheral blood and hyperplasia of bone marrow in both sexes of rats. In mice, liver toxicity, including individual liver cell abnormalities, focal areas of cellular alteration, and focal and nodular areas of hyperplasia were commonly present. Regarding carcinogenic activity, TCP caused leukemias/lymphomas in male rats, and possibly in female rats and female mice as well, and induced liver tumors in male and female mice. **Using NTP categories of evidence indicates ‘clear evidence of carcinogenicity’ for male rats [hematopoietic system tumors]; ‘equivocal evidence of carcinogenicity’ for female rats [hematopoietic system tumors]; ‘clear evidence of carcinogenicity’ for male and female mice [liver tumors].**

HSE agrees with the evaluation of the EU peer-review process that this review confirms 2,4,6-TCP was carcinogenic in male F344 rats, inducing lymphomas or leukemias from the lowest dose of 258 mg/kg

bw/d. It was also carcinogenic in both sexes of B6C3F1 mice, inducing liver hepatocellular carcinomas and/or adenomas from the lowest dose of 650 mg/kg bw/d.

(Huff J, 2012)

## REPROTOXICITY STUDIES

**EFSA Request for additional information (February 2018), Question 42:** Applicant to provide more detailed results of the reproductive toxicity study with 2,4,6-TCP (██████, 1986).

Additional tables demonstrating the lack of significant treatment-related differences via 2,4,6-TCP have been provided by the applicant in the summary below.

<b>Report:</b>	K-CA 5.8.1/43 Blackburn K. <i>et al.</i> , (1986). Evaluation of the Reproductive Toxicology of 2,4,6-Trichlorophenol in Male and Female Rats. Department of Environmental Health. University of Cincinnati Medical Centre. Published: Blackburn K, Zenick H, Hope E, Manson J, George E, Smith M (1986). Evaluation of the Reproductive Toxicology of 2,4,6-Trichlorophenol in Male and Female Rats. Fundamental and Applied Toxicology 6, 233-239. Syngenta File No. NA_13761.
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**STUDY TYPE:** Reproductive toxicology study in male and female rats.

**Guidelines:** No globally or regionally accepted guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (99%).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The present study was designed to evaluate the reproductive effects of 2,4,6-trichlorophenol (2,4,6-TCP), in male and female rats. Adult males were treated with 0, 100, 500, or 1000 mg/kg of 2,4,6-TCP (gavage) for 10 weeks (5 days/week), at which time semen evaluations were conducted on ejaculates recovered from the genital tract of receptive untreated females. Fertility was assessed in the 0 and 1000 mg/kg groups.

Females were treated with identical doses for 2 weeks prior to pregnancy (5 days/week) then throughout gestation (7 days/week). Females were mated with untreated males. Dams were allowed to litter and pup development was monitored until Day 42 postpartum.

2,4,6-TCP had no effect on any sperm parameter or male fertility. Treatment of females with 1000 mg/kg of 2,4,6-TCP produced gross maternal toxicity as reflected in increased lethality and decreased weight gain in the dams. However no treatment-related differences were seen in litter sizes or pup survival. Male and female birth weights were significantly depressed in the 500 and 1000 mg/kg groups; these differences disappeared by Day 4 postpartum suggesting that they were a reflection of maternal toxicity.

Under the conditions of the study, the reproductive processes of male and female rats do not appear to be a primary target for the effects of 2,4,6-TCP.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Source:</b>	Fisher Scientific and re-purified to 99%
<b>Purity:</b>	99%
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

**Vehicle and/or positive control:** The test substance was administered dissolved in corn oil, by gentle heating, and administered in a volume of 2ml/kg bw. Control animals received the same volume of corn oil alone.

<b>Test Animals:</b>	
<b>Species</b>	Rat
<b>Strain</b>	Long-Evans hooded (LEH)
<b>Age/weight at dosing</b>	Males: Dosed at 100 days of age Females: Obtained at 60 days of age, dosed after 2 weeks of cycle monitoring
<b>Source</b>	
<b>Housing</b>	Male fertility segment: Individually Female fertility segment: 3 females to 1 male (breeding period) Individually from day 14 of gestation
<b>Acclimatisation period</b>	Males: 30 days Females: 2 weeks (period of cycle monitoring)
<b>Diet</b>	Purina Lab Chow 5001 <i>ad libitum</i>
<b>Water</b>	Tap water <i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Photoperiod: 12/12 hours light/dark cycle

### Study Design and Methods:

**In-life dates:** Not indicated

### Dose selection rationale:

Doses were selected on the basis of a 3 week pilot study wherein males and females receiving 1000 mg/kg exhibited approximately a 5% weight loss. It was concluded that higher doses would produce more pronounced and unacceptable weight losses given the lengthened dosing periods in the present study.

### Male reproductive segment

At 100 days of age, pre-treatment (baseline) copulatory and semen evaluations were conducted on the males. These methods have been described in detail (Zenick et al. 1984). Behavioural evaluations included recording mount and ejaculation latencies and number of mounts and intromissions. Semen evaluations were conducted on ejaculates recovered from the genital tract of a receptive female (15 min postcopulation) and included assessments of sperm count, motility, and morphology. Animals having baseline ejaculated sperm counts of less than 20 million sperm or ejaculation latencies longer than 30

mins were eliminated from the study. Remaining animals were assigned to treatment groups (0, 100, 500 or 1000 mg/kg 2,4,6-TCP) so that base line group mean sperm counts and ejaculation latencies were matched across groups. Fifteen animals per group were included in the 0, 100, and 500 mg/kg groups with 25 assigned to the 1000 mg/kg group. The additional animals in this group were necessary to ensure adequate sample size for the distribution and kinetic studies conducted at the end of treatment. Animals were dosed by gavage 5 days/week for 11 weeks.

Following 10 weeks of treatment, copulatory behaviour and semen profiles were again evaluated. On the 11<sup>th</sup> week males from the high-dose and control groups were mated to unexposed females for fertility evaluations. Females selected had regular oestrus cycles as determined by evaluation of vaginal smears for 3 weeks prior to mating. A male was housed with a female until mating was confirmed by a copulatory plug and/or presence of sperm in the vaginal lavage.

Once mating was confirmed females were removed to individual cages. On Day 18 of gestation, females were sacrificed by CO<sub>2</sub> asphyxiation. The sex, weight and variability of all foetuses were assessed. The number of resorption sites was recorded after staining the uteri in 10% ammonium sulfide for 10 min.

One week later, all males were sacrificed by CO<sub>2</sub> asphyxiation. Blood was drawn from the heart and plasma samples were frozen for testosterone analyses. Testosterone analyses were conducted using a Diagnostic Products Kit. In addition, one cauda epididymis from each animal was minced, filtered through a 20-gauge brass screen, diluted to 50 ml, and the sperm count was determined in a haemocytometer. Although not detailed in the materials and methods section of the paper, the results indicate that the following organs, from all groups, were weighed at necropsy: liver, kidney, lung, adrenal, spleen, heart, testis, prostate, seminal vesicles, vas deferens and epididymides.

#### **Female reproductive segment**

Female rats were obtained at 60 days of age. Males were obtained from the same source for the breeding segment. The females employed were selected from a larger breeding colony population. Oestrus cycles were monitored for 2 weeks by vaginal lavage and females not cycling were eliminated. Animals were assigned to treatment groups (0, 100, 500 or 1000 mg/kg 2,4,6-TCP) so that mean body weights were matched across groups. Forty animals were assigned to the 0 and 1000 mg/kg groups with 30 assigned to the 100 and 500 mg/kg groups.

Animals were dosed by gavage for 5 days/week for 2 weeks and then mated. Three females were housed with one male and allowed to cohabit until mating was confirmed by presence of sperm in the vaginal lavage or for a maximum of 10 days (males failing to impregnate a female were replaced after 5 days). Daily intubations continued from the beginning of the mating trials through Day 21 of gestation.

Body weights were recorded daily from the beginning of the treatment period until delivery. Animals were individually housed in plastic shoe-box cages on Day 14 of gestation. Those females which had not delivered by Day 24 of gestation were sacrificed by CO<sub>2</sub> asphyxiation. Their ovaries were examined and uteri stained with 10% ammonium sulfide for 10 min and then evaluated for signs of postimplantation loss.

For animals that delivered, date of delivery, sex ratio of the litter and body weights of male and female offspring were recorded. Litters were culled to eight pups on Day 4 postpartum with four males and four females retained when possible. At weaning, litters were culled to two males and two females. Body weights by sex were recorded on Days 1, 4, 7, 14, 21, 28, 35 and 42 postpartum. The establishment of vaginal patency was monitored beginning at Day 28. On Day 42 postpartum all pups were sacrificed.

#### **Statistical analysis:**

Body weights (adult male, adult female and pups postpartum by sex) were analysed first by a repeated measures analysis of variance with one between factor (dose) and one within factor (time). Subsequently, data were analysed week by week using a one-way analysis of variance procedure. One-

way analysis of variance procedures were also used to evaluate percentage of weight increase data. Differences between group means were assessed using Duncan's new multiple-range test.

Male mating behaviour and semen parameters were analysed by one-way analysis of variance procedures. Data on each animal were expressed as percentage of his baseline value (i.e. Week 10 value/baseline). Organ weights, plasma testosterone and cauda epididymal sperm counts at sacrifice were also evaluated using one-way analysis of variance procedures.

Litter sizes, sex ratios and resorption data were analysed using a Kruskal Wallis test. Foetal and pup weights and mean times of vaginal opening were analysed using a one-way analysis of variance. Litter means were the units of analysis for these variables. Female breeding outcomes (e.g. percentage pregnant, percentage sperm positive) were evaluated using  $\chi^2$ .

## RESULTS

### Male reproductive segment

#### Morbidity and Mortality

Death due to treatment occurred in the high-dose group alone during the first 4 weeks of treatment (two, three, one, and two deaths respectively). Deaths in the other groups (one each in the low and mid dose groups) were the result of intubation errors as evidenced by sudden death minutes after gavaging the animal and confirmed at time of necropsy. The only other indication of toxicity was a reduction in rate of weight gain in the 1000 mg/kg group which was significant at Week 3 alone. The only consistent clinical indication noted was urogenital staining in all 2,4,6-TCP treated groups. No warning signs were noted in the appearance and behaviour of the animals which subsequently died. Due to autolysis, necropsy of these animals was limited to an examination of the potential contribution of the intubation procedure to death.

#### Copulatory Behaviour and Semen Evaluations and Sacrifice Data

No treatment related differences were seen in any of the copulatory behaviours or semen parameters measured. Seminal plug weight increased over the treatment period for all groups, a maturational increase which has been previously noted. No significant treatment-related differences were seen in male organ weights, plasma testosterone, or caudal sperm counts obtained at sacrifice (Table 6.8.1-90).

**Table 6.8.1-90: Organ weights and epididymal sperm counts following 11 weeks of TCP exposure.**

Treatment (mg/kg TCP)	0 (N=15)	100 (N=14)	500 (N=14)	1000 (N=17)
Liver	18.26 ± 3.01 <sup>a</sup>	19.30 ± 3.69	18.89 ± 5.89	19.04 ± 1.89
Kidney	1.97 ± 0.22	2.00 ± 0.33	1.89 ± 0.37	2.04 ± 0.19
Lung	1.13 ± 0.25	1.20 ± 0.20	1.04 ± 0.34	1.11 ± 0.11
Adrenal	0.05 ± 0.01	0.06 ± 0.05	0.05 ± 0.05	0.05 ± 0.02
Spleen	0.75 ± 0.15	0.82 ± 0.22	0.70 ± 0.17	0.68 ± 0.11
Heart	1.41 ± 0.17	1.53 ± 0.18	1.33 ± 0.27	1.39 ± 0.11
Testis	1.69 ± 0.18	1.80 ± 0.18	1.58 ± 0.30	1.62 ± 0.19
Prostate	0.76 ± 0.25	0.66 ± 0.19	0.59 ± 0.14	0.59 ± 0.11
Seminal vesicle	2.73 ± 0.54	2.69 ± 0.30	2.39 ± 0.67	2.48 ± 0.30
Vas deferens	0.23 ± 0.03	0.25 ± 0.07	0.22 ± 0.03	0.22 ± 0.03
Epididymis	0.63 ± 0.08	0.70 ± 0.07	0.64 ± 0.10	0.61 ± 0.09
Sperms counts/g cauda epididymis (x10 <sup>6</sup> )	760 ± 150	682 ± 206	795 ± 250	658 ± 119

<sup>a</sup> Mean and standard deviations

### Fertility and Foetal Outcome Evaluations

No male in either the control or 1000 mg/kg group required more than 5 days to mate as noted by the presence of copulatory plugs and positive sperm lavages. Females were sacrificed on Day 18 postconception. Ten of thirteen females bred to control males and all females bred to high dose males ( $n = 14$ ) carried litters. Litter size, sex ratio, mean pup weight by sex, number of dead fetuses and number of resorption and implantation sites were recorded for each litter. No treatment-related differences were seen in any of these outcomes (Table 6.8.1-91).

**Table 6.8.1-91: Male reproductive segment: fetal outcome parameters obtained on Day 18 of pregnancy**

Treatment (mg/kg TCP)	Number litters	Litter size <sup>a</sup>	% Post-implantation loss <sup>b</sup>	Sex ratio <sup>c</sup>	Pup body weights (g)	
					Male	Female
0	10	12.9 ± 3.4	9.6 ± 4.8	1.39 ± 0.63	1.64 ± 0.17	1.61 ± 0.14
1000	14	12.9 ± 2.3	6.9 ± 8.9	1.32 ± 0.53	1.53 ± 0.23	1.45 ± 0.22

a Number of viable fetuses per litter

b (No. resorptions) / (No. implantations) x 100

c (No. males/litter) / (No. females/litter)

## Female reproductive segment

### Morbidity and Mortality

The survival of females through the entire treatment period was as follows: 38/39 control animals; 29/29 100 mg/kg animals; 25/30 500-mg/kg animals; and 24/40 1000 mg/kg animals. The cause of death appeared to be primarily intubation errors, with females in the high-dose group exhibiting marked resistance to intubation in the hands of technicians highly experienced in this procedure. Three of the sixteen deaths in the high-dose group appeared to be the result of 2,4,6-TCP toxicity since postmortem necropsy eliminated intubation error as a cause. As with the males, females which died as an apparent result of 2,4,6-TCP toxicity did not exhibit differences in appearance or behaviour which were not apparent in other members of the treatment group (urogenital staining and weight loss). Due to autolysis, necropsy of these animals was confined to an examination of the potential contribution of the intubation procedure to death. Repeated measures analysis of variance showed a significant effect of dose on female body weight ( $p < 0.05$ ) at the end of both the first and second week of dosing as well as Days 1, 7, and 14 of pregnancy. Comparison of group means using Duncan's new multiple-range test showed that only the 1000 mg/kg group differed significantly from the control group. No significant effects were seen on Day 21 of pregnancy. As a consequence of the "catch up" in weight between Days 14 and 21 of pregnancy in the 1000 mg/kg group, no group differences were seen in percentage of weight gain during gestation. Mean percent weight gains during gestation were 30, 29, 29, and 28% for the 0, 100, 500, and 1000 mg/kg groups, respectively.

### Reproductive performance

Breeding success was somewhat low across all groups. The percentage of females that were pregnant was 63, 72, 60, and 50% for the 0, 100, 500, and 1000 mg/kg groups respectively. These differences were not significantly different ( $\chi^2 = 2.87$ ,  $p = 0.40$ ). Historically, control breeding data range from 70 to 85% in this laboratory. There did not appear to be any differences in housing, season, diet, or protocol that would account for the lower rates seen in the present study. Furthermore, only cycling females were selected for inclusion in the study. That the general condition of the animal colony was adequate is also supported by the fact that breeding in the concurrent male reproductive segment was quite good (76-100%).

### Evaluation of Offspring

No treatment-related differences were apparent. Both the 500 and 1000 mg/kg groups showed significantly depressed body weights for both sexes on Day 1 postpartum. By Day 4 no differences were detectable. No treatment-related differences were seen in survival or pup body weights from weaning through postnatal Day 42. No treatment-related differences were seen in mean day of vaginal patency of female pups. Means and standard deviations were  $34.0 \pm 2.0$ ,  $32.9 \pm 1.8$ ,  $33.3 \pm 1.7$ , and  $34.5 \pm 2.8$



days postnatal for the 0, 100, 500, and 1000 mg/kg 2,4,6-TCP groups, respectively (Table 6.8.1-92, -93).

**Table 6.8.1-92: Female reproductive segment: litter size and survival from birth to Day 4 postpartum**

Dam dose (mg/kg TCP)	N	Litter size at birth ( $\pm$ SD)	Percentage survival on day 4 ( $\pm$ SD)
0	22	10.2 (3.7)	95.2 (10.6)
100	20	10.6 (3.6)	98.9 (2.8)
500	15	12.3 (2.5)	96.1 (7.5)
1000	11	11.5 (2.4)	99.3 (2.3)

**Table 6.8.1-93: Female reproductive exposure segment: mean litter weights for male and female pups from birth until weaning (mean ( $\pm$  SD))**

Dam dose group (mg/kg TCP)	N	Postpartum day				
		1	4	7	14	21
Males						
0	22	6.94 (0.77)	9.02 (0.94)	14.10 (2.05)	30.93 (3.47)	50.08 (5.74)
100	20	6.57 (0.72)	9.38 (1.03)	14.64 (1.46)	30.71 (2.44)	47.70 (4.09)
500	15	6.24 (0.35)*	9.35 (1.71)	14.35 (1.33)	30.50 (1.98)	48.76 (3.00)
1000	11	6.21 (0.62)*	8.70 (1.26)	13.85 (1.11)	30.69 (1.25)	46.43 (6.73)
Females						
0	22	6.57 (0.94)	8.61 (0.96)	13.38 (2.06)	29.87 (3.64)	47.07 (5.09)
100	20	6.22 (0.61)	8.97 (0.84)	14.11 (1.44)	29.43 (2.39)	44.65 (5.91)
500	15	5.88 (0.31)*	8.54 (0.95)	13.63 (1.21)	29.32 (1.71)	46.42 (2.95)
1000	11	5.91 (0.59)*	8.05 (1.48)	13.40 (1.09)	29.69 (1.77)	47.00 (2.81)

\* Significantly different from control  $P \leq 0.05$ .

## CONCLUSION:

In a publication from 1986, the reproductive effects of 2,4,6-TCP were investigated in male and female Long-Evans rats. Adult males (15-22, depending on group) were treated with 0, 100, 500, or 1000 mg/kg bw/d 2,4,6-TCP (gavage) for 10 weeks (5 days/week), at which time semen evaluations were conducted on ejaculates recovered from the genital tract of receptive untreated females. Fertility was assessed in the 0 and 1000 mg/kg bw groups. Females (15-22, depending on group) were treated with identical doses for 2 weeks prior to pregnancy (5 days/week) then throughout gestation (7 days/week). Females were mated with untreated males. Dams were allowed to litter and pup development was monitored until Day 42 post-partum.

2,4,6-TCP had no effect on any sperm parameter or male fertility. Treatment of females with 1000 mg/kg bw/d of 2,4,6-TCP produced maternal toxicity as reflected in increased lethality and decreased weight gain in the dams. However no treatment-related differences were seen in litter sizes or pup survival. Male and female birth weights were significantly depressed in the 500 and 1000 mg/kg bw/d groups; these differences disappeared by Day 4 post-partum suggesting that they were a reflection of maternal toxicity.

Overall, in this limited study, the reproductive processes of male and female rats do not appear to be a primary target of 2,4,6-TCP up to the limit dose of 1000 mg/kg bw/d. HSE notes that a NOAEL of 100 mg/kg bw/d could be identified for generalised offspring toxicity and a NOAEL of 500 mg/kg bw/d could be identified for parental toxicity.

## REFERENCES:

Zenick H, Blackburn K, Hope E and Baldwin D (1984). An assessment of the copulatory endocrinologic and spermatotoxic effects of carbon disulfide. *Toxicol. App. Pharmacol.* 73, 275-283.

(Blackburn K *et al*, 1986)

<b>Report:</b>	K-CA 5.8.1/44 Exon J.H. and Koller L.D. (1985). Toxicity of 2-Chlorophenol, 2,4-Dichlorophenol and 2,4,6-Trichlorophenol. University of Idaho, USA. Published: Exon J. H. Koller L.D. (1985). Toxicity of 2-chlorophenol, 2,4-Dichlorophenol and 2,4,6-Trichlorophenol. Water Chlorination: Chem., Environ. Impact Health Eff., Proc. Conf., 5th, (1985) Chapter 25. pp. 307-330. Syngenta File No. NA_13804.
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**STUDY TYPE:** Reproductive and Immunological toxicology study in male and female rats.

**Guidelines:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3 - The results and conclusion statements in the paper do not match.**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The purpose of the study was to explore the toxic effects of 2,4,6-Trichlorophenol (TCP) produced in laboratory rats following oral exposure (via drinking water) as monitored by bodyweight, histopathology changes in major organs and effects on reproduction and immune competence.

Weanling female Sprague Dawley (SD) rats were placed on 2,4,6-TCP treatment (either 3, 30 or 300 ppm administered in drinking water) from 3 weeks of age through to breeding and parturition. The rats were mated with untreated males at 90 days of age and conception, mean litter size, % stillborn, birth weight and survival to weaning were measured. The study reported that exposure to 2,4,6-TCP at 300 ppm significantly ( $p < 0.1$ ) produced smaller litter sizes with respect to the negative control. No other significant effects were reported on any of the other parameters measured in this part of the study.

Ten randomly selected pups from each groups of the female rats exposed to 2,4,6-TCP were obtained at 3 weeks of age and continued on treatment for 12-15 weeks. The immunological competence was assessed in each animal by measuring the following parameters: humoral immunity, cell mediated immunity and macrophage function. Body and organ (liver, spleen and thymus) weights were also recorded. No significant effects were observed for any of the immunological parameters measured. A significant increase ( $p < 0.05$ ) in spleen (at 300 ppm) and liver (at 30 and 300 ppm) weights were observed at the end of the study.

**In the present study, treatment with 2,4,6-TCP was not found to have an effect on reproduction of female Sprague-Dawley rats, apart from litter size in the highest dose group (300 ppm) where a significant decrease was observed ( $P < 0.1$ ) with respect to the control group. The authors concluded that 2,4,6-TCP may be embryotoxic when administered transplacentally. The authors have reported that  $p < 0.1$  to be significant however it is unlikely that this decrease is significant. No significant effect was observed on any of the immunological parameters measured in the study following exposure to 2,4,6-TCP. A significant increase was observed in spleen and liver weights following exposure to 2,4,6-TCP. However authors of the study concluded that the immune system appears to be a sensitive target to toxic insult induced by chronic exposure to 2,4,6-TCP. The conclusion made regarding immunological toxicity of 2,4,6-TCP does not match the results**

reported in the paper. From the presented results it is unlikely that 2,4,6- TCP targets the immune system based on the parameters investigated in this study.

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Source:</b>	Aldrich (No 75530-1)
<b>Purity:</b>	98%
<b>Stability of test compound:</b>	Not indicated

**Vehicle and/or positive control:** The test substance was administered dissolved in the drinking water. Fresh batches were made weekly.

<b>Test Animals:</b>	
<b>Species</b>	Rat
<b>Strain</b>	Sprague-Dawley
<b>Age/weight at dosing</b>	Females: Dosed from 3 weeks of age
<b>Source</b>	
<b>Housing</b>	4 per cage in racks of stainless steel hanging wire cages, except for pregnant female rats and their pre-weaning pups, which were housed in polycarbonate cages containing heat-treated hardwood shavings.
<b>Acclimatisation period</b>	Not indicated
<b>Diet</b>	A commercial rodent chow was available <i>ad libitum</i>
<b>Water</b>	Deionised water <i>ad libitum</i> in bottles
<b>Environmental conditions</b>	Temperature: 20-23 °C (68-73 °F) Humidity: Not indicated Air changes: Not indicated Photoperiod: 12/12 hours light/dark cycle

### Study Design and Methods:

**In-life dates:** Not indicated

### Female reproductive segment

Weanling female S-D rats were placed on 2,4,6-TCP treatment from 3 weeks of age through to breeding and parturition (pre-natal exposure). The rats were treated with either a low (3 ppm), medium (30 ppm) or high dose (300 ppm) of 2,4,6-TCP in drinking water, a negative control group was also treated concurrently. The group size was 12-14 dams per group. The females were bred to untreated males at 90 days of age. The parameters of reproduction recorded included per-cent conception, litter size, numbers of stillborn births, weaning weight and survival to weaning.

### Immunological Assays

Ten randomly selected progeny of female rats exposed from weaning age through to breeding at 90 day and parturition and lactation were obtained at 3 weeks of age per group and continued on 2,4,6-TCP treatment for an addition 12 to 15 weeks. Immunologic competence was assessed in each animal by measuring the ability to elicit three major types of immune responses: humoral immunity, cell mediated immunity (CMI) and macrophage function. Humoral immune responses were assessed using an enzyme-linked immunosorbant assay (ELISA) to quantitate specific serum IgG antibody levels to the T-cell-dependent protein antigens bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH). CMI responses were measured by a delayed-type hypersensitivity (DTH) response to either oxazolone

applied to the ears, or heat aggregated BSA, injected into the foot pads. Macrophage function was assessed by the ability of peritoneal cavity-derived, elicited, glass-adherent cells to phagocytize sheep red blood cell in vitro. Body and organ (liver, spleen and thymus) weights were recorded from all rats for which immunological competence was assessed.

#### Statistical analysis:

The data were analysed compared to the respective controls by analysis of variance and least square means.

## RESULTS

### Female reproductive segment

Litter sizes of female rats treated with 300 ppm 2,4,6-TCP were reported to be significantly smaller ( $p < 0.1$ ) than the respective controls. The percentage of stillborn pups tended to be increased in all groups that received 2,4,6-TCP, however this was not found to be significant (Table 6.8.1-94).

**Table 6.8.1-94: Summary of reproductive effects of female rats on exposure to 2,4,6-TCP. (a) Inclusive of still born pups; (b) Non-inclusive of still born pups; (c)  $p < 0.1$  compared with controls by analysis of variance and least square means**

Treatment 2,4,6-TCP (ppm)	Conception (%)	Litter size (mean +/- SE) <sup>a</sup>	Stillborn (%)	Birth weight (Mean +/- SE) <sup>a</sup>	Survival to weaning (%) <sup>c</sup>
Controls	77	12.1 +/- 1.1	0	2.9 +/- 0.4	100
3	69	11.3 +/- 1.1	2	2.7 +/- 0.3	99
30	85	11.2 +/- 1.0	3	2.8 +/- 0.5	99
300	77	9.1 +/- 0.9 <sup>d</sup>	3	2.6 +/- 0.5	97

### Immunological Results

No significant effects of 2,4,6-TCP treatment on immune responses were observed. However antibody levels, DTH reactions and macrophage numbers were consistently greater in the 2,4,6-TCP exposed animals compared to the controls (Table 6.8.1-95).

**Table 6.8.1-95: Effects of pre- and post-natal exposure to 2,4,6-TCP on immune system parameters of Sprague-Dawley rats\* (\*rats were exposed to 2,4,6-TCP in the drinking water continuously from 3 weeks through parturition (bred at 90 days) and lactation. Ten randomly selected pups from each group were weaned at 3 weeks and continued on 2,4,6-TCP treatment for 12 weeks)**

Treatment (ppm)	Antibody Production	DTH Response	Phagocytic Activity	PEC/Rat (No.)
	Mean (+/-SE) absorbance at 405nm	Mean (+/- SE) footpad swelling (mm)	Mean (+/-SE) cpm/100 µg protein	Mean (+/- SE) x 10 <sup>7</sup>
Controls	1.37 +/- 0.11	1.59 +/- 0.20	34.1 +/- 1.5	3.3 +/- 1.7
3	1.38 +/- 0.11	1.64 +/- 0.12	36.5 +/- 1.7	4.2 +/- 1.8
30	1.50 +/- 0.11	1.69 +/- 0.1	38.8 +/- 1.7	3.9 +/- 1.6
300	1.51 +/- 0.10	1.74 +/- 0.2	35.4 +/- 1.7	4.0 +/- 1.9

The body, liver, spleen and thymus weights were recorded from rats whose immunological competence was assessed. The body weights of rats exposed pre-and post-natally to 2,4,6-TCP were not significantly different to the controls. The liver weights were significantly increased in the rats that received 30 and 300 ppm 2,4,6-TCP with respect to the controls. The spleen weights for the rats receiving 300pm dose were also significantly increased. (Table 6.8.1-96)

**Table 6.8.1-96: Effects on body and organ weights of pre- and post-natal exposure of rats to 2,4,6-TCP. (a)  $P \leq 0.05$  compared with the controls by analysis of variance and least-square means**

Treatment (ppm)	Mean weight (g +/- SE)			
	Body	Thymus	Spleen	Liver
Control	271 +/- 4	0.38 +/- 0.08	0.93 +/- 0.09	10.9 +/- 0.4
3	282 +/- 5	0.36 +/- 0.01	0.95 +/- 0.04	11.9 +/- 0.3
30	256 +/- 8	0.32 +/- 0.08	0.89 +/- 0.03	12.5 +/- 0.5 <sup>a</sup>
300	262 +/- 4	0.40 +/- 0.08	1.07 +/- 0.07 <sup>a</sup>	14.1 +/- 0.6 <sup>a</sup>

### CONCLUSION:

HSE agrees with the evaluation of the EU peer-review process that this study is significantly different from a modern OECD guideline 416. It is also noted that the results and conclusion statements in the paper do not match. Therefore the reliability of the study is questioned.

In a publication from 1985, the toxic effects of 2,4,6-TCP on bodyweight, histopathology of major organs and reproduction and immune competence were investigated.

Weanling female Sprague Dawley rats were placed on 2,4,6-TCP treatment (either 3, 30 or 300 ppm administered in drinking water) from 3 weeks of age through to breeding and parturition. The rats were mated with untreated males at 90 days of age and conception, mean litter size, % stillborn, birth weight and survival to weaning were measured. The study reported that exposure to 2,4,6-TCP at 300 ppm produced statistically significant smaller litter sizes compared to the negative control. No other significant effects were reported on any of the other parameters measured in this part of the study.

Ten randomly selected pups from each groups of the female rats exposed to 2,4,6-TCP were obtained at 3 weeks of age and continued on treatment for 12-15 weeks. The immunological competence was assessed in each animal by measuring the following parameters: humoral immunity, cell mediated immunity and macrophage function. Body and organ (liver, spleen and thymus) weights were also recorded. No significant effects were observed for any of the immunological parameters measured. A statistically significant increase in spleen (at 300 ppm) and liver (at 30 and 300 ppm) weights were observed at the end of the study.

Overall, in this study, treatment with 2,4,6-TCP was found not to have an effect on reproduction of female Sprague-Dawley rats, apart from litter size in the highest dose group (300 ppm) where a significant decrease was observed ( $P < 0.1$ ). The authors concluded that 2,4,6-TCP may be embryotoxic. The authors reported that  $p < 0.1$  is significant; however it is unlikely that this decrease was significant. No significant effect was observed on any of the immunological parameters measured in the study following exposure to 2,4,6-TCP. A significant increase was observed in spleen and liver weights following exposure to 2,4,6-TCP. The authors concluded that the immune system appeared to be a sensitive target of exposure to 2,4,6-TCP. The conclusion made regarding immunological toxicity of 2,4,6-TCP does not match the results reported in the paper. From the results presented in the paper, it is unlikely that 2,4,6-TCP targets the immune system.

(Exon J.H and Koller L.D 1985)

### OTHER TOXICITY STUDIES

<b>Report:</b>	K-CA 5.8.1/45 Arrhenius E. <i>et al.</i> , (1977). Disturbance of Microsomal Detoxication Mechanisms in Liver by Chlorophenol Pesticides. Environment Toxicology Unit, Wallenberg Laboratory, University of Stockholm, Lilla Frescati, S-104 05 Stockholm 50, Sweden. Published: Arrhenius E, Renberg L, Johansson L, Zetterqvist M (1977). Disturbance of Microsomal Detoxication Mechanisms in Liver by Chlorophenol Pesticides. Chem. Biol. Interactions. 18:35-46. Syngenta File No. NA_13764.
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**STUDY TYPE:** Investigation into the effects on microsomal detoxication mechanisms in liver.

**Guidelines:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (stated only as purified).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

Livers were isolated from rats, following which, mitochondria and microsomes were prepared 2,4,6-trichlorophenol (2,4,6-TCP) was tested at various concentrations (0.03-3.0 M), alongside the internal standard pentachlorophenol (PCP).

There was a marked inhibition of C-oxygenation at relatively low concentrations and less marked effects on the N-oxygenation favouring an increased N/C-oxygenation quotient at higher concentration.

2,4,6-TCP produced a weaker inhibition of C-oxygenation than PCP.

**Under the conditions of the study reported, the test substance 2,4,6-TCP was shown to disturb liver microsomal detoxication functions. It was comparatively weak compared to the internal standard PCP.**

## MATERIALS AND METHODS

### Materials:

<b>Non-Radiolabelled Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	'Purified'
<b>Contaminants:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated
<b>Source:</b>	Not indicated

<b>Test Animals:</b>	
<b>Species:</b>	Rat (male)
<b>Strain:</b>	Wistar
<b>Age/weight at dosing:</b>	150-200 g
<b>Source:</b>	Bred in house
<b>Housing:</b>	Not indicated
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	Fors R 3 pellets until 16-20 h before sacrifice
<b>Water:</b>	Not indicated
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Lighting: Not indicated

### Study Design and Methods:

**In-life dates:** Not indicated

2,4,6-TCP was tested using PCP as an internal positive control. 2,4,6-TCP concentrations tested were 0.03, 0.1, 1.0 and 3.0 mM.

### Preparations of subcellular fractions

Mitochondria and microsomes were prepared from rat livers as described before (Estabrook *et al.*, 1967 and Arrhenius E *et al.*, 1969/70). The homogenization medium was 0.25 M sucrose for mitochondria, and 0.2 M potassium phosphate buffer pH 7.5 for microsomes. The microsomes were washed by layering the mitochondria-free homogenate over 0.3 M sucrose in 0.2 M phosphate buffer and centrifuging at 115 000 g for 60 min.

### *In vitro* assays

#### Measurement of subcellular functions

Oxygen consumption in mitochondrial preparations was measured using a Clark polarographic electrode at 35°C (Estabrook *et al.*, 1967). The microsomal incubation system for N- and C-oxygenation of DMA (N,N-dimethylaniline) was as described (Arrhenius E *et al.*, 1969/70) except that the nicotinamide concentration was reduced to 60 µmoles/2 ml, NADP was 0.5 µmole/2 ml and glucose-6-phosphate dehydrogenase was 0.50 units/2 ml. At this concentration the inhibition of mixed function oxygenases was negligible, whereas the protection against cleavage of the nicotinamide-ribose bound in NADP was still complete. 2,4,6-TCP (0.03 - 3.0 mM) was dissolved in ethanol and added to the incubation systems, the amount of ethanol never exceeding 20 µl/2 ml incubation mixture. This concentration did not affect N- and C-oxygenation of DMA. N-oxygenation was measured as the DMA N-oxide produced, and C-oxygenation as formaldehyde produced (Arrhenius E *et al.*, 1969/70). Protein was measured by the method of Lowry (Lowry *et al.*, 1951).

## RESULTS

### Microsomal N and C oxygenation

2,4,6-TCP was tested with PCP as an internal positive control. There was a marked inhibition of C-oxygenation at relatively low concentrations and less marked effects on the N-oxygenation favouring an increased N/C-oxygenation quotient at higher concentration.

2,4,6-TCP produced a weaker inhibition of C-oxygenation than PCP. Also the stimulatory effect on N-oxygenation is reached only at higher concentrations. Thus the maximum in the N/C-oxygenation ratio which presumably is obtained also with 2,4,6-TCP, is not reached within the concentration range used in the experiments described (0.03 - 3.0 mM).

**CONCLUSION:**

In a publication from 1977, mitochondria and microsomes prepared from rat livers were treated with 2,4,6-TCP at various concentrations (0.03-3.0 M) to investigate detoxication functions. 2,4,6-TCP was shown to disturb liver microsomal detoxication functions. It was comparatively weak compared to the internal standard PCP.

**REFERENCES:**

*Estabrook R (1967). Mitochondrial respiratory control and the polarographic measurement of ADP : O ratios. Methods in Enzymology, Vol X, Academic Press, New York, p. 41.*

*Arrhenius E (1969/70). Effects of various in vitro conditions on hepatic microsomal N- and C-oxygenation of aromatic amines. Chem. Biol. Interact. 1 361.*

*Lowry O, Rosebrough N, Farr A and Randall R (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem., 193 265.*

(Arrhenius E *et al*, 1977)

<b>Report:</b>	K-CA 5.8.1/46 Carlson G. (1978). Effect of Trichlorophenols on Xenobiotic Metabolism in the Rat. Department of Pharmacology and Toxicology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Ind. 47907 (U.S.A.). Published: Carlson G (1978). Effect of Trichlorophenols on Xenobiotic Metabolism in the Rat. Toxicology 11:145-151. Syngenta File No. NA_13803.
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**STUDY TYPE:** Effect of compound exposure on xenobiotic metabolism in the rat.

**Guidelines:** No globally or regionally accepted guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

2,4,6-trichlorophenol (2,4,6-TCP) was dosed to male rats at doses as high as 400 mg/kg by oral gavage, daily for 14 days, to investigate effects on various markers of xenobiotic metabolism and hepatotoxicity.

2,4,6-TCP did not alter ethyl p-nitrophenyl thionobenzenephosphonate (EPN) detoxification, NADPH-cytochrome c reductase activity or the cytochrome P450 content in samples derived from 2,4,6-TCP



administred rats. No evidence of hepatotoxicity was observed, as measured by hepatic glucose 6-phosphatase activity and serum sorbitol dehydrogenase activity.

2,4,6-TCP inhibited EPN detoxification and the demethylation of p-nitroanisole *in vitro*.

**Under the conditions of the study reported, the test substance 2,4,6-TCP had no effect on xenobiotic metabolism, did not alter glucuronyltransferase activity and was not hepatotoxic in the male rat. 2,4,6-TCP showed inhibition of both p-nitroanisole demethylation and EPN detoxification *in vitro*, toxicological relevance of this finding was not discussed.**

## MATERIALS AND METHODS

### Materials:

<b>Non-Radiolabelled Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>Contaminants:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated
<b>Source:</b>	Not indicated

**Vehicle:** Corn oil

**Preparation of dosing solutions:** 2,4,6- was administered by oral gavage, after formulation in corn oil.

<b>Test Animals:</b>	
<b>Species:</b>	Rat (male)
<b>Strain:</b>	Sprague-Dawley
<b>Age/weight at dosing:</b>	Adult
<b>Source:</b>	
<b>Housing:</b>	Not indicated
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	<i>ad libitum</i> (diet not specified)
<b>Water:</b>	Tap water <i>ad libitum</i>
<b>Environmental conditions:</b>	Temperature: Controlled Humidity: Not indicated Air changes: Not indicated Light controlled rooms

### Study Design and Methods:

**In-life dates:** Not indicated

### Study design:

Test Group	Dose level (mg/kg)	Dose route	Number/sex	Dosing period
<b>EPN detoxification, NADPH-cytochrome c reductase activity and cytochrome P-450 content</b>				

Control (corn oil)	0	gavage	6/males	14 days
Low	25		6/males	14 days
Middle	100		6/males	14 days
High	400		6/males	14 days
Glucuronyltransferase activity				
Control	0	gavage	5/males	14 days
200 mg/kg	200			
Hepatic glucose 6- phosphatase and serum sorbitol dehydrogenase activity				
Control (corn oil)	0	gavage	4/males	14 days
200 mg/kg	200			

### Dosing and sample collection:

#### EPN detoxification, NADPH-cytochrome c reductase activity and cytochrome P450 content

Groups of 6 animals were administered 25, 100 or 400 mg/kg 2,4,6-TCP by intubation daily for 14 days. Control animals received corn oil which was used as the vehicle. 24 hours after the last dose the animals were lightly anaesthetized with ether. A small portion of the liver was removed for determination of EPN detoxification using previously described methods (Neal *et al.* 1965, as modified by Kinoshita *et al.* 1966). The livers were then perfused *in situ* with cold isotonic saline.

Microsomal fractions were prepared as previously described (Carlson *et al.* 1975). NADPH-cytochrome c reductase activity and cytochrome P450 content were measured as previously described (Dallner *et al.* 1963) and proteins by the method of Lowry (Lowry *et al.* 1951).

For the *in vitro* studies, 2,4,6-TCP was dissolved in benzene and added so that 50 µl gave a final concentration of  $2.5 \times 10^{-4}$  M. The benzene itself had no significant effect on activity. p-Nitroanisole demethylation was determined using previously described methods (Netter *et al.* 1964, modified by Kinoshita *et al.* 1966).

#### Glucuronyltransferase activity

The influence of the compounds on glucuronyltransferase activity was assessed following both *in vivo* and *in vitro* administration of 2,4,6-TCP. Groups of 5 rats received 200 mg/kg 2,4,6-TCP daily for 14 days by oral gavage. Controls received corn oil. At termination (24 hours after the last dose) microsomes were prepared from a small piece of liver and glucuronyltransferase activity measured using naphthol as the substrate according to previously described methodology (Lucier *et al.* 1971).

*In vitro*, 2,4,6-TCP was dissolved in benzene and added to give a final concentration of  $1 \times 10^{-4}$  M. The control sample received an equivalent volume (50 µL) of benzene.

#### Hepatic glucose 6- phosphatase and serum sorbitol dehydrogenase activity

2,4,6-TCP was administered at a dose of 200 mg/kg by oral gavage to groups of 4 rats daily for 14 days. The animals were lightly anaesthetized with ether, and tail vein blood samples were taken for measurement of serum sorbitol dehydrogenase (Gerlach *et al.* 1965). Details of blood sampling times are not reported, it is assumed they are taken at scheduled termination. The rats were sacrificed and portions of the livers removed for glucose-6-phosphatase determinations (Harper *et al.* 1965).

#### Statistical analysis

In the *in vivo* studies comparisons were made among the doses as well as with the control using Duncan's new multiple range finding test. For the *in vitro* studies a paired Student's t-test was used. In both cases the level of significance chosen was  $p < 0.05$ .

### RESULTS

2,4,6-TCP did not induce xenobiotic metabolism, as measured by EPN detoxification, cytochrome c reductase activity or cytochrome p450 content.

The *in vitro* addition of 2,4,6-TCP indicated inhibition of both p-nitroanisole demethylation and EPN detoxification.

**Table 6.8.1-97: Effect of 2,4,6-TCP in vitro on p-nitroanisole o-demethylation and EPN detoxification**

Concentration	p-Nitroanisole O-demethylation (µg p-nitrophenol/50mg/30min)	EPN detoxification (µg p-nitrophenol/50 mg/30min)
Control	6.64 ± 0.68	8.39 ± 0.34
2.5 × 10 <sup>-4</sup> M 2,4,6-TCP	2.33 ± 0.19*	4.03 ± 1.17*

\*Significantly different from control (P < 0.05).

The ability of 2,4,6-TCP to alter the conjugation ability of liver microsomes was determined using naphthol as the substrate (glucuronyltransferase activity). No significant inhibition of this reaction was observed when 2,4,6-TCP was administered daily for 14 days at a dose of 200 mg/kg. This correlated with the *in vitro* data generated, that again showed 2,4,6-TCP had no effect on glucuronyltransferase activity.

2,4,6-TCP was not hepatotoxic when administered by oral gavage at a dose of 200 mg/kg daily for 14 days by gavage, as measured by hepatic glucose 6- phosphatase activity and serum sorbitol dehydrogenase activity.

## CONCLUSION:

In a publication from 1978, 2,4,6-TCP was dosed to male rats at doses as high as 400 mg/kg bw by oral gavage, daily for 14 days, to investigate effects on various markers of xenobiotic metabolism and hepatotoxicity. 2,4,6-TCP had no effect on xenobiotic metabolism, did not alter glucuronyltransferase activity and was not hepatotoxic in the male rat.

## REFERENCES:

- Neal RA and DuBois KP (1965). *Studies on the mechanism of detoxification of cholinergic phosphorothioates. Pharmacol. Exp Ther* 148 185.
- Kinoshita FK, Frawley JP and DuBois KP (1966). *Quantitative measurement of induction of hepatic microsomal enzymes by various levels of DDT and toxaphene in the rat. Toxicol. Appl. Pharmacol.*, 9 505.
- Carlson GP (1975) *Protection against carbon tetrachloride-induced hepatotoxicity by pretreatment with methylmercury hydroxide Toxicology*, 4 83.
- Dallner G (1963). *Studies on the structural and enzymic organisation of the membranous elements of liver microsomes. Acta Pathol. Microbiol. Scand., Suppl.* 166 7
- Lowry GH, Rosebrough NJ, Farr AL and Randall RJ (1951). *Protein measurement with the folin phenol reagent. J. Biol. Chem.*, 193 265.
- Netter KJ and Seidel G (1964). *An adaptively stimulated o-demethylating system in rat liver microsomes and its kinetic properties. J. Pharmacol. Exp. Ther.*, 146 61.
- Lucier GW, McDaniel OS and Matthews HB (1971). *Microsomal rat liver UDP glucuronyltransferase: effects of piperonyl butoxide and other factors on enzyme activity. Arch. Biochem. Biophys.*, 145 520.
- Gerlach U (1965). *Sorbitol Dehydrogenase*, in H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Academic Press, New York, p. 761.

Harper AE (1965) Glucose-6-phosphatase, in H.U. Bergmeyer (Ed.), *Methods of Enzymatic Analysis*, Academic Press, New York, p. 788.

(Carlson G *et al*, 1978)

<b>Report:</b>	K-CA 5.8.1/47 Götz R. <i>et al.</i> , (1980). Effects of Pentachlorophenol and 2,4,6-Trichlorophenol on the Disposition of Sulfobromophthalein and Respiration of Isolated Liver Cells. Gesellschaft für Strahlen- und Umweltforschung, D-8042 Neuherberg/München, Federal Republic of Germany. Published: Götz R, Schwarz L, Greim H (1980). Effects of Pentachlorophenol and 2,4,6-Trichlorophenol on the Disposition of Sulfobromophthalein and Respiration of Isolated Liver Cells. Arch. Toxicol. 44:147-155. Syngenta File No. NA_13798.
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**STUDY TYPE:** *In Vitro* Assay Investigating Chemical Effect on Disposition of Sulfobromophthalein and Respiration of Isolated Liver Cells

**Guideline:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (highly purified at laboratory).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The effect of 2,4,6-trichlorophenol (2,4,6-TCP) on the disposition of the hepatodiagnostic dye, sulfobromophthalein (BSP) was studied in isolated liver cells.

2,4,6-TCP (50-100 µM) interferes with the disposition of BSP. The main effect apparently occurs at the secretion step as the test item severely impaired the release of the glutathione conjugate of BSP into the medium. As a consequence, BSP and its conjugate accumulate in the cell.

The effect of 2,4,6-TCP on mitochondrial respiration was also investigated. Concentrations which interfere with the secretion of BSP also completely uncouple the oxidative phosphorylation of hepatocellular mitochondria. The dysfunction of liver cells described here may therefore be explained by the effect 2,4,6-TCP on the energy production of the cells.

**In the assays described, 2,4,6-TCP interfered with the disposition of BSP in isolated liver cells. The test item also caused uncoupling of the oxidative phosphorylation of hepatocellular mitochondria, as measured by altered oxygen consumption of the cells. This data indicates 2,4,6-TCP has an effect on the energy production of the cells.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	'Highly purified', free of chlorodibenzo-p-dioxines at laboratory
<b>Stability of test compound:</b>	Not indicated
<b>Source:</b>	Dr Parlar (Institut für ökologische chemie, GSF, Attachingen, FRG)

<b>Control Materials:</b>	
<b>Negative:</b>	Not indicated
<b>Solvent control (final concentration):</b>	
<b>Positive control:</b>	Not indicated (CCP for mitochondrial respiration studies)

### Concentrations of 2,4,6-TCP used:

Effect on BSP kinetics and metabolism	5, 50 and 100 µM
Effect on the conjugation of BSP with glutathione	100 and 1000 µM
Uncoupling of mitochondrial respiration	25, 50, 75, 100 µM

<b>Test Animals:</b>	
<b>Species:</b>	Rat (male)
<b>Strain:</b>	Sprague Dawley
<b>Age/weight at dosing:</b>	200 g on arrival
<b>Source:</b>	Bred in house
<b>Housing:</b>	Not indicated
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	Standard lab chow <i>ad libitum</i>
<b>Water:</b>	<i>Ad libitum</i>
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated Lighting: Not indicated

### Study Design and Methods:

**In-life dates:** Not indicated

### Test performance:

#### Isolation and viability of liver cells

Liver cells were isolated from male Sprague Dawley rats, as previously described (Baur *et al.*, 1975) but without hyaluronidase (Schwenk *et al.*, 1977). The viability of the hepatocytes was tested as described earlier (Baur *et al.*, 1975). Only cell preparations were used when respiratory control ratio was above 2.0 at 37°C and stimulation by succinate was below 20%. Susceptibility of the cell preparations to trypan blue staining ranged from 5-8%.

#### Hepatocyte biochemical assays

Hepatocytes, equivalent to 4.5 mg protein/mL, were incubated at 37°C in Leibowitz L-15 medium containing 40 mM hepes buffer. The oxygen supply under these conditions was maintained by streaming oxygen over the surface of the cell suspension. After a preincubation period of 10 min (<sup>35</sup>S)- BSP was

added in a final concentration of 25  $\mu\text{M}$ . One minute prior to the addition of the dye various concentrations 2,4,6-TCP were added. After 1, 2, 5, 10, 20, 30, 45, and 60 min, 200  $\mu\text{L}$  aliquots of the cell suspension were with-drawn, placed into 0.5 mL polyethylene tubes previously filled with 100  $\mu\text{L}$  silicone oil (AR 200/AR 20; 1/1) and centrifuged in a Beckman Microfuge 124 for rapid separation of the cells from the medium. Immediately after centrifugation 100  $\mu\text{L}$  of the supernatant was mixed with the three-fold volume of chilled methanol, while the tips containing the cell pellet were frozen in liquid nitrogen to prevent further metabolism. BSP and its glutathione conjugate (BSP-GS) was extracted twice from the cell pellet with 100  $\mu\text{L}$  methanol-water (3:1). After removal of lipids by extraction into 100  $\mu\text{L}$  heptane, BSP and its glutathione conjugate were separated on thin layer chromatography foils according to Schulze and Czok (1975). Chromatograms were developed in tert-butyl alcohol/water (3/1) according to Sardini *et al.* (1969).

*In vitro* conjugation of BSP with glutathione was performed with 100,000 g supernatants of rat liver homogenates following the method of Klaassen and Plaa (1967). Lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase and glutamate dehydrogenase leakage from the cells was determined in the supernatant of centrifuged samples at zero time and after an incubation period of 1 h. The total enzyme activities of the samples were determined after breaking the cells by 0.1% Triton X-100 (Baur *et al.*, 1975). The enzyme activities were measured according to Bergmeyer and Bernt (1970). Cellular protein was determined by a modified biuret method (Szarkowska and Klingenberg, 1963). Trypan blue staining was performed by mixing equal volumes of cell suspension and 0.4% trypan blue in incubation medium. Oxygen consumption was measured with a Clark oxygen electrode at pH 7.4 and 37°C.

**Statistical Methods:** None indicated.

**Evaluation Criteria:** None provided.

## RESULTS

### Effects 2,4,6-TCP on the disposition of BSP

The effect of 2,4,6-TCP was only described in comparison to the effects of pentachlorophenol (PCP), which was tested in parallel in this publication.

After treatment with PCP, the concentration of BSP in the medium decreases exponentially with time. Within 20 min of incubation more than 90% of the initial amount of BSP has disappeared from the medium. Correspondingly, the intracellular concentration of unconjugated BSP increases rapidly and reaches a maximum after 10 min. Thereafter the intracellular concentration decreases due to conjugation of the dye and secretion of the metabolite into the medium. The conjugate is transiently accumulated in the cells indicating a temporary overload of the secretory mechanism for BSP-GS. Elimination of the dye proceeds with a relative constant rate of 0.1 nmol/mg prot.  $\times$  min.

Up to 2  $\mu\text{M}$ , PCP did not affect any of the individual steps of the BSP turnover; however 4  $\mu\text{M}$  PCP, and even more pronounced, 6  $\mu\text{M}$  PCP, caused a marked disturbance of the disposition of the dye mainly because of interference with the secretion step. The initial rates of uptake seem to be only slightly affected up to 6  $\mu\text{M}$  PCP as demonstrated by the disappearance of BSP from the medium during the first minutes. At higher time values the concentration of the unconjugated dye in the medium is increased. In the cellular compartment the maxima of the BSP curves are shifted from about 10 min to 20 min and the subsequent intracellular disappearance of the dye is much less expressed. The latter effect is even more expressed in respect to the time course of the intracellular BSP-GS. The metabolite seems to accumulate in the cells apparently due to its impaired secretion into the medium.

2,4,6-TCP produces effects which are similar to those of PCP only at concentrations from 50-100  $\mu\text{M}$ . At 5  $\mu\text{M}$  where PCP interferes strongly with the disposition of BSP, 2,4,6-TCP has no effect.

Additionally the rates of BSP conjugation by 100,000 g supernatant were determined. PCP did not inhibit the reaction up to a concentration of 10  $\mu$ M. 100  $\mu$ M of the drug decreased conjugation activity by 25%. At this concentration 2,4,6-T diminished conjugation by 5% .

### Mitochondrial respiration

Oxygen consumption of the isolated hepatocytes was measured and amounted to 12.5 natom oxygen/min  $\times$  mg in the absence of the toxic agents. Increasing concentrations of PCP or 2,4,6-TCP by steps of 2  $\mu$ M and 25  $\mu$ M, respectively, successively lead to an increased respiration of the cells, indicating uncoupling of the mitochondrial electron transport. Above 75  $\mu$ M 2,4,6-TCP no further stimulation of the respiration occurred. A similar stimulation was achieved with CCP at a concentration of 2  $\mu$ M, which is known to uncouple the mitochondrial respiration of cells completely (Baur *et al.*, 1975).

### CONCLUSION:

In a publication from 1980, the effect of 2,4,6-TCP on mitochondrial respiration and on the disposition of the hepatodiagnostic dye, sulfobromophthalein (BSP) was studied in isolated liver cells. 2,4,6-TCP (50-100  $\mu$ M) interferes with the disposition of BSP. As a consequence, BSP and its conjugate accumulate in the cell. Concentrations which interfere with the secretion of BSP also completely uncouple the oxidative phosphorylation of hepatocellular mitochondria. This data indicates 2,4,6-TCP has an effect on the energy production of liver cells in vitro.

### REFERENCES:

Baur H, Kasperek S, Pfaff E (1975). Criteria of viability of isolated liver cells. *Hoppe-Seylers Z. Physiol. Chem.* 356:827-838

Bergmeyer U, Bernt E (1970) In *Methoden der Enzymatischen Analyse*, Bergmeyer, H. U. (Hrsg.), S. 533--538. 2. Ausg., Bd. 1. Weinheim: Verlag Chemie

Klaassen C, Plan G (1967). Species variation in metabolism, storage and excretion of sulfobromophthalein. *Am. J. Physiol.* 213:1322-1326

Sardini D, Barbi G, Bastoni F, Marzo A (1969). Urinary metabolites of bromosulfophthalein in normal rat. *Experientia* 25:1250

Schulze P, Czok G (1975). Reduced bile flow in rats during sulfobromophthalein infusion. *Toxicol. Appl. Pharmacol.* 32, 213-224

Schwenk M, Burr R, Schwarz L, Pfaff E (1976). Uptake of bromosulfophthalein by isolated liver cells. *Eur. J. Biochem.* 64:189-197

Szarkowska L, Klingenberg M (1963). On the role of ubiquinone in mitochondria. *Biochem. Z.* 338, 674-697.

(Götz R *et al.*, 1980)

<b>Report:</b>	K-CA 5.8.1/48 Heil J. and Reifferscheid G. (1992). Detection of Mammalian Carcinogens with an Immunological DNA Synthesis-Inhibition Test. Department of Environmental and Molecular Genotoxicity (AMMUG), University of Mainz and Academy of Sciences and Literature, Obere Zahlbacher Strasse 63, 6500 Mainz, Germany. Published: Heil J and Reifferscheid G (1992). Detection of mammalian carcinogens with an immunological DNA synthesis-inhibition test. <i>Carcinogenesis</i> 13:2389-2394. Syngenta File No. NA_13785.
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**STUDY TYPE:** *In Vitro* DNA synthesis-inhibition test (DIT) in HeLa S3 cells.

**Guideline:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The potential for 2,4,6-trichlorophenol (2,4,6-TCP) to cause inhibition of DNA synthesis was investigated in HeLa S3 cells *in vitro*.

2,4,6-TCP was tested up to 10 mM, the limit of solubility of the compound in the test system.

2,4,6-TCP was inactive in the assay up to the limit of solubility. The  $DI_{50}$  for 2,4,6-TCP was therefore given as >10 mM. The  $DI_{50}$  is the concentration of an agent in mol/L which inhibits DNA synthesis by 50%.

**Under the conditions of the test described, 2,4,6-TCP did not cause a greater than 50% inhibition of DNA synthesis over the concentration range tested.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>Source:</b>	Not defined (Merck, Darmstadt or Sigma, Deisenhofen, Germany)
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated

<b>Control Materials:</b>	
<b>Negative:</b>	Not specified
<b>Solvent control (final concentration):</b>	Not specified
<b>Positive control:</b>	Not specified

In all assays, appropriate positive and negative controls were used, although these were not specified.



**Test cells: mammalian cells in culture**

HeLa S3 (ATCC, Rockville, MD, USA) cells were cultured in Eagle's minimal essential medium (MEM) supplemented with 10% foetal calf serum, 20 mM HEPES and 100 mg/1 kanamycin in an incubator at 37°C; subcultivation was every 4 days.

**Test compound concentrations used:**

Absence of S9 mix                      Not specified. Up to 10 mM

**Study Design and Methods:**

**In-life dates:** Not specified

**Test performance:****Cell treatment:**

A 4 day old culture of logarithmically growing HeLa S3 cells was transferred into a single cell suspension by gently detaching the cells with EDTA (250 mg/1 PBS (phosphate-buffered saline)). Trypsination should be avoided. Then the cells were seeded into 96 well microplates at a density of  $2 \times 10^4$  cells/well. The next day (not < 16 h later because of recovery of the cells from that procedure) the monolayers of the HeLa cells were exposed for 90 min to the agents to be tested. The authors used dimethylsulfoxide (DMSO) as a solvent for the testing of a wide range of substances when required; the final DMSO concentration in serum-free MEM, did not exceed 1.5%. However it was not specified whether 2,4,6-TCP had to be dissolved in a solvent for use in the assay.

Then the agent was washed away by two rinses with fresh, pre-warmed medium, and the cells were allowed to recover in complete medium for 2 h. This was followed by addition of BrdU and equimolar amounts of 2-deoxycytidine (decreased BrdU toxicity) to the cells in a final concentration of 20 µM for 60 min. Subsequently, the cells were fixed with ethanol:acetic acid:water (90:5:5) for 30 min at room temperature.

**Immunological assay:**

Thereafter the alcohol was poured off and 4 N HCl was added to the fixed cells for 10 min (this denatures the DNA and produces single-stranded molecules). Excess acid was washed away by rinsing the microplate twice with tap water. Then 50 µl/well of a 1:1500 dilution of a monoclonal anti-BrdU antibody (Bio Cell Consulting, Reinach, Switzerland) in PBT (PBS + 0.5% Tween<sup>R</sup> 20) containing 50 % sheep serum (in order to minimize unspecific binding of the antibody) was added to the cells for 30 min. After washing the cells three times with tap water, a 1:500 dilution of peroxidase-conjugated F(ab')<sub>2</sub>-sheep-anti-mouse IgG antibody (Sigma) in PBT containing 10 % sheep serum was added for another 30 min. The cells were washed three times with tap water, and 100 µL/well of a freshly prepared peroxidase substrate solution (300 mg *o*-phenylenediamine (OPD)/100 mL citrate phosphate buffer (50 mM, pH 5.0), 0.015 % H<sub>2</sub>O<sub>2</sub> was added. This was incubated at room temperature until the required colour intensity was achieved. The colour development was stopped with 50 µL/well of a stop solution (H<sub>2</sub>SO<sub>4</sub>, 1 M). The absorbance of the wells was measured at 495 nm using an ELISA reader. Cell counts were determined by sulforhodamine B (SRB) adsorption (0.1 g SRB/L acetic acid (1 %)), followed by elution of the dye with Tris buffer (10 mM) and colorimetric measurement at 564 nm. The OPD/SRB ratios for 2,4,6-TCP-treated cells were expressed as percentages of control incorporation into DNA. This ratio will be low in genotoxically damaged cells and maximal in undamaged controls.

Tests performed in 96 well microplates were handled with an automated laboratory workstation (Biomek 1000, Beckman).

In all assays, appropriate positive and negative controls were used.

**Data evaluation:**

From the data generated,  $DI_{50}$  values (i.e. the concentration of 2,4,6-TCP in mol/L which inhibits DNA synthesis by 50%) were calculated. If no  $DI_{50}$  value could be derived, the highest possible well soluble concentration was specified. In all assays, appropriate positive and negative controls were used.

**Evaluation Criteria:** Criteria for positive/negative responses in the assay were not specified

**RESULTS**

**Preliminary toxicity assay:** Not performed.

**DNA synthesis-inhibition assay:**

2,4,6-TCP was tested up to 10 mM, the limit of solubility in the assay.

2,4,6-TCP was inactive in the assay up to the limit of solubility. The  $DI_{50}$  for 2,4,6-TCP was therefore given as >10 mM.

**CONCLUSION:**

In a publication from 1992, the potential for 2,4,6-TCP to cause inhibition of DNA synthesis was investigated in HeLa S3 cells in vitro. 2,4,6-TCP was tested up to 10 mM, the limit of solubility of the compound in the test system. 2,4,6-TCP was inactive in the assay up to the limit of solubility.

(Heil J and Reifferscheidt G, 1992)

<b>Report:</b>	K-CA 5.8.1/49 Kitchin K. Brown J. (1994). Dose-Response Relationships for Rat Liver DNA Damage Caused by 49 Rodent Carcinogens. Carcinogenesis and Metabolism Branch, Genetic Toxicology Division, Health Effects Research Laboratory, US Environmental Protection Agency, Research Triangle Park, NC 27711, USA. Published: Kitchin K and Brown J (1994). Dose-response relationships for rat liver DNA damage caused by 49 rodent carcinogens. Toxicology 88:31-49. Syngenta File No. NA_13808.
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**STUDY TYPE:** Dose-response relationships for rat liver DNA damage caused by 49 rodent carcinogens

**Guidelines:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (Purity not indicated)

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

2,4,6 trichlorophenol (2,4,6-TCP) was dosed by oral gavage to 90 day old female rats between 164 and 500 mg/kg at 21 and 4 hours prior to kill to determine rat liver DNA damage using rat hepatic DNA damage assay (alkaline elution method). At the highest dose tested (500 mg/kg), no rat liver DNA damage was observed.

**Under the conditions of the reported study, 2,4,6-TCP was not found to induce DNA damage in rat livers at a concentration of 500 mg/kg as measured using rat hepatic DNA damage assay.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>Source</b>	Not defined (US National Toxicology Program repository managed by Radian Corporation or standard commercial sources.
<b>Contaminants:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated

**Vehicle:** 1:1:1-volume mixture of Tween 80, Triton X-100 and dimethylsulfoxide (DMSO).

**Preparation of dosing solutions:** 2,4,6-TCP was administered by oral gavage in a 1:1:1-volume-mixture of Tween 80, Triton X-100 and DMSO. Further details of preparation method, or dose analysis for achieved concentration/homogeneity or stability are not provided. Details of 2,4,6-TCP concentrations in the dose preparation and dose volume are not provided.

<b>Test Animals:</b>	
<b>Species:</b>	Female Rat
<b>Strain:</b>	Sprague-Dawley (CD)
<b>Age/weight at dosing:</b>	90 day old
<b>Source:</b>	
<b>Housing:</b>	3 per cage
<b>Acclimatisation period:</b>	Not indicated
<b>Diet:</b>	Available <i>ad libitum</i>
<b>Water:</b>	Available <i>ad libitum</i>
<b>Environmental conditions:</b>	Temperature: Not indicated Humidity: Not indicated Air changes: Not indicated

### Study Design and Methods:

**In-life dates:** Not indicated

### Animal treatment

Adult female rats were given two oral doses of 2,4,6-TCP formulated in 1:1:1 volume mixture of Tween 80:Triton X-100:DMSO at 21 and 4 hours before sacrifice. The lowest dose of 2,4,6-TCP was 164 mg/kg and was selected as 1/5 of the oral LD<sub>50</sub> in the rat. The high dose of 2,4,6-TCP was 500 mg/kg as the 'highest tested negative dose'; however it was not reported if any higher or intermediate doses were also tested. A group of vehicle-only treated rats were also employed in the study and used for statistical analysis.

There was no indication of how many animals were dosed per dose level, the number of dose levels or if a positive control was employed in the study.

### Liver tissue preparation

The liver tissue preparation was performed according to a previously reported study (Kitchin and Brown, 1989). To prepare subcellular tissue fractions, liver tissue (1.5 g) was homogenized in 6 ml of ice-cold pH 7.5 buffer containing NaCl (136 mM), KCl (5.4 mM), HEPES (20 mM), dithioerythritol (5mM), EDTA (4 mM) and pyridoxal-5'-phosphate (0.08 mM). Liver samples were homogenized with six strokes with a size C Potter-Elvehjem homogenizer (clearance 0.15-0.23 mm) operated at 300 rpm. After a 10-min settling period at 4°C, 75 µL of the whole liver homogenate were used for alkaline elution.

### DNA elution

The DNA elution assay was performed according to a previously reported study (Kitchin and Brown, 1989). The Stout and Becker (Stout and Becker, 1982) modification of the Kohn et al (Kohn *et al*, 1981) basic alkaline elution procedure minimizing protein adsorption was employed. A 48-h delay in the SDS-lysis step (Nicolini *et. al* 1985), addition of 0.06% Sarkosyl to the first EDTA wash, and addition of 5 mM phosphate buffer to increase the buffering capacity of the pH 12.10 eluting solution were the additional modifications of the alkaline elution technique. Alkaline elution data are expressed as the fraction of DNA eluted during the 14-h period. A fraction of 1.00 DNA eluted means 100 % of the DNA was eluted from the filter.

### Statistical analysis

Statistical analysis employed analysis of variance and where statistical differences were observed data were evaluated using a Students *t* test.

## RESULTS

No evidence of DNA damage in the liver was observed in the DNA alkaline elution assay after administration of 2,4,6-TCP at 500 mg/kg dose. However the alkaline elution data is not reported in the study.

### CONCLUSION:

In a publication from 1994, 2,4,6-TCP was dosed by oral gavage to female rats between 164 and 500 mg/kg bw at 21 and 4 hours prior to kill to determine rat liver DNA damage using a DNA damage assay (alkaline elution method). At the highest dose tested (500 mg/kg bw), no rat liver DNA damage was observed.

### REFERENCES:

- Kitchin K and Brown J. (1989). *Biochemical effects of 3 carcinogenic chlorinated methanes in rat livers. Teratogenesis, Carcinogenesis and Mutagenesis*; 9:61-69.
- Kohn K, Ewig R, Erickson L and Zwelling L. *DNA Repair, A Laboratory Manual of Research Procedures* (P. C. Hanawalt and E. C. Friedberg, eds.) (Marcel-Dekker, New York, 1981), pp. 379-401.
- Stout D and Becker F (1982). *Fluorometric quantitation of single stranded DNA: A method applicable to the technique of alkaline elution. Analytical Biochemistry* 127:302-307.
- Nicolini C, Robbiano L, Pino A, Maura A, Finollo R and Brambilla G (1985). *Higher sensitivity for the detection of chemically-induced DNA damage: role of DNA unfolding in determining alkaline elution rate. Carcinogenesis* 6: 385-389.

(Kitchin K and Brown J, 1994)

<b>Report:</b>	K-CA 5.8.1/50 Pereira M. <i>et al.</i> , (1982). Initiation/Promotion Bioassay in Rat Liver: use of Gamma Glutamyltranspeptidase-Positive Foci to Indicate Carcinogenic Activity. Toxicology and Microbiology Division, Health Effects Research Laboratory, U.S. Environmental Protection Agency, 26 W. St. Clair Street, Cincinnati, Ohio 45268. Published: Pereira M, Herren S, Britt A, Khoury M (1982). Initiation/promotion bioassay in rat liver: use of gamma glutamyltranspeptidase-positive foci to indicate carcinogenic activity. Toxicological Pathology 10(2):11-18. Syngenta File No. NA_13776.
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**STUDY TYPE:** Initiation/promotion bioassay in rat liver

**Guidelines:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 3**

**HSE comment:** supportive study

## EXECUTIVE SUMMARY

The tumour initiation potential of 2,4,6-trichlorophenol (2,4,6-TCP) in the liver of male Sprague Dawley rats was investigated.  $\frac{2}{3}$  hepatectomised rats were administered 2,4,6-TCP (dose route and vehicle not specified), following which, they were administered 500 ppm sodium phenobarbital in their drinking water for a total of 49 days (promotion). Seven days after the cessation of the phenobarbital treatment the rats were sacrificed and GGT-ase positive foci were examined in the liver.

2,4,6-TCP did not increase the number of GGT-ase positive foci in the livers of rats, and was therefore classified as not having tumour initiation potential in the model.

**Under the conditions of the study, 2,4,6-TCP did not cause an increase in GGT-ase positive foci in the livers of Fischer rats. 2,4,6-TCP was therefore classed as negative for tumour initiation in the study.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Not indicated
<b>Source:</b>	Not indicated
<b>Stability of test compound:</b>	Not indicated

**Vehicle and/or positive control:** The choice of vehicle was not specified, although it is presumed from a choice of water, DMSO, Tricapryline or corn oil for which data is presented as controls. No positive control was included on this study. Details are not provided as to the administration route for 2,4,6-TCP or vehicle. Details of preparation method, or dose analysis for achieved concentration/homogeneity or

stability are not provided. Details of 2,4,6-TCP concentrations in the dose preparation and dose volume are not provided.

<b>Test Animals:</b>	
<b>Species</b>	Rat (male)
<b>Strain</b>	Fischer 344; Wistar-lewis and Sprague Dawley
<b>Age/weight at dosing</b>	175-200 g at dosing
<b>Source</b>	
<b>Housing</b>	Not indicated
<b>Acclimatisation period</b>	Not indicated
<b>Diet</b>	Rodent Laboratory chow ad libitum
<b>Water</b>	Tap water ad libitum
<b>Environmental conditions</b>	In accordance with the standards set forth in the “Guide for Care and Use of Laboratory Animals” of the Institute of Laboratory Animal Research, National Research Council

Note: Animal details as above are provided in the materials and methods, but it is not clear which rat strain was used for the study. The results section describes that Fischer F344 male rats have high background incidence of GGTase positive foci and due to convenience of supply and historical use in hepatic biochemical studies, Sprague Dawley rats were used in the validation study.

#### Study Design and Methods:

**In-life dates:** Not indicated

#### Study design

Test group	Dose to animal (mmol/kg)	# male	# female
2,4,6-TCP	1.0	10	0

#### Protocol of Rat Liver Foci Bioassay

The protocol of the rat liver foci bioassay used in these studies has previously been described in detail (Ford *et al.*, 1980 and Herren *et al.* 1982). Groups of 8 to 12 rats each received either a 2/3 partial hepatectomy or a sham operation. Twenty-four hours later the rats received 2,4,6-TCP (initiator) or vehicle control. Seven days after the partial hepatectomy, the rats received 500 ppm sodium phenobarbital in their drinking water for a total of 49 days (promotion). Seven days after the cessation of the phenobarbital treatment the rats were sacrificed.

#### Determination of GGTase-Positive Foci

Liver sections were scored for the presence of GGTase-positive foci as previously described (Ford *et al.*, 1980 and Herren *et al.* 1982). Briefly, the sections were stained for GGTase activity according to the procedure of Rutenburg (Rutenburg *et al.*, 1969), counterstained with hematoxylin and scored for focal areas of GGTase activity that contained 9 or more nuclei.

**Evaluation criteria:** Not specified

## RESULTS

After dosing (presumed orally, although route not specified) of  $\frac{2}{3}$  hepatectomised rats with 2,4,6-TCP (1.0 mmol/kg), to investigate tumour initiator potential of the test substance, no increase in the number of GGTase-Positive liver foci were observed.

Although the vehicle used to formulate 2,4,6-TCP dosing preparations was not specified, the GGTase-positive foci incidence for 2,4,6-TCP was comparable to all 4 controls used by the authors for formulation of various compound (for which data not discussed) (Table 6.8.1-98).

**Table 6.8.1-98: An overview of GGT-ase foci in the livers of Sprague Dawley rats administered control vehicles (corn oil, DMSO, tricapryline or water) or 2,4,6-TCP at 1.0 mmol/kg.**

Compound	Dose (mmol/kg)	Foci/cm <sup>2</sup>
Corn oil	-	0.18 ± 0.10
DMSO	-	0.31 ± 0.11
Tricapryline	-	0.85 ± 0.29
Water	-	0.52 ± 0.23
2,4,6-TCP	1.0	0.59 ± 0.20

Mean ± SD

### CONCLUSION:

In a publication from 1982, the tumour initiation potential of 2,4,6-TCP in the liver of male Sprague Dawley rats was investigated.  $\frac{2}{3}$  hepatectomised rats were administered 2,4,6-TCP (dose route and vehicle not specified), following which, they were administered 500 ppm sodium phenobarbital in their drinking water for a total of 49 days (promotion). Seven days after the cessation of the phenobarbital treatment the rats were sacrificed and GGT-ase positive foci were examined in the liver.

2,4,6-TCP did not increase the number of GGT-ase positive foci in the livers of rats, and was therefore classified as not having tumour initiation potential in the model.

### REFERENCES:

Ford J, Pereira M (1980). Short-term in vivo initiation/promotion bioassay for hepatocarcinogens. *J Environ. Pathol. Toxicol.* 4:39-46.

Herren S, Pereira M, Britt A, Khoury M (1982). Initiation/promotion assay for chemical carcinogens in rat liver. *Toxicol Letters* 12143-150.

Rutenburg A, Kim H, Fishbein J, Hanker S, Wasserkrug H, Seligman A (1969). Histochemical and ultrastructural demonstration of gammaglutamyl transpeptidase activity. *Histochem Cytochem* 17:517-522.

(Pereira M *et al*, 1982)

<b>Report:</b>	K-CA 5.8.1/51 Stoner G. <i>et al.</i> , (1986). Comparison of Two Routes of Chemical Administration on the Lung Adenoma Response in Strain A/J Mice. Department of Pathology, Medical College of Ohio, Toledo, Ohio 43614. Published: Stoner G, Conran P, Greisiger E, Stober J, Morgan M and Periera M (1986). Comparison of Two Routes of Chemical Administration on the Lung Adenoma Response in Strain A/J Mice. <i>Toxicol. Appl. Pharmacol.</i> 82:19-31. Syngenta File No. NA_13799.
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**STUDY TYPE:** Carcinogenicity study in A/J mice via both oral gavage and intraperitoneal injection dosing routes.

**Guidelines:** No globally or regionally accepted regulatory guidelines were followed.

**TEST MATERIAL (PURITY):** 2,4,6-TCP (not indicated).

**GLP:** No claim of GLP compliance was made and Quality Assurance statements were not provided.

**Only data and discussion relevant to the testing of 2,4,6-TCP is discussed in this document.**

**KLIMISCH SCORE: 1**

**HSE comment:** supportive study

**EXECUTIVE SUMMARY**

The potential of 2,4,6-trichlorophenol (2,4,6-TCP) to induce lung tumors in strain A/J mice after either intraperitoneal or gavage administration was investigated.

When administered 2,4,6-TCP by gavage (1200 mg/kg) or intraperitoneal (300, 600, 1200 mg/kg) for 24 weeks, dosing three times a week, no increase in the number of animals with lung tumours was observed when compared to concurrent control groups.

No effect on survival was seen in the groups that received 2,4,6-TCP *via* either dosing route.

**Under the experimental conditions reported, 2,4,6-TCP did not cause an increase in the number of A/J mice with lung tumours, when dosed by either gavage or intraperitoneal.**

**MATERIALS AND METHODS****Materials:**

<b>Test Material:</b>	2,4,6-TCP
<b>Description:</b>	Not indicated
<b>Lot/Batch number:</b>	Not indicated
<b>Purity:</b>	Reagent grade
<b>Source:</b>	Sigma Chemical Company, St Louis, Missouri, USA
<b>CAS#:</b>	88-06-2
<b>Stability of test compound:</b>	Not indicated
<b>Storage:</b>	4°C in dark

**Vehicle and/or positive control:**

With sterile technique, 2,4,6-TCP was administered either ip by injection or po by gavage as 0.1 ml/mouse in tricaprylin (glycerol trioctanoate from Eastman Kodak, Rochester, N.Y.). Solutions were freshly prepared in amber bottles before administration. Details of preparation method, or dose analysis for achieved concentration/homogeneity or stability are not provided. Details of 2,4,6-TCP concentrations in the dose preparation and dose volume are not provided.



<b>Test Animals:</b>	
<b>Species</b>	Mouse (male and female)
<b>Strain</b>	A/J
<b>Age/weight at dosing</b>	6-8 weeks old
<b>Source</b>	From a breeding colony, derived from A/J mice purchased from the [REDACTED]
<b>Housing</b>	Not indicated
<b>Acclimatisation period</b>	Not indicated
<b>Diet</b>	Purina Certified Rodent Chow No. 5002 <i>ad libitum</i>
<b>Water</b>	<i>ad libitum</i>
<b>Environmental conditions</b>	Temperature: 22 ± 2°C Humidity: 50% Air changes: Not indicated Photoperiod: 12 h light/dark cycle

**Study Design and Methods:****In-life dates:** Not indicated**Preliminary toxicology.**

The maximum tolerated dose (MTD) for 2,4,6-TCP was determined. Serial two-fold dilutions were administered either intraperitoneal or oral gavage to groups of eight mice (four males and four females). The MTD was the maximum dose that all eight mice tolerated (survived) without loss of body weight after receiving three intraperitoneal or gavage treatments over a 1-week period. Animals were held for 1 month before experimental groups were initiated to detect delayed toxicity.

**Bioassays.**

2,4,6-TCP was tested via intraperitoneal route at three doses: the MTD, 0.5 MTD, and 0.2 MTD, and orally (gavage) at the MTD only. There were 32 animals per dose (equal number of males and females). The compound was administered via the intraperitoneal or gavage three times per week for 8 weeks and for a total of 24 injections. Control groups consisted of vehicle (tricaptylin) treated mice by intraperitoneal or gavage. The dose administered was calculated as a total cumulative dose (mg/kg).

**Table 6.8.1-99: Study design, dose levels of 2,4,6-TCP**

Test group	Dose route	Dose of 2,4,6-TCP to animal (mg/kg)	# male	# female
Control	IP	0	16	16
Low	IP	300	16	16
Mid	IP	600	16	16
High	IP	1200	16	16
Control	PO	0	16	16
High	PO	1200	16	16

**Bodyweight:**

Animal weights were obtained every 2 weeks during the injection period and at monthly intervals thereafter.

**Investigations *post mortem*:****Macroscopic examination:**

Chemically treated and control mice were killed by cervical dislocation 24 weeks after the initiation of the bioassay. Their lungs were removed and fixed in 70% ethanol containing 5% glacial acetic acid and 5% formaldehyde. Lung tumors, which appeared as pearly white nodules on the surface of the lungs, were counted under a dissecting microscope by two technicians working independently.

**Microscopic examination:**

Random samples of nodules were taken from the lungs of all chemically treated and control groups for histopathological evaluation and confirmation of adenoma.

During necropsy, the liver, kidneys, spleen, intestines, stomach, thymus, and the salivary and endocrine glands were examined grossly. If gross lesions were observed, they were documented and examined histologically for the presence of neoplasms. In addition, the livers of all control and chemically treated mice were fixed in 10% buffered Formalin (pH 7.4) embedded in paraffin, and at least six sections per liver were stained with hematoxylin and eosin. The livers were examined with a compound microscope for the presence of foci of altered cells, adenomas, and adenocarcinomas.

### Statistical analysis

The lung tumor responses were examined by statistical tests for two different type hypotheses. The first was the Janckheere nonparametric trend test (Hollander et al., 1973) for an increasing tumor occurrence with an increasing dose of compound. The second was the Wilcoxon nonparametric rank test (Hollander et al., 1973) a pairwise comparison test used to test for significant differences between each dose group and the respective control group. Each test was one sided (testing for an increased response only) and was performed separately for males and females. For a given compound, differences between routes of exposure and between sexes were analyzed by the Mantel- Haenszel test (Fleiss, 1981) with dose as a blocking factor. Since multiple comparisons were made with the same vehicle control, statistical significance was set at  $p < 0.01$  for a single dose and  $< 0.05$  for two consecutive doses.

## RESULTS

### Survival

After exposure to 2,4,6-TCP, via both intraperitoneal and oral administration, no effect on survival of the mice compared to concurrent controls was seen.

2,4,6-TCP did not cause an increase in lung tumours following intraperitoneal or gavage administration in either sex.

**Table 6.8.1-100: Lung tumour incidences in mice receiving 2,4,6-TCP via oral or intraperitoneal administration**

Treatment	Duration of experiment (weeks)	aTotal dose 2,4,6-TCP (mg/kg)	Sex	Number of animals (initial/survivors)	Survivors with lung tumours				
					No.	%	( $\bar{x} \pm SD$ )	SE	P value
Tricaprylin PO	24	0	M	16/15	-	20	$0.27 \pm 0.59$	(0.15)	-
			F	16/14	-	14	$0.14 \pm 0.36$	(0.10)	-
Tricaprylin IP	24	0	M	16/15	-	30	$0.33 \pm 0.49$	(0.13)	-
			F	16/15	-	30	$0.33 \pm 0.49$	(0.13)	-
2,4,6-TCP PO	24	1200	M	16/14	3	21	$0.21 \pm 0.43$	(0.11)	NS
			F	16/14	1	7	$0.14 \pm 0.53$	(0.14)	NS
2,4,6-TCP IP	24	1200	M	16/15	5	33	$0.33 \pm 0.49$	(0.13)	NS
			F	16/15	2	13	$0.13 \pm 0.35$	(0.09)	NS
		600	M	16/16	5	31	$0.38 \pm 0.62$	(0.15)	NS
			F	16/15	8	53	$0.67 \pm 0.82$	(0.21)	NS

		240	M	16/16	4	25	0.31 ± 0.60	(0.15)	NS
			F	16/16	3	19	0.25 ± 0.58	(0.14)	NS

<sup>a</sup> = Total cumulative dose per animal.

### CONCLUSION:

In a publication from 1986, the potential of 2,4,6-TCP to induce lung tumors in strain A/J mice after either intraperitoneal or gavage administration was investigated. When administered 2,4,6-TCP by gavage (1200 mg/kg bw) or intraperitoneal (300, 600, 1200 mg/kg bw) for 24 weeks (three times a week), no increase in the number of animals with lung tumours was observed when compared to concurrent control groups. No effect on survival was seen in the groups that received 2,4,6-TCP *via* either dosing route.

### REFERENCES:

*Fleiss L (1981). Statistical Methods for Rates and Proportions. John Wiley and Sons, New York.*

*Hollander H and Wolfe D (1973). Nonparametric Statistical Methods. John Wiley and Sons, New York.*

(Stoner G *et al*, 1986)

The following publications on 2,4,6-TCP are cell based assays. Full summaries have not been included.

Reference:	K-CA 5.8.1/52 Butt C M, Wang D, Stapleton H M (2011). Halogenated Phenolic Contaminants Inhibit the In Vitro Activity of the Thyroid-Regulating Deiodinases in Human Liver. <i>Toxicological Sciences</i> 124:339-347. Syngenta File No. NA_13784.
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Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Deiodinase (DI) enzyme assay	Human liver microsomes incubated with pooled thyroxine (T4) or triiodothyronine (rT3) in the presence of 2,4,6-TCP and the production of T3, rT3, 3,3#-diiodothyronine, and 3-monoiodothyronine was analysed by LC-MS/MS.	T3: IC <sub>50</sub> = 130 µM rT3: IC <sub>50</sub> = 470 µM 3,3'-T2: IC <sub>50</sub> = 70 µM

### KLIMISCH SCORE: 1

**CONCLUSION:** 2,4,6-Trichlorophenol (2,4,6-TCP) inhibited deiodinase in a dose-dependent manner *in vitro*.

Reference	K-CA 5.8.1/53 Cascorbi I, Ahlers J (1989). Correlation between the lipophilicity of substituted phenols and their inhibition of the Na <sup>+</sup> /K <sup>+</sup> -ATPase of Chinese hamster ovary cells. <i>Toxicology</i> 58:197-210. Syngenta File No. NA_13802.
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Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Enzyme assay to measure the inhibition of Na <sup>+</sup> /K <sup>+</sup> -ATPase as a	Plasma membrane was obtained from CHO cells. To measure the ATPase activity, the amount of free phosphate	EC <sub>20</sub> 0.78 mmol/L EC <sub>50</sub> 1.33 mmol/L

measure of toxicity in Chinese Hamster Ovary (CHO) cells	released over 10 mins. Reduced ATPase activity is associated with cytotoxicity.	
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**KLIMISCH SCORE: 3**

**CONCLUSION:** The authors concluded that the concentration of 2,4,6-trichlorophenol (2,4,6 TCP) which inhibits 20% and 50% of ATPase activity was 0.78 and 1.33 mmol/L, respectively. The authors also concluded that due to the concentration of 2,4,6-TCP in the membrane and also the cytoplasm that it induces changes in membrane permeability or could inhibit membrane bound enzymes/transport systems.

Reference:	K-CA 5.8.1/54 Ekwall B, Selling J, Johnels D. (1987). Toxicity of chlorophenols to HeLa cells as measured in the MIT-24 system. ATLA, Alternatives to Laboratory Animals 14:178-81. Syngenta File No. NA_13767.
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Only data on 2,4,6-TCP are included.

Assay	Study design	Result
MIT-24 assay	HeLa cells were treated with low dose (0.5-2 mg/L) and high dose (10-100 mg/L) of 2,4,6-TCP. After 24 h the spreading ability was determined (round cells = inhibition, spindle cell = normal). After 7 d inhibition of anaerobic glycolysis was analysed by the pH-shift and a change in colour of the pH indicator in the medium.	24 h: IC <sub>50</sub> = 160 mg/L 7 d: IC <sub>50</sub> = 27 mg/L

**KLIMISCH SCORE: 3**

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) inhibited spreading of HeLa cells with an IC<sub>50</sub> = 27 mg/L and inhibited anaerobic glycolysis with an IC<sub>50</sub> = 27 mg/L.

Reference:	K-CA 5.8.1/55 Henschler R, Appel KE, Heyworth CM, Glatt H (2001). Proliferation and differentiation of murine haemopoietic progenitor cells in stroma-free culture in the presence of metabolites of chlorinated pesticides. Toxicology In Vitro 15:31-37 Syngenta File No. NA_13780.
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Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Radio-labelled cytotoxicity assay on multipotent progenitor cell line (FDCP-mix) and a primary lineage-depleted bone marrow cell line.	<p>FDCP-mix cells (multipotent progenitor cell line) and primary lineage-depleted bone marrow cells were differentiated in the presence of 2,4,6-TCP to either granulocytes / macrophages, erythrocytes and thrombocytes.</p> <p>Proliferation was assessed by [<sup>3</sup>H]thymidine incorporation.</p> <p>The second assay investigated the influence of the test compound on cell differentiation and measuring the differentiation of the cell line into either granulocytic, macrophages, erythrocytic or megakaryocytic cells.</p>	<p>FDCP assay- IC<sub>50</sub> under self-renewal culture conditions 160 µM; under differentiation conditions 200 µM.</p> <p>Bone marrow cell assay: 2,4,6 TCP reduces number of cells with increasing concentrations. At all concentrations the % of granulocytic cells was significantly greater in treated cells than control (P&lt;0.05). 2,4,6-TCP exposure stimulated erythrocytic and megakaryocytic lineages at low concentrations.</p>

**KLIMISCH SCORE: 3**

**CONCLUSION:** IC<sub>50</sub> of 2,4,6-trichlorophenol (2,4,6-TCP) in a multipotent progenitor cell line was 160 µM under self-renewal culture conditions and 200 µM under differentiation conditions. 2,4,6-TCP was found to have an effect on cell differentiation of a primary lineage-depleted bone marrow cell line. At all concentrations, the % of granulocytic cells formed under the conditions of the assay were significantly greater (P<0.05) following exposure to 2,4,6-TCP.

Reference: K-CA 5.8.1/56 Jansson K, Jansson V (1993). The Toxicity of Chlorophenols in V79 Chinese Hamster Cells. Toxicol.Lett.69:289-94. Syngenta File No. NA_13771.
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Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Giemsa cells survival assay	<p>V79 Chinese hamster cells were treated with 2,4,6-TCP for 24 h and stained with Giemsa. survival was calculated as compared to the solvent control cloning efficiency. The negative logarithm of the chlorophenol concentration required to reduce the survival to 50% of control (pLC<sub>50</sub>) was estimated by linear regression analysis.</p>	<p>pLC<sub>50</sub> = 3.3</p> <p>Toxicity of chlorophenols was linearly related to both hydrophobicity, log D, and electronic effects.</p>

**KLIMISCH SCORE: 3**

**CONCLUSION:** Toxicity of 2,4,6-trichlorophenol (2,4,6-TCP) in V79 cells depends not only on hydrophobicity but also on electron-withdrawing substituent effects.

Reference:	K-CA 5.8.1/57 Jia R-W, Liao T-T, Shi Y-L, Wang L, Xia J, Lu B, Xu D-S (2010). Cytotoxic characteristics and plasma membrane component analysis in Vero cells exposed to 2,4,6-trichlorophenol. J. Environ. Health, 270(4):302-305. Syngenta File No. NA_13806.
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Only data on 2,4,6-TCP are included.

The original publication was in Chinese and a translation to English obtained.

Assay	Study design	Result
Cell morphology, membrane viability and cytotoxicity assay	<p>Vero cells were treated with 2,4,6-TCP at varying concentrations.</p> <p>Flow cytometry was utilised for cell viability</p> <p>Cell membranes were extracted and protein and phospholipid content was determined.</p>	<p>Following 24 and 48 hours exposure to 2,4,6-TCP the number of viable cells were significantly reduced (<math>P&lt;0.05</math>) and the number of dead cells and early apoptotic cells were significantly increased (<math>P&lt;0.05</math>) at all concentrations tested (1-50 mg/L TCP).</p> <p>The Vero cell plasma membrane protein and lipid content were significantly reduced (<math>P&lt;0.05</math>) wrt the control at all concentrations (1-125mg/L TCP). The protein and phospholipid content reduced to a greater extent as the concentration of 2,4,6-TCP increased.</p>

#### KLIMISCH SCORE: 1

**CONCLUSION:** Under the conditions of the study, 2,4,6-trichlorophenol (2,4,6-TCP) was found to lead to a reduction in the protein and phospholipid content and increase the protein: phospholipid ratio in Vero cell plasma membranes. The authors therefore concluded that 2,4,6-TCP disrupts the structure of the cell membrane in Vero cells and this is mechanism is responsible for the cytotoxicity observed in these cells.

Reference:	K-CA 5.8.1/58 Judis J (1982). Binding of selected phenol derivatives to human serum proteins. Journal of Pharmaceutical Sciences 71:1145-47. Syngenta File No. NA_13794.
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Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Protein-binding assay using radiolabelled ( $^{14}\text{C}$ ) phenol derivatives	Equilibrium and dynamic dialysis methods were used to measure % binding of $^{14}\text{C}$ labelled 2,4,6-TCP to various human serum proteins and to determine binding parameters to albumin	Ligand % Binding: 2,4,6-TCP was found to bind to all human serum proteins tested to varying degrees: Albumin (94.1%), $\alpha$ -Globulin IV-1 (8.09), $\alpha$ -Globulin IV-4 (55.2%), $\beta$ -Globulin III (13.0%), $\gamma$ -Globulin II (6.4%) and human serum (95.8%)  Albumin Binding Parameters: $K_1$ (Association constant) ((litres per mol) 30,200,000, $n_1$ 0.2940, $K_2$ 1,470,000; $n_2$ 0.323 (n- number of binding sites)

**KLIMISCH SCORE: 3**

**CONCLUSION:** 2,4,6-Trichlorophenol (2,4,6-TCP) had a much higher binding affinity for albumin than 2,4 dichlorophenol and *p*-tert-amylphenol. The authors conclude that the binding is related to molecular weight and Hansch  $\pi$  values. Binding to albumin appeared to primarily involve hydrophobic bonds rather than polar bonds.

Reference: K-CA 5.8.1/59 Ma Y, Liu C, Lam P.K.S, Wu R.S.S., Giesy J.P, Hecker M., Zhang X, Zhou B (2011). Modulation of steroidogenic gene expression and hormone synthesis in H295R cells exposed to PCP and TCP. Toxicology, 282:146-53. Syngenta File No. NA\_13797.

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Cell viability assay	Human adrenocortical carcinoma cells (H295R cells) were exposed to 0.4 to 3.4 $\mu\text{M}$ 2,4,6-TCP for 48 h. Cell viability was determined by measuring lactate dehydrogenase (LDH) activity.	There was no induction of LDH activity.
Expression of mRNA steroidogenic genes	H295R cells were exposed to 0.4 to 3.4 $\mu\text{M}$ 2,4,6-TCP for 48 h. RT-PCR analysis for gene expression of STAR, CYP11A, CYP11, CYP17, 3 $\beta$ HSD2, 17 $\beta$ HSD4 was performed.	Dose- and time-dependent reduction in RNA levels of all tested steroidogenic genes, statistically significant only at top dose. No statistically significance for the reduction of 17CYP.
Hormone assays (testosterone (T) and 17 $\beta$ -estradiol (E2))	H295R cells were exposed to 0.4 to 3.4 $\mu\text{M}$ 2,4,6-TCP for 48 h. Expression of hormones was measured by quantitative ELISA.	Dose- and time dependent decrease of T and E2, statistically significant only at 3.4 $\mu\text{M}$ ( $p < 0.05$ ).
Concentrations of cAMP	H295R cells were exposed to 0.4 to 3.4 $\mu\text{M}$ 2,4,6-TCP for 48 h. cAMP levels were analysed in the lysate using a commercial kit.	Dose-dependent decrease of cAMP levels, statistically significant at all concentrations tested ( $p < 0.01$ at 3.4 $\mu\text{M}$ ).

**KLIMISCH SCORE: 1**

**CONCLUSION:** While 2,4,6-trichlorophenol (2,4,6-TCP) did not alter cell viability of Human adrenocortical carcinoma cells (H295R cells), 2,4,6-TCP treatment led to decreased production of both testosterone and 17 $\beta$ -estradiol and induced a dose-dependent decrease of cellular cAMP. 2,4,6-TCP exposure also decreased steroidogenic gene expressions in a dose- and time-dependent manner.

Reference: K-CA 5.8.1/60 Murayama J-I, Ishiwata, F. M, Utsumi H, Hamada A (1990). Comparative acute cytotoxicities of 37 xenobiotics detected in drinking water to rat hepatocyte primary culture. Eisei Kagaku 36:267-276. Syngenta File No. NA\_13774.

**The original publication was in Japanese and a translation to English obtained.**

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Cytotoxicity assay in primary rat hepatocytes	Hepatocytes were isolated from the liver of Wistar rats and treated with up to 1 mM 2,4,6-TCP for 20 h. Cells were lysed and LDH and glycogenic activity were measured.	Primary rat hepatocytes showed strong morphological changes in culture when treated with 2,4,6-TCP. 2,4,6-TCP inhibited LDH release by 93.5 % in a dose dependent manner (IC <sub>50</sub> = 230 $\mu$ M). Glycogenic activity was almost undetectable after 2,4,6-TCP treatment.

#### KLIMISCH SCORE: 1

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) was highly cytotoxic to primary rat hepatocytes *in vitro*.

Reference: K-CA 5.8.1/61 Qin H, Liu J, Zhang Z, Li J, Gao G, Yang Y, Yuan X, Wu D (2014). In situ electrochemical assessment of cytotoxicity of chlorophenols in MCF-7 and HeLa cells. Analytical Biochemistry 462:60-66. Syngenta File No. NA\_13768.

Only data on 2,4,6-TCP are included.



Assay	Study design	Result
Cell viability assay (Electrochemical method)	MCF-7 and HeLa cells were treated with up to 500 µmol/L 2,4,6-TCP and the electrochemical behaviours of the cell suspension was measured by linear sweep voltammetry (LSV) for 36 h.	MCF-7: EC <sub>50</sub> = 179.64 µmol/L HeLa: EC <sub>50</sub> = 157.07 µmol/L
Cell viability assay (MTT assay)	MCF-7 and HeLa cells were treated with up to 500 µmol/L 2,4,6-TCP for 30 h. Cell viability was assessed in an MTT assay.	MCF-7: EC <sub>50</sub> = 232.38 µmol/L HeLa: EC <sub>50</sub> = 185.52 µmol/L

**KLIMISCH SCORE: 1**

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) was cytotoxic to MCF-7 and HeLa cells *in vitro*.

Reference: K-CA 5.8.1/62 Sakazaki H, Ueno H, Umetani K, Utsumi H, Nakamuro K (2001). Immunotoxicological evaluation of environmental chemicals utilizing mouse lymphocyte mitogenesis test. Journal of Health Science 47: 258-271. Syngenta File No. NA\_13772.

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Mouse lymphocyte mitogenesis test	Spleen cells were isolated from C3H/He mouse for B cell test and from BALBc mouse for T cell test. The cells were treated with mitogen (LPS for B cell test; ConA for T cell test) and 2,4,6-TCP for 4 d. DNA content of the cells was fluorometrically quantified by EtBr staining and the mitogenic rate calculated.	B cells mitogenesis: IC <sub>50</sub> = 6.0 x 10 <sup>-5</sup> mmol/L T cells mitogenesis: IC <sub>50</sub> = 6.6 x 10 <sup>-5</sup> mmol/L  2,4,6-TCP was cytotoxic to B and T cells.

**KLIMISCH SCORE: 3**

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) was cytotoxic to splenic mouse B and T cells.

Reference: K-CA 5.8.1/63 Shannon R D, Boardman G D, Dietrich A M, Bevan D R (1991). Mitochondrial response to chlorophenols as a short-term toxicity assay. Environmental Toxicology and Chemistry 10:57-66. Syngenta File No. NA\_13770.

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Mitochondrial respiration assay	Mitochondria were isolated from the liver of Sprague-Dawley rats and treated with up to 80 µM 2,4,6-TCP. Mitochondrial respiration rates were measured polarographically and the reduction in the respiratory control ratio (RCR) was calculated.	2,4,6-TCP treatment led to a dose-dependent decrease in RCR.

**KLIMISCH SCORE: 1**

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) is an uncoupler of oxidative phosphorylation in mitochondria when analyzed *in vitro*.

Reference: K-CA 5.8.1/64 Utsumi H, Hakoda M, Kiyoshige K, Manabe H, Mitade C, Murayama J, Han S K, Hamada A (1992). Cytotoxicity and mutagenicity of micropollutants in drinking water. Water Science and Technology 25:325-332. Syngenta File No. NA\_13779.

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Cytotoxicity assay HL-60 cells	HL-60 were exposed to 1mM 2,4,6-TCP for 20 h and compared to responses of the tumour promoter PMA. Morphological changes were analysed by phase contrast microscopy. Viability was determined by trypan-blue exclusion assay.	No changes in morphology occurred.  No viable cells were found after 2,4,6-TCP treatment.
Macrophage activity assay	Peritoneal mouse macrophages were treated <i>in vitro</i> with 1mM 2,4,6-TCP and phagocytic activity was measured by the uptake of latex beads and densitometry analysis.	Phagocytosis was reduced to 26%. Viability was not determined.
Metabolic inhibition and lethality test in hepatocytes	Rat liver hepatocytes isolated from Wistar rats were treated with 1mM 2,4,6-TCP for 20h. Viability was measured by the release of lactate dehydrogenase (LHD). Glycogenolytic activity (GLC) was measured by glucose release after incubation with glucagon in glucose-free medium.	The release of LHD was reduced to 6.5%  GLC was 0.1 µmol/min/dish compared to 9.6 µmol/min/dish in DMSO treated cells.

**KLIMISCH SCORE: 3**

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) showed effects in all 3 cell systems tested. 2,4,6-TCP is highly cytotoxic to HL60 cells. 2,4,6-TCP inhibits phagocytosis by peritoneal mouse macrophages *in vitro*. 2,4,6-TCP reduces the viability of rat hepatocytes *in vitro* and inhibits glycogenolytic activity.

Reference: K-CA 5.8.1/65 van Delft J.H.M., Van Agen, E, van Breda S.G.J, Herwijnen M.H, Staal Y.C.M., Kleinjans J.C.S. (2004). Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling. *Carcinogenesis* 25:1265-1276.

van Delft J.H.M., Van Agen, E, van Breda S.G.J, Herwijnen M.H, Staal Y.C.M., Kleinjans J.C.S. (2005). Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling. [Erratum]. *Carcinogenesis* 26:511. Syngenta File No. NA\_13786.

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
Cytotoxicity assay	Human HepG2 cells were treated with up to 4000 µM 2,4,6-TCP for 24h and cytotoxicity was measured in an MTT assay.	sudden decrease in survival from 100 to 15 % at dose levels higher than 500 µM
Microarray analysis	HepG2 cell were treated with 500 µM 2,4,6-TCP for 24 h. Total RNA was isolated and a Microarray analysis was performed analysing for genotoxic and non-genotoxic. The candidate genes were selected by the authors.	2,4,6-TCP showed a difference in expression levels of 71 genes. The induced profile of changes was non-genotoxic.

#### KLIMISCH SCORE: 1

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) is cytotoxic to HepG2 cells. The changes in gene expression levels have a non-genotoxic profile.

Reference: K-CA 5.8.1/66 van den Berg K..J (1990). Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin and thyroid binding globulin. *Chemico-Biological Interactions* 76:63-75. Syngenta File No. NA\_13782.

Only data on 2,4,6-TCP are included.

Assay	Study design	Result
T4 binding site competition assay	2,4,6-TCP was mixed with radiolabeled thyroxine (T4) and transthyretin (TTR). TTR-bound radioactivity was then analysed.	IC <sub>50</sub> = 12 x 10 <sup>-7</sup> M relative affinity 0.33

#### KLIMISCH SCORE: 1

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) is able to compete with thyroxine for transthyretin binding in a dose-dependent manner.

Reference: K-CA 5.8.1/67 Shi Y-L, Wang L, Zhou Q, (2008). Cytotoxic response characteristics and sensitivity in mammalian Vero cells exposed to 2,4,6-Trichlorophenol. *Asian Journal of Ecotoxicity* 3:479-487. Syngenta File No. NA\_13754.

Assay	Study design	Result
MTT assay and Annexin V-FITC/PI double staining flow cytometry on Vero CCL-81 cells exposed to 2,4,6-TCP	<p>The cell morphology, optical density and % inhibition of proliferation using the MTT assay were determined at various concentrations of MTT and compared to negative and positive (ZnSO<sub>4</sub>) control.</p> <p>An Annexin V-FITC/PI double staining flow cytometry was also carried out.</p>	<p>Altered cell morphology was observed using a microscope at concentrations &gt;0.5 mg/L</p> <p>The Optical density for the MTT assay was significantly reduced wrt to the negative control at concentrations &gt; 5mg/L and this gave an inhibition rate of 18.6%.</p> <p>The flow cytometry plots showed an increase in the number of the cells in the upper left quadrant of the plot after the first 24 hours of exposure. The authors concluded that this suggests a mechanical injury to the cells such as damage to the cell membrane. 48 hours after exposure the number of apoptotic and necrotic cells increased.</p>

**KLIMISCH SCORE: 2**

**CONCLUSION:** At concentrations >0.5 mg/L a noticeable change was observed in cell morphology following exposure to 2,4,6-trichlorophenol (2,4,6-TCP). 2,4,6-TCP causes damage to the cell membrane in Vero cells in the first 24 hours following exposure and the effects tended to be reversible. After 48 hours of exposure, cell apoptosis and necrosis are more evident suggesting that damage to the cell membrane is the initial mechanism of 2,4,6-TCP on Vero cells in the conditions of this study. In addition, the study found that after 48 hours of exposure the effects were not reversible compared to the shorter contact time.

Reference: K-CA 5.8.1/68 Zhang X, Zhang X, Niu Z, Qi Y, Huang D, Zhang Y (2014). 2,4,6-Trichlorophenol cytotoxicity involves oxidative stress, endoplasmic reticulum stress, and apoptosis. *International Journal of Toxicology*, 33(6):532-41. Syngenta File No. NA\_13755.

Assay	Study design	Result
Cell viability	Mouse embryonic fibroblasts (MEF) were dosed with up 1.0 mmol/L 2,4,6-TCP for 24h and analysed in an MTT assay.	<p>Decrease in cell viability:</p> <p>0.5 mmol/L: 13.88% ( P &lt; 0.05)</p> <p>0.75 mmol/L: 21.93% ( P &lt; 0.01)</p> <p>1.0 mmol/L: 31.01% ( P &lt; 0.01)</p> <p>morphological changes, including cellular shrinkage and cell rounding</p>

Assay	Study design	Result
Apoptosis	<p>Apoptotic staining: MEFs were dosed with 0.5 to 1.0 mmol/L 2,4,6-TCP for 24h and analysed by AnnexinV / Propidium-Iodide staining by flow cytometry.</p> <p>Caspase-3 assay: In a time course MEFs were exposed to 1.0mmol/L 2,4,6-TCP and Caspase-3 activation was analysed in a peptide cleavage assay.</p>	<p>Apoptosis was increased in a dose dependent manner (<math>P &lt; 0.05</math>, all concentrations).</p> <p>Caspase-3 activity was increased by 1.56- and 1.51-fold after 12 and 24 h exposure respectively (<math>P &lt; 0.05</math>)</p>
Mitochondrial stress response	<p>Mitochondrial membrane potential (MMP) assay: MEFs were either dosed with 1.0 mmol/L for 1, 2, 4 and 6 h or 0.5, 0.75, and 1.0 mmol/L for 24 h and analysed in an 5,5,6,6-Tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) assay.</p> <p>Bax/Bcl-2 mRNA ratio: MEFs were dosed with 0.5 to 1.0 mmol/L for 24 h. Gene expression was analysed by qRT-PCR.</p>	<p>MMP was reduced at 1.0 mmol/L (<math>P &lt; 0.01</math>).</p> <p>Concentration-dependent increase for the Bax/Bcl-2 ratio (<math>P &lt; 0.01</math> at 1.0 mmol/L)</p>
Reactive oxygen species production (ROS) and expression levels of Heme Oxygenase 1 (HMOX1)	<p>ROS production: MEFs were either dosed with 1.0 mmol/L for 1, 2, 4 and 6 h or 0.5, 0.75, and 1.0 mmol/L for 24 h and analysed in an dichlorodihydrofluorescein diacetate (DCFH-DA) assay.</p> <p>HMOX1 expression: MEFs were dosed up to 1.0mmol/ L 2,4,6-TCP for 12 hours. HMOX1 levels were analysed by quantitative PCR.</p>	<p>2,4,6-TCP induced biphasic ROS production, with an early ROS burst occurring within 1 h of exposure followed a transient drop within 1-2 h, and then, a second ROS burst occurring within 2-5 h</p> <p>HMOX1 expression was up regulated on RNA level (1.0 mmol/L, <math>P &lt; 0.01</math>)</p>
Expression and nuclear translocation of nuclear factor-E2-related factor (Nrf2)	<p>Nrf2 expression: MEFs were dosed up to 1.0mmol/ L 2,4,6-TCP for 12 hours. Nrf2 expression was analysed by quantitative PCR and Western blotting.</p> <p>Nrf2 translocation: MEFs were treated with 0.5 mmol/L 2,4,6-TCP for 12 h and analysed by immune staining and fluorescence microscopy.</p>	<p>Nrf2 expression was up regulated on RNA level (1.0 mmol/L, <math>P &lt; 0.01</math>).</p> <p>Nrf2 translocated to the nucleus after 2,4,6-TCP treatment.</p>

Assay	Study design	Result
Endoplasmic Reticulum (ER) stress response	<p>Expression levels of ER stress markers: MEFs were dosed from 0.5 to 1.0mmol/L 2,4,6-TCP for 12 hours. Expression levels of, inositol-requiring enzyme/endonuclease 1<math>\alpha</math> (IRE1<math>\alpha</math>), binding immunoglobulin protein (Bip), C/EBP homologous protein (CHOP), were analysed by quantitative PCR and Western blotting.</p> <p>Xbp1 mRNA Splicing: MEFs were dosed from 0.5 to 1.0mmol/L 2,4,6-TCP for 12 hours and analysed for Xbp1 splicing by RT-PCR.</p>	<p>Changes in RNA expression levels at 1.0mmol/L:</p> <p>↑ Bip (P &lt; 0.01)          ↑ CHOP (P &lt; 0.01)          ↑ IRE1<math>\alpha</math> (P &lt; 0.05)</p> <p>Changes in protein expression at 1.0mmol/L:</p> <p>↑ Bip (P &lt; 0.05)          ↑ CHOP (P &lt; 0.01)          ↑ IRE1<math>\alpha</math> (P &lt; 0.05 at 0.5 and 0.75mmol/L, not at 1.0mmol/L)</p> <p>2,4,6-TCP induced Xbp1 splicing at all concentrations tested (P &lt; 0.01 at 1mmol/L)</p>

**KLIMISCH SCORE: 1**

**CONCLUSION:** 2,4,6-trichlorophenol (2,4,6-TCP) induces mitochondrial and ER stress as well as apoptosis in mouse embryonic fibroblasts.

**Cell based assays of varying quality**

HSE agrees with the evaluation of the EU peer-review process that these cell-based assays provide only very limited information on the toxicological profile of 2,4,6-TCP.

**Summary of data on 2,4,6-TCP****Summary of data (including additional information provided by the applicant following the EU peer-review process) on 2,4,6-TCP sulphate and reference value setting**

Metabolite 2,4,6-TCP sulphate is a livestock metabolite. In the human gastro-intestinal tract, the sulphate will be easily cleaved, leading to systemic exposure to the aglycon, 2,4,6-TCP. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugate. In addition, it is noted that 2,4,6-TCP sulphate is a major rat metabolite covered by the parent.

**ADME:** In rats, on repeat oral dosing with radiolabel 2,4,6-TCP, >90% of the dose was excreted in urine. 2,4,6-TCP was not significantly degraded, but conjugated and to some extent isomerized (Bahig, 1981). In a study by Pekari et al. (1986) in which 2,4,6-TCP was administered by ip to male rats, the highest concentrations of 2,4,6-TCP were found in the kidney, blood and liver. 2,4,6-TCP was rapidly and extensively conjugated and excreted (half times were similar in all tissues (1.4 to 1.8 hours)). Additional data by Balikova et al. (1988) support urine as the main route of elimination of 2,4,6-TCP in rats with short half-life.

**Acute toxicity:** An oral LD50 of 820 mg/kg bw is cited in NCI (1979). No further data were identified in the search of the published literature. However 2,4,6-TCP has the following harmonised EU classification (and GB mandatory classification) entry for acute endpoints: Acute Tox 4 (H302); Skin Irrit 2 (H315) and Eye Irrit. 2 (H319).

**Short-term toxicity:** The short-term toxicity of pydiflumetofen has been investigated in three publications, a 90-day study in rats and preliminary 7-wk studies in rats and mice. The preliminary studies do not allow the identification of robust NOAELs and hence they are not described further. In addition, as the 90-day study had some limitations, the Applicant recently generated and submitted two regulatory studies, a 14-day range finder and a 28-day study in the rat.

In the 90-day study (Bercz et al., 1990), male and female Sprague-Dawley rats (10/sex/dose) were gavaged with 2,4,6-TCP administered in corn oil for 90 consecutive days at dose levels of 0, 80, 240, and 720 mg/kg bw/d. No mortality or significant effects were observed at any dose level for body weight, food consumption, ophthalmic lesions, haematology, gross pathology, or histopathology. Treatment-related effects were observed at the highest dose (720 mg/kg bw/d) and consisted of salivation, urine stains on the fur, increase in absolute and relative weights of the kidneys, liver, adrenal glands, and testes. At this dose, increases were seen in serum protein, albumin, and alanine aminotransferase (ALT), with a decrease in urinary pH. At 240 mg/kg bw/d there were increases in the absolute and relative weights of the liver and adrenal glands in females, relative liver weights in males, and an increase in serum albumin in males. No treatment-related effects were observed at 80 mg/kg bw/d. Based on these findings, in this 90-day study in the rat, a LOAEL was identified at 240 mg/kg bw/d and the **NOAEL was set at 80 mg/kg bw/d**.

In the recently submitted GLP and guideline 28-day gavage study (Tilley, 2021) in which rats were given 0, 100, 250 or 500 mg/kg bw/d 2,4,6-TCP, the only relevant adverse effects were seen at the top dose on the weight of the liver (females), thyroid (males) and uterus (females). Based on these effects a **NOAEL of 250 mg/kg bw/d** could be identified from the study. The applicant proposed a NOAEL of 500 mg/kg bw/d as the observed organ weight changes were regarded to be non-adverse in the absence of any associated histopathology. It is HSE's view, that despite the lack of histopathology, the adversity of these organ changes cannot be disregarded, given their magnitude.

**Genotoxicity:** A wide range of in vitro and in vivo studies were identified in the published literature. These were in the main non-standard studies with several limitations and showing inconsistent results. Following the EU peer-review process, it was concluded that the genotoxic potential of 2,4,6-TCP was inconclusive, based on positive results observed in vitro and inconsistent results observed in vivo, and needed to be clarified. On this basis, Syngenta recently submitted a modern package of three in vitro tests (Ames, micronucleus and mammalian cell gene mutation tests) and an in vivo TGR (Transgenic rodent) assay in rats. These well conducted tests have demonstrated that 2,4,6-TCP is not genotoxic in vitro or in vivo.

**Long-term toxicity and carcinogenicity:** The chronic toxicity and carcinogenic potential of 2,4,6-TCP was investigated in rats and mice (NCI, 1979). 2,4,6-TCP was carcinogenic in male F344 rats, inducing lymphomas or leukemias from the lowest dose of **258 mg/kg bw/d (LOAEL)**. It was also carcinogenic in both sexes of B6C3F1 mice, inducing liver hepatocellular carcinomas and/or adenomas from the lowest dose of 650 mg/kg bw/d. Based on these findings, 2,4,6-TCP has harmonised classification in the EU and mandatory classification in GB with Carc. Cat 2 (H351).

**Reproductive toxicity:** The reproductive toxicity potential of 2,4,6-TCP was investigated in two limited one-generation studies from the open literature. In a publication from 1986 (Blackburn et al., 1986), the reproductive effects of 2,4,6-TCP were investigated in male and female Long-Evans rats. Adult males (15-22, depending on group) were treated with 0, 100, 500, or 1000 mg/kg bw/d 2,4,6-TCP (gavage) for 10 weeks (5 days/week), at which time semen evaluations were conducted on ejaculates recovered from the genital tract of receptive untreated females. Fertility was assessed in the 0 and 1000 mg/kg bw groups. Females (15-22, depending on group) were treated with identical doses for 2 weeks prior to pregnancy (5 days/week) then throughout gestation (7 days/week). Females were mated with untreated males. Dams were allowed to litter and pup development was monitored until Day 42 post-partum. 2,4,6-TCP had no effect on any sperm parameter or male fertility. Treatment of females with 1000 mg/kg bw/d of 2,4,6-TCP produced maternal toxicity as reflected in increased lethality and decreased weight

gain in the dams. However no treatment-related differences were seen in litter sizes or pup survival. Male and female birth weights were significantly depressed in the 500 and 1000 mg/kg bw/d groups; these differences disappeared by Day 4 post-partum suggesting that they were a reflection of maternal toxicity.

Overall, in this limited study, the reproductive processes of male and female rats did not appear to be a primary target of 2,4,6-TCP up to the limit dose of 1000 mg/kg bw/d. HSE notes that a NOAEL of 100 mg/kg bw/d could be identified for generalised offspring toxicity and a NOAEL of 500 mg/kg bw/d could be identified for parental toxicity.

In a publication from 1985, the toxic effects of 2,4,6-TCP on bodyweight, histopathology of major organs and reproduction and immune competence were investigated. Treatment with 2,4,6-TCP was found not to have an effect on reproduction of female Sprague-Dawley rats, apart from litter size in the highest dose group (300 ppm) where a significant decrease was observed ( $P < 0.1$ ). The authors concluded that 2,4,6-TCP may be embryotoxic. The authors reported that  $p < 0.1$  is significant; however it is unlikely that this decrease was significant. No significant effect was observed on any of the immunological parameters measured in the study following exposure to 2,4,6-TCP. A significant increase was observed in spleen and liver weights following exposure to 2,4,6-TCP. The authors concluded that the immune system appeared to be a sensitive target of exposure to 2,4,6-TCP. The conclusion made regarding immunological toxicity of 2,4,6-TCP does not match the results reported in the paper. From the results presented in the paper, it is unlikely that 2,4,6-TCP targets the immune system.

**Other toxicity and mechanistic studies:** There are numerous publications describing non-standard toxicity and mechanistic investigations. These add no useful information to the toxicity profile of 2,4,6-TCP.

### **Dietary reference values**

Although 2,4,6-TCP sulphate is a major rat metabolite of pydiflumetofen and could be considered 'covered' by the parent, HSE notes that there is a significant dataset on the substance showing that 2,4,6-TCP has a different toxicity profile compared to pydiflumetofen. Therefore, the specific toxicological data on 2,4,6-TCP should take priority and be used to establish specific reference values.

The table below gives an overview of the acceptable studies relevant to reference value setting.

Study	Doses (mg/kg bw/d)	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Effects at the LOAEL
90-day study (Bercz et al., 1990)  Rat  Acceptable study	0, 80, 240, 720	80	240	Liver, kidney and adrenal wt changes
28-day study (Tilley, 2021)  Rat	0, 100, 250, 500	250	500	Liver, thyroid and uterus wt changes



GLP and guideline study				
Cancer bioassay (NCI, 1979)  Rat  Acceptable study	0, 258, 544	< 258	258	Leukemia and lymphoma in males
Cancer bioassay (NCI, 1979)  Mouse  Acceptable study	0, 650, 1300	< 650	650	Liver tumours in both sexes
One-generation study (Blackburn et al., 1986)  Rat  Acceptable study	0, 100, 500, 1000	<i>Reproductive and parental</i> 1000  <i>Offspring</i> <b>100</b>  <i>Parental</i> 500	<i>Reproductive and parental</i> >1000  <i>Offspring</i> 500  <i>Parental</i> 1000	<i>Reproductive and parental</i> No adverse effects  <i>Offspring</i> Reduced pup bw at birth  <i>Parental</i> Effects on body weights and mortality

The most appropriate POD for the derivation of the ADI is the NOAEL of 80 mg/kg bw/d for effects on organ weights at 240 mg/kg bw/d from the rat 90-day study. By applying the standard default factor of 100 and an additional factor of 2 as tumours were seen at the LOAEL of 258 mg/kg bw/d, and **ADI of 0.4 mg/kg bw/d** is derived. No further assessment factors are required as chronic/carcinogenicity studies and reproductive toxicity studies are available.

Considering that 2,4,6-TCP is acutely toxic by the oral route, an ARfD should be derived. An appropriate POD for the ARfD is the offspring NOAEL of 100 mg/kg bw/d for reduced pup body weights at birth in the reproductive toxicity study. By applying the standard default factor of 100, an **ARfD of 1 mg/kg bw** can be established.

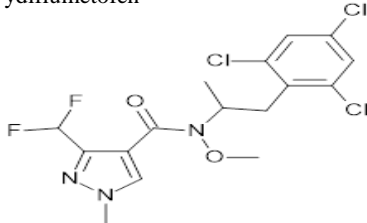
These reference values compared to those of the parent substance indicate that 2,4,6-TCP is not more toxic than pydiflumetofen. Therefore, if 2,4,6-TCP sulphate needs to be included in the RD for risk assessment from an exposure perspective, it could be either added to the parent and assessed against the parent dietary reference values or a separate and specific RD could be set for it, utilising the specific reference values set for 2,4,6-TCP sulphate in the dietary risk assessment.

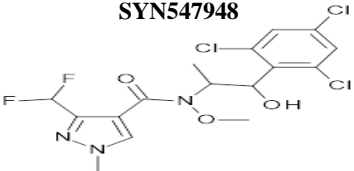
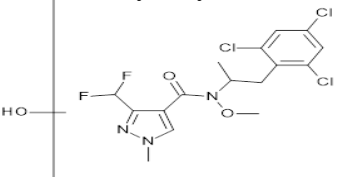
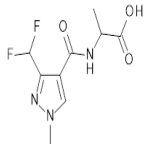
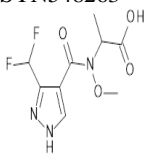
**B.6.8.1.8. Metabolites SYN547948, CSCD745176 (hydroxylated parent), SYN548264 glucuronide/sulphate**

Metabolite SYN548264 glucuronide/sulphate is a livestock metabolite. In the human gastro-intestinal tract, the glucuronide/sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN548264. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

HSE requested toxicological information on three further dietary metabolites (SYN547948, CSCD745176 and SYN548264). The applicant provided genotoxicity information based on QSAR analysis (DEREK Nexus v6.1.0, CAESAR v2.1.13 and OECD QSAR Toolbox v4.4) and read-across from suitable analogues (Parr-Dobrzanski & Bridgwood, 2021; Parr-Dobrzanski & Mason, 2021).

The table below summarises the results of the genotoxicity QSAR analysis and read-across (comparative QSAR analysis) of the three additional dietary metabolites described above.

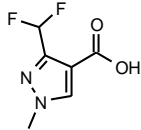
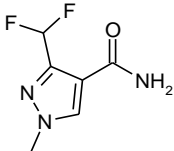
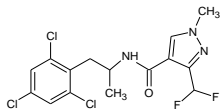
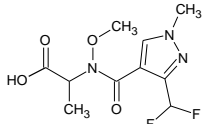
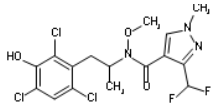
Metabolite/ Chemical Structure	Genotoxicity studies	DEREK NEXUS (v6.1.0)	VEGA (CAESAR Ames mutagenicity, v2.1.13)	QSAR toolbox V4.4	QSAR/Read across conclusion
Pydiflumetofen 	Ames -ve x4 L51 -ve x2 IVC +ve x2 IVC -ve x1 CA -ve x1 MNA -ve x3	No alerts	Outside the Applicability Domain Non-Mutagenic	DNA binding by OASIS v1.7 AN2 >> Schiff base formation by aldehyde formed after metabolic activation >> Geminal Polyhaloalkane Derivatives; Radical >> Radical mechanism via ROS formation (indirect) >> Geminal Polyhaloalkane Derivatives; SN2 >> Acylation involving a leaving group after metabolic activation >> Geminal Polyhaloalkane Derivatives; SN2 >> Nucleophilic substitution at sp <sup>3</sup> carbon atom after thiol (glutathione) conjugation >> Geminal Polyhaloalkane Derivatives  in vivo mutagenicity (Micronucleus) alerts by ISS v2.5 H-acceptor-path3-H-acceptor	Full gentoxcity package available. <b>Pydiflumetofen is not genotoxic</b>

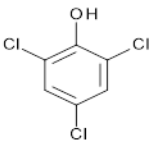
Metabolite/ Chemical Structure	Genotoxicity studies	DEREK NEXUS (v6.1.0)	VEGA (CAESAR Ames mutagenicity, v2.1.13)	QSAR toolbox V4.4	QSAR/Read across conclusion
<b>SYN547948</b> 	None available	No alerts	Outside the Applicability Domain Non-Mutagenic	Same alerts as parent	Same QSAR alerts and chemically similar with respect to genotoxicity to parent. <b>SYN547948 is not genotoxic based on read across to parent</b>
<b>CSCD745176 (hydroxylated SYN545974)</b> 	None available	No alerts	Outside the Applicability Domain Mutagenic	Same alerts as parent	Same QSAR alerts and chemically similar with respect to genotoxicity to parent. <b>CSCD745176 is not genotoxic based on read across to parent</b>
<b>SYN548264</b> 	None available	No alerts	Outside the Applicability Domain Mutagenic	Same alerts as parent	Same QSAR alerts and chemically similar with respect to genotoxicity to SYN548263 (see below). <b>SYN548264 is not genotoxic based on read across to SYN548263 (genotoxic data available)</b>
<b>SYN548263</b> 	Ames -ve MCGM -ve In vitro MN -ve	No alerts	Outside the Applicability Domain Mutagenic	Same alerts as parent	Full in vitro genotoxic package. <b>SYN548263 is not genotoxic</b>

Metabolites SYN547948 and CSCD745176 have the same genotoxicity QSAR alerts of the parent and are structurally similar to parent. Therefore, read-across from parent (pydiflumetofen) is supported. As pydiflumetofen has a full negative genotoxicity package, SYN547948 and CSCD745176 can be considered not genotoxic. Metabolite SYN548264 has the same genotoxicity QSAR alerts as SYN548263 and is structurally similar to SYN548263. Therefore, read-across from SYN548263 is supported. As SYN548263 has a full negative in vitro genotoxicity package, SYN548264 can be considered not genotoxic. Metabolites SYN547948, CSCD745176 and SYN548264 are not major rat metabolites and hence are not covered by the parent dataset. Based on these considerations, if a dietary risk assessment were required for **SYN547948, CSCD745176 and SYN548264 glucuronide/sulphate, the TTC Cramer Class III values** (chronic value = 1.5 µg/kg bw/d and acute value = 5 µg/kg bw) could be used.

#### B.6.8.1.9. Summary data on metabolites

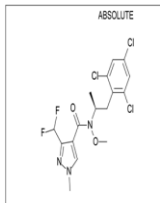
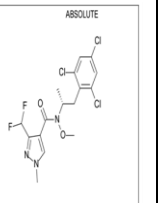
A summary of toxicity studies/data on pydiflumetofen metabolites is presented in the table below

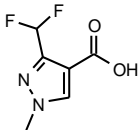
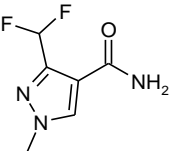
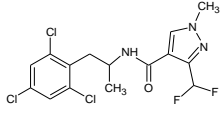
Metabolite	Structure	Detected in rat metabolism Yes/No	Available toxicological data
CSAA798670 (=NOA449410)		No	<ul style="list-style-type: none"> <li>- LD<sub>50</sub> &gt; 2000 mg/kg</li> <li>- Not genotoxic (Ames, in vitro CA, MLA TK, in vivo MN)</li> <li>- 28D rat: NOAEL = 1000 mg/kg bw/d (highest dose)</li> <li>- 90D rat: NOAEL = 1000 mg/kg bw/d (highest dose)</li> <li>- Prenatal development, Rabbit: NOAEL mat/dev = 250 mg/kg bw/d (higher dose)</li> </ul>
SYN508272		Yes <b>Major pyrazole specific metabolite</b> detected for up to 14,8% AUC in rat blood	<ul style="list-style-type: none"> <li>- Rat oral LD<sub>50</sub> &gt; 500 &lt; 2000 mg/kg bw./d</li> <li>- Genotox: In vitro: Ames negative, MLA TK negative, CA positive In vivo: MN negative (proof of blood exposure available) =&gt; Overall conclusion: not genotoxic</li> <li>- 28D rat: NOAEL = 37-42.5 mg/kg bw./d (M-F).</li> </ul>
SYN545547		Yes but minor metabolite (<10% AD) Intermediary metabolite (found at 1.3% total excreta)	<ul style="list-style-type: none"> <li>o Genotox: In vitro tests: Ames, MN (human lymphocyte) and MLA Tk (L5178Y cells) were negative</li> <li>o QSAR analysis: No alert for genotoxicity (derek nexus, CAESAR, ToxTree, OECD toolbox)</li> </ul>
SYN548263		Yes but <10% AD Detected at 8.9% in urine and 7% AUC in blood Precursor of SYN508272 found at 14.8% TRA in blood	<ul style="list-style-type: none"> <li>o Genotox: In vitro tests: Ames, MN (human lymphocyte) and MLA Tk (L5178Y cells) were negative</li> </ul>
SYN547897		Yes but <10% AD Detected at 0.9% in urine and 4.3% TRA in plasma	QSAR and read across analysis (genotoxicity end-point only): no alerts highlighted for both SYN547897 and the parent pydiflumetofen

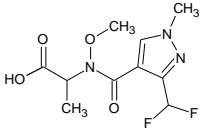
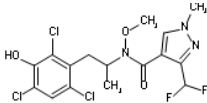
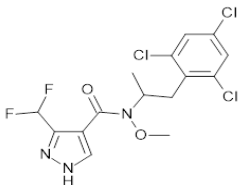
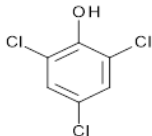
2,4,6 - TCP		Yes <b>Major phenol specific metabolite</b> detected for up to 44% TRA in plasma (2,4,6-TCP and conjugates)	Toxicity data available were from literature
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### Summary of toxicological profiles of pydiflumetofen dietary metabolites

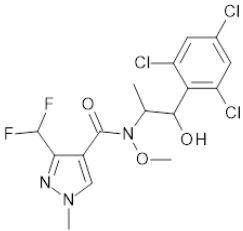
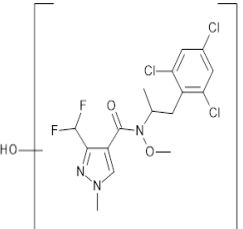
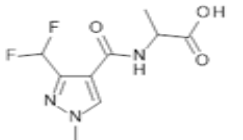
The table below gives an overview of the toxicological profile, including reference values for a number of pydiflumetofen dietary metabolites and for the parent substance itself.

Metabolite	Structure of aglycon	Detected in rat metabolism Yes/No	Available relevant toxicological data	Conclusion for dietary risk assessment
Parent (pydiflumetofen)	<div style="display: flex; justify-content: space-around;"> <div style="text-align: center;"> <p>ABSOLUTE</p>  <p>SYN54098 (S)-3-Difluoromethyl-1-methyl-1H-pyrazolo-4-carboxylic acid methyl-[(1-methyl-3,4,5-trichlorophenyl)-ethyl]-amide</p> </div> <div style="text-align: center;"> <p>ABSOLUTE</p>  <p>SYN54099 (R)-3-Difluoromethyl-1-methyl-1H-pyrazolo-4-carboxylic acid methyl-[(1-methyl-3,4,5-trichlorophenyl)-ethyl]-amide</p> </div> </div>	Not relevant	<ul style="list-style-type: none"> <li>- Rat oral LD<sub>50</sub> &gt; 5000 mg/kg bw</li> <li>- Not genotoxic (negative Ames and MCGM; weakly positive in vitro CA, but negative in vivo MN and CA)</li> <li>- 28D rat: NOAEL = 43/40 mg/kg bw/d (M/F) based on liver effects at 343/342 mg/kg bw/d</li> <li>- 90D rat: NOAEL = 18/6/21.6 mg/kg bw/d (M/F) based on liver and thyroid effects at 111/127 mg/kg bw/d</li> <li>- PNDT, rabbit: NOAEL<sub>mat</sub> = 500 mg/kg bw/d (highest dose); NOAEL<sub>dev</sub> = 10 mg/kg bw/d based on increased incidence of variant at 100 mg/kg bw/d (although no dose-response)</li> </ul>	<p><b>ADI = 0.09 mg/kg bw/d</b> (based on NOAEL of 9 mg/kg bw/d from mouse cancer study)</p> <p><b>ARfD = 0.3 mg/kg bw</b> (based on NOAEL<sub>mat</sub> of 30 mg/kg bw/d from rat PNDT study)</p>

CSAA798670 glucuronide/sulphate (=NOA449410)		No	<ul style="list-style-type: none"> <li>- Rat oral LD<sub>50</sub> &gt; 2000 mg/kg</li> <li>- Not genotoxic (Ames, in vitro CA, MLA TK, in vivo MN)</li> <li>- 28D rat: NOAEL = 1000 mg/kg bw/d (highest dose)</li> <li>- 90D rat: NOAEL = 1000 mg/kg bw/d (highest dose)</li> <li>- PNDT, Rabbit: NOAEL mat/dev = 250 mg/kg bw/d (highest dose)</li> </ul>	<b>Less toxic than parent;</b> Specific ADI = 0.25 mg/kg bw/d set at EU level, but <b>parent reference values may be more appropriate</b>
SYN508272 glucuronide/sulphate		Yes <b>Major pyrazole specific metabolite</b> detected for up to 14,8% AUC in rat blood	<ul style="list-style-type: none"> <li>- Rat oral LD<sub>50</sub> &gt; 500 &lt; 2000 mg/kg bw/d</li> <li>- Genotox: In vitro: Ames negative, MLA TK negative, CA positive. In vivo: MN negative (proof of blood exposure available) =&gt; Overall conclusion: not genotoxic</li> <li>- 28D rat: NOAEL = 37-42.5 mg/kg bw./d (M-F).</li> </ul>	<b>More toxic than parent;</b> Hence, specific <b>ADI = 0.04 mg/kg bw/d</b> set even though can be considered covered by parent (major rat metabolite)  <b>Could be included in RD-RA with parent using RPF of 2.25</b>
SYN545547 glucuronide/sulphate		Yes but minor metabolite (<10% AD) Intermediary metabolite (found at 1.3% total excreta)	<ul style="list-style-type: none"> <li>Genotox: In vitro tests: Ames, MN (human lymphocyte) and MLA Tk (L5178Y cells) were negative. Not genotoxic</li> </ul>	Not major rat metabolite. Not genotoxic, hence <b>TTC CCH values</b> (1.5 and 5 µg/kg bw/d) could be used in the dietary risk assessment

SYN548263 glucuronide/sulphate		Yes but <10% AD Detected at 8.9% in urine and 7% AUC in blood Precursor of SYN508272 found at 14.8% TRA in blood	Genotox: In vitro tests: Ames, MN (human lymphocyte) and MLA Tk (L5178Y cells) were negative. Not genotoxic	Major rat metabolite (as a precursor of a major rat metabolite) Not genotoxic <b>Covered by parent. Hence, parent reference values</b> should be used in the risk assessment
SYN547897		Yes but minor (<10% AD) Detected at 0.9% in urine and 4.3% TRA in plasma	QSAR and read across analysis (genotoxicity end-point only): no alerts highlighted for both SYN547897 and the parent pydiflumetofen. Not genotoxic	Not major rat metabolite. Not genotoxic based on QSAR and read-across, hence <b>TTC CCIII values</b> (1.5 and 5 µg/kg bw/d) could be used in the dietary risk assessment
SYN547891 glucuronide/sulphate		Yes, but minor (<10% AD)	QSAR and read across analysis (genotoxicity end-point only): no alerts highlighted for both SYN547891 and the parent pydiflumetofen. Not genotoxic	Not major rat metabolite. Not genotoxic based on QSAR and read-across, hence <b>TTC CCIII values</b> (1.5 and 5 µg/kg bw/d) could be used in the dietary risk assessment
2,4,6 – TCP sulphate		Yes <b>Major phenol specific metabolite</b> detected for up to 44% TRA in plasma (2,4,6-TCP	Toxicity data available from literature and from applicant (full genotoxicity package and 28-day study)	Although major rat metabolite, extensive dataset should take priority. Less toxic than parent. Not genotoxic.



		and conjugates)		Specific ADI = 0.4 mg/kg bw/d Specific ARfD = 1 mg/kg bw. <b>As less toxic than parent, the parent reference values should be used in the risk assessment</b>
SYN547948		Yes, but minor (<10% AD)	QSAR and read across analysis (genotoxicity end-point only): no alerts highlighted for both SYN547948 and the parent pydiflumetofen. Not genotoxic.	Not major rat metabolite. Not genotoxic based on QSAR and read-across, hence <b>TTC CCIII values</b> (1.5 and 5 µg/kg bw/d) could be used in the dietary risk assessment
CSCD745176 (hydroxylated parent)		Yes, but minor (<10% AD)	QSAR and read across analysis (genotoxicity end-point only): no alerts highlighted for both CSCD745176 and the parent pydiflumetofen. Not genotoxic.	Not major rat metabolite. Not genotoxic based on QSAR and read-across, hence <b>TTC CCIII values</b> (1.5 and 5 µg/kg bw/d) could be used in the dietary risk assessment
SYN548264 glucuronide/sulphate		Yes, but minor (<10% AD)	QSAR and read across analysis (genotoxicity end-point only): no alerts highlighted for both SYN548264 and metabolite SYN548263 for which there is a negative in vitro genotox	Not major rat metabolite. Not genotoxic based on QSAR and read-across, hence <b>TTC CCIII values</b> (1.5 and 5 µg/kg bw/d) could be used in

			package. Not genotoxic.	the dietary risk assessment
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#### **CSAA798670 glucuronide/sulphate**

**CSAA798670** is a common metabolite to a number of SDHI molecules and toxicity studies performed on this metabolite have been assessed during the peer-review of other pyrazole active substances (sedaxane, fluxapyroxade, benzovendiflupyr). Metabolite **CSAA798670 glucuronide/sulphate** is a livestock metabolite of pydiflumetofen. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, CSAA798670. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

A number of GLP and OECD compliant toxicity studies (acute oral toxicity study, 28-d study, 90-d study, rabbit PNDT study) and standard in vitro genotoxicity assays are available on metabolite CSAA798670. The metabolite did not show any genotoxic potential in the standard three in vitro genotoxicity tests. It was of low acute oral toxicity ( $LD_{50} > 2000$  mg/kg bw) in the rat and did not show any adverse effects up to the limit dose of 1000 mg/kg bw/d in a 28-day and 90-day study in the rat. In addition, no maternal toxicity or developmental toxicity was seen in rabbits up to the top dose of 250 mg/kg bw/d. However, significant maternal toxicity was noted at doses of 500 mg/kg bw/d and above in a range-finding study in pregnant rabbits. In conclusion, **CSAA798670 is of significantly lower toxicity than the parent substance** (parent 28-day rat NOAEL = 43/40 mg/kg bw/d based on liver effects and parent 90-day rat NOAEL = 18.6/21.6 mg/kg bw/d based on liver effects). From a toxicological point of view, CSAA798670 might not be needed to be included in the residue definition for risk assessment (RD-RA). Alternatively, if inclusion is required from a residue perspective, the parent dietary reference values could be used on a conservative basis. At EU level, a specific ADI of 0.25 mg/kg bw/d was derived from the NOAEL of 250 mg/kg bw/d from the rabbit PNDT study with an UF of 1000 (extra assessment factor of 10 to account for the limited database, as no long-term, multigeneration or rat developmental toxicity studies are available). An ARfD was not established, but if required, it could be set at the same level of the ADI. HSE is of the view, that if this metabolite needs to be taken into account in the dietary risk assessment, then it would be more appropriate to include it in the RD-RA together with the parent (and **applying the parent reference values**) rather than setting a separate RD-RA and applying the metabolite-specific ADI of 0.25 mg/kg bw/d.

#### **SYN508272 glucuronide/sulphate**

Metabolite **SYN508272 glucuronide/sulphate** is a livestock metabolite of pydiflumetofen. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN508272. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

Several GLP and OECD compliant toxicity studies (acute oral toxicity study and 28-d study) and standard in vitro and in vivo genotoxicity assays are available on metabolite **SYN508272**. The metabolite was positive in the in vitro chromosome aberration test, but this result was not confirmed in vivo in a valid rat bone marrow micronucleus study. It was of moderate acute oral toxicity ( $500 < LD_{50} < 2000$  mg/kg bw) in the rat and a NOAEL of 500 ppm (37.4/42.5 mg/kg bw/d in males/females) was identified from a 28-day study in the rat based on effects on body weights and food consumption at the next dose level of 2000/4000 ppm (143.1/243.5 mg/kg bw/d in males/females). In conclusion, **SYN508272 appears of higher toxicity than the parent substance**, with moderate acute oral toxicity compared to the low acute toxicity of the parent ( $LD_{50} > 5000$  mg/kg bw). In the 28-day toxicity study in the rat, reductions in body weight gain and food consumption were observed at 143 mg/kg bw/d (males)/243.5 mg/kg bw/d (females). In comparison, the same effect (decrease BW gains) was observed at 10 fold higher dosage (i.e. 1322 mg/kg bw/d) in the equivalent 28-d study in rat performed with pydiflumetofen. One explanation of these differences may be a higher oral absorption of the metabolite compared to the parent. Indeed, ADME studies demonstrated that oral absorption of pydiflumetofen is

limited by the dose level: 19-24% at 300 mg/kg bw in males and 50-55% at 100 mg/kg bw in females. Therefore, from a toxicological point of view, SYN508272 needs to be considered in the residue definition for risk assessment (RD-RA). SYN508272 is a major rat metabolite of pydiflumetofen as it was detected in plasma accounting for up to 14.8% of the total radioactivity AUC (TRA). On this basis, its toxicological profile can be considered covered by that of the parent and the parent dietary reference values could be used in the risk assessment. However, HSE agrees with the EU, that given its higher toxicity potential compared to the parent, it would be more appropriate to set metabolite specific reference values on the basis of the available data. An **ADI of 0.04 mg/kg bw/d** was set at EU level from the NOAEL of the 28-day study with the application of an UF of 1000 (extra assessment factor of 10 to account for the limited database, as no long-term, multigeneration or developmental toxicity studies are available). The ArfD was set at the same level of the ADI. It should be noted that this metabolite-specific ADI is lower than the parent ADI (0.09 mg/kg bw/d), confirming the relative higher toxicity of the metabolite. If from a residue perspective, a dietary risk assessment is required for this metabolite, **SYN508272 glucuronide/sulphate could be included in the RD-RA together with parent by applying a Relative Potency Factor (RPF) of 2.25**. Alternatively, a separate RD-RA could be set for this metabolite using its specific ADI and ArfD.

#### **SYN545547 glucuronide/sulphate**

Metabolite **SYN545547 glucuronide/sulphate** is a livestock metabolite of pydiflumetofen. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN545547. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

GLP and OECD compliant in vitro genotoxicity assays (supported by a comparative genotoxicity QSAR analysis) are available on metabolite **SYN545547**. The metabolite was negative in the standard battery of 3 in vitro tests and therefore it is considered to be non-genotoxic. It is noted that SYN545547 is only a minor rat metabolite; therefore it is not covered by the parent dataset. However, based on the available data, if a dietary risk assessment were to be required, **the TTC Cramer Class III values (chronic value = 1.5 µg/kg bw/d and acute value = 5 µg/kg bw)** could be used. This is in contrast to the advice given by the EU peer-review process.

#### **SYN548263 glucuronide/sulphate**

Metabolite **SYN548263 glucuronide/sulphate** is a livestock metabolite of pydiflumetofen. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN548263. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

Overall, GLP and OECD compliant in vitro genotoxicity assays are available on metabolite **SYN548263**. The metabolite was negative in the standard battery of 3 in vitro tests and therefore it is considered to be non-genotoxic. It is noted that SYN548263 is only a minor rat metabolite (< 10% AD in urine and plasma); however, it is a direct precursor of SYN508272, which is a major rat metabolite (14.8% TRA in blood). On this basis, it can be assumed that at some point, SYN548263 must have also been present at similar levels in plasma; thus it can be considered a major rat metabolite, **covered by the parent dataset**. Therefore, if a dietary risk assessment were to be required, **the dietary reference values of the parent could be used**. This is in contrast to the advice given by the EU peer-review process.

#### **SYN547897**

**SYN547897** is not a major rat metabolite; therefore it cannot be considered covered by the parent dataset. However, given the lack of genotoxicity based on a comparative QSAR analysis with the parent, HSE concludes that, if required, **the TTC Cramer Class values (chronic value = 1.5 µg/kg bw/day and acute value = 5 µg/kg bw) can be used in the dietary risk assessment**. This is in contrast to the EU decision not to set a toxicological reference values for SYN547897.

**SYN547891 glucuronide/sulphate**

Metabolite **SYN547891 glucuronide/sulphate** is a livestock metabolite of pydiflumetofen. In the human gastro-intestinal tract, the glucuronide and/or sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN547891. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

**SYN547891** is not a major rat metabolite; therefore it cannot be considered covered by the parent dataset. However, given the lack of genotoxicity based on a comparative QSAR analysis with the parent, HSE concludes that, if required, the **TTC Cramer Class values (chronic value = 1.5 µg/kg bw/day and acute value = 5 µg/kg bw) can be used in the dietary risk assessment.**

**2,4,6-TCP sulphate**

Metabolite **2,4,6-TCP sulphate** is a livestock metabolite. In the human gastro-intestinal tract, the sulphate will be easily cleaved, leading to systemic exposure to the aglycon, 2,4,6-TCP. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugate. In addition, it is noted that 2,4,6-TCP sulphate is a major rat metabolite covered by the parent.

**ADME:** In rats, oral absorption was very extensive (>90% of the dose). 2,4,6-TCP was rapidly and extensively conjugated and excreted in urine. The highest concentrations of 2,4,6-TCP were found in the kidney, blood and liver.

**Acute toxicity:** An oral LD50 of 820 mg/kg bw is cited in NCI (1979). No further data were identified in the search of the published literature. However 2,4,6-TCP has the following harmonised EU classification (and GB mandatory classification) entry for acute endpoints: Acute Tox 4 (H302); Skin Irrit 2 (H315) and Eye Irrit. 2 (H319).

**Short-term toxicity:** The short-term toxicity of pydiflumetofen has been investigated in three publications, a 90-day study in rats and preliminary 7-wk studies in rats and mice. The preliminary studies do not allow the identification of robust NOAELs and hence they are not described further. In addition, as the 90-day study had some limitations, the Applicant recently generated and submitted two regulatory studies, a 14-day range finder and a 28-day study in the rat. In the 90-day study, a LOAEL was identified at 240 mg/kg bw/d for changes in the weights of liver, kidney and adrenals and the **NOAEL was set at 80 mg/kg bw/d**. In the recently submitted GLP and guideline 28-day gavage study adverse effects were seen at the top dose (500 mg/kg bw/d) on the weight of the liver (females), thyroid (males) and uterus (females). Based on these effects a **NOAEL of 250 mg/kg bw/d** could be identified from the study.

**Genotoxicity:** A wide range of in vitro and in vivo studies were identified in the published literature. These were in the main non-standard studies with several limitations and showing inconsistent results. Following the EU peer-review process, it was concluded that the genotoxic potential of 2,4,6-TCP was inconclusive, based on positive results observed in vitro and inconsistent results observed in vivo, and needed to be clarified. On this basis, Syngenta recently submitted a modern package of three in vitro tests (Ames, micronucleus and mammalian cell gene mutation tests) and an in vivo TGR (Transgenic rodent) assay in rats. These well conducted tests have demonstrated that 2,4,6-TCP is not genotoxic in vitro or in vivo.

**Long-term toxicity and carcinogenicity:** The chronic toxicity and carcinogenic potential of 2,4,6-TCP was investigated in rats and mice (NCI, 1979). 2,4,6-TCP was carcinogenic in male F344 rats, inducing lymphomas or leukemias from the lowest dose of **258 mg/kg bw/d (LOAEL)**. It was also carcinogenic in both sexes of B6C3F1 mice, inducing liver hepatocellular carcinomas and/or adenomas from the lowest dose of 650 mg/kg bw/d. Based on these findings, 2,4,6-TCP has harmonised classification in the EU and mandatory classification in GB with Carc. Cat 2 (H351).

**Reproductive toxicity:** The reproductive toxicity potential of 2,4,6-TCP was investigated in two limited rat one-generation studies from the open literature, but only the first one is considered reliable. In a publication from 1986, 2,4,6-TCP had no effect on any sperm parameter or male fertility. Treatment of females with 1000 mg/kg bw/d of 2,4,6-TCP produced maternal toxicity as reflected in increased lethality and decreased weight gain in the dams. However no treatment-related differences were seen in litter sizes or pup survival. Male and female birth weights were significantly depressed in the 500 and 1000 mg/kg bw/d groups; these differences disappeared by Day 4 post-partum suggesting that they were a reflection of maternal toxicity. Overall, in this limited study, the reproductive processes of male and female rats do not appear to be a primary target of 2,4,6-TCP up to the limit dose of 1000 mg/kg bw/d. HSE notes that a **NOAEL of 100 mg/kg bw/d could be identified for generalised offspring toxicity and a NOAEL of 500 mg/kg bw/d could be identified for parental toxicity.**

**Dietary reference values:** Although 2,4,6-TCP is a major rat metabolite of pydiflumetofen and could be considered 'covered' by the parent, HSE notes that there is a significant dataset on the substance showing that 2,4,6-TCP has a different toxicity profile compared to pydiflumetofen. Therefore, the specific toxicological data on 2,4,6-TCP should take priority and be used to establish specific reference values. The most appropriate POD for the derivation of the ADI is the NOAEL of 80 mg/kg bw/d for effects on organ weights at 240 mg/kg bw/d from the rat 90-day study. By applying the standard default factor of 100 and an additional factor of 2 as tumours were seen at the LOAEL of 258 mg/kg bw/d, and **ADI of 0.4 mg/kg bw/d** is derived. No further assessment factors are required as chronic/carcinogenicity studies and reproductive toxicity studies are available. Considering that 2,4,6-TCP is acutely toxic by the oral route, an ARfD should be derived. An appropriate POD for the ARfD is the offspring NOAEL of 100 mg/kg bw/d for reduced pup body weights at birth in the reproductive toxicity study. By applying the standard default factor of 100, an **ARfD of 1 mg/kg bw** can be established. These reference values compared to those of the parent substance indicate that 2,4,6-TCP is **not more toxic than pydiflumetofen**. Therefore, if 2,4,6-TCP sulphate needs to be included in the RD for risk assessment from an exposure perspective, it could be either added to the parent and **assessed against the parent dietary reference values or a separate and specific RD could be set for it, utilising the specific reference values set for 2,4,6-TCP in the dietary risk assessment.**

**SYN547948, CSCD745176 (hydroxylated parent) and SYN548264 glucuronide/sulphate**

Metabolite **SYN548264 glucuronide/sulphate** is a livestock metabolite of pydiflumetofen. In the human gastro-intestinal tract, the glucuronide/sulphate will be easily cleaved, leading to systemic exposure to the aglycon, SYN548264. Therefore, the toxicological properties determined for the aglycon can be extrapolated to the conjugates.

Metabolites **SYN547948, CSCD745176 (hydroxylated parent) and SYN548263** are not genotoxic based on QSAR and read-across analysis. Metabolites SYN547948, CSCD745176 and SYN548264 are not major rat metabolites and hence are not covered by the parent dataset. Based on these considerations, if a dietary risk assessment were required for **SYN547948, CSCD745176 and SYN548264, the TTC Cramer Class III values** (chronic value = 1.5 µg/kg bw/d and acute value = 5 µg/kg bw) could be used.

**B.6.8.2. Supplementary studies on the active substance**

A small number of supplementary studies have been conducted using SYN545974 as the test item, these are summarised in Table 6.8.2-1.

**Table 6.8.2-1: Summary of supplementary studies on SYN545974**

Study	Mode of dosing	Test material & dose levels	Results
Immunotoxicity review. [REDACTED], (2016). Report No. TK0288608.	N/A	N/A	Review of the SYN545974 database shows no evidence of an adverse effect on the immune system in rats, mice or dogs. SYN545974 has no immunotoxic potential.
Hepatic microsomal UDP-GT activity in livers of male rats. [REDACTED], (2014). Report No 5522/1/2/2014	Samples derived from male rats on 90 day dietary study ([REDACTED] and [REDACTED], 2015)	SYN545974 99.5% 0, 250, 1500, 8000 & 16000 ppm	SYN545974 is an inducer of hepatic microsomal UDP-GT in male rats.
Thyroid peroxidase activity in rats. [REDACTED], (2014). Report No. 5523/1/2/2014.	<i>In vitro</i>	SYN545974 98.5% 0 to 10 µM	SYN545974 is not an inhibitor of rat thyroid peroxidase activity <i>in vitro</i> .

**Immunotoxicity**

According to Commission Regulation (EU) No 283/2013 supplementary studies on the immunotoxicological potential are required for an active substance when they are necessary to further clarify observed effects on the immune system. SYN545974 does not fulfil these criteria and specific studies on immunotoxicity would not be required.

A review of the currently available toxicity studies SYN545974 has been undertaken and endpoints considered relevant for the identification of potential immunotoxicity have been evaluated and it was concluded that SYN545974 has no immunotoxic potential. All of the reports discussed in the following review are fully summarised under the appropriate data point within this dossier.

<b>Report:</b>	K-CA 5.8.2/01 [REDACTED] (2016). SYN545974: Position Statement Concerning Immunotoxicity Potential. Syngenta Ltd. Jealott's Hill International Research, Bracknell, Berks RG42 6EY. Laboratory Report No. TK0288608, 07 January 2016. Unpublished. Syngenta File No. SYN545974_10361.
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**Guidelines:** This was a review article with no applicable guidelines.

**GLP:** Not applicable as no experimental work conducted.

**EXECUTIVE SUMMARY**

A detailed review of parameters related to immune function has been conducted on the toxicity database for SYN545974. Repeat-dose studies in rats, mice and dogs were reviewed for any treatment-related changes in a variety of indicators of potential immunotoxicity including leukocyte counts, lymphocyte counts, globulin concentration, macroscopic findings (lymph nodes, thymus, and spleen), organ weights (spleen, thymus and adrenals), and microscopic findings (bone marrow, lymph nodes, spleen, thymus and adrenals).

A thorough review of the toxicology database for SYN545974 has shown no evidence of adverse effects on the immune system in rats, mice or dogs. In addition, SYN545974 does not belong to a class of chemicals (e.g., the organotin, heavy metals, or halogenated aromatic hydrocarbons) that would be

expected to be immunotoxic. **Therefore, it can be concluded that SYN545974 has no immunotoxic potential.**

**Position statement concerning immunotoxicity potential (██████████, 2016)**

Overall, HSE agrees with the evaluation of the EU peer-review process.

No specific studies on immunotoxicity are available for pydiflumetofen and none are required.

There were no treatment-related changes indicative of immunotoxic potential in rats, mice or dogs following repeated exposure to pydiflumetofen. There was no effect on haematological (leukocyte/lymphocyte counts) or clinical chemistry (globulin concentration) parameters. There were no unusual macroscopic or microscopic findings related to those tissues of the immune system that were examined (lymph nodes, thymus, spleen, bone marrow and adrenals). Additionally, the spleen, thymus and adrenals were weighed, and no treatment-related changes were seen.

Furthermore, pydiflumetofen does not belong to any class of chemicals which are known to have immunotoxic properties (e.g., halogenated aromatic hydrocarbons). Therefore, no studies to further elucidate the immunotoxic potential of pydiflumetofen are required.

**It can be concluded from the available data that pydiflumetofen has no immunotoxic potential and that further investigation is not required.**

(██████████, 2016)

**Mechanistic studies on potential thyroid effects in the rat**

Two mechanistic studies performed to investigate the potential thyroid toxicity of the active substance seen in rats were conducted.

<b>Report:</b>	K-CA 5.8.2/02 ██████████ (2015). SYN545974: Effect on Hepatic UDPglucuronosyltransferase Activity Towards Thyroxine as Substrate after Dietary Administration for 90 Days to Male Rats. ██████████ ██████████. Laboratory Report No. 5522/1/2/2014 (Amendment 1), 14 August 2015. Unpublished. Syngenta File No. SYN545974_10264.
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**Guidelines:** This was a research study with no applicable regulatory guidelines.

**GLP:** Signed and dated GLP and Quality Assurance statements were provided.

There were no deviations considered to compromise the scientific validity of the study.

**HSE comment:** as a mechanistic study, this study was considered supportive

**EXECUTIVE SUMMARY**

The aim of this study was to evaluate the effect of treatment with SYN545974 on hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine (tetraiodothyronine; T4) as substrate in the livers of male rats. The male rat liver samples analysed were taken at termination of a dietary toxicity study where the rats were given diets containing 0 (control), 250, 1500 or 8000 ppm SYN545974 for 90 days. Liver microsomes from control and SYN545974 treated rats were assayed for protein content and UDPglucuronosyltransferase activity towards thyroxine as substrate. Enzyme activity was expressed as

specific activity (i.e. per unit of microsomal protein), per gram of liver, per total liver and per relative liver weight.

The treatment of male rats with 1500 and 8000 ppm SYN545974 for 90 days significantly increased hepatic microsomal protein content to 114 and 122% of control, respectively. The treatment of male rats with 250 ppm SYN545974 for 90 days significantly increased hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate expressed as specific activity and per gram of liver to 152 and 162% of control, respectively. Hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate expressed as specific activity, per gram of liver, per total liver and per relative liver weight was significantly increased to 171, 194, 224 and 239% of control, respectively, by treatment with 1500 ppm SYN545974 and to 288, 347, 421 and 486% of control, respectively, by treatment with 8000 ppm SYN545974.

**Overall, the data obtained in this study demonstrate that SYN545974 is an inducer of hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate in male rats. Treatment with 250 ppm SYN545974 for 90 days resulted in significant increases in hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate expressed as specific activity and per gram of liver, whereas treatment with 1500 and 8000 ppm SYN545974 resulted in significant increases in enzyme activity expressed as specific activity, per gram of liver, per total liver and per relative liver weight.**

## MATERIALS AND METHODS

### Materials:

**Liver samples:** Liver samples were collected from a 90 day dietary study in male rats (Study No. 520843) performed at ( ). Samples were collected from 10 rats at each of the following dose levels: 250 ppm SYN545974, 1500 ppm SYN545974 and 8000 ppm SYN545974 and from 9 control rats.. The liver samples were snap-frozen in liquid nitrogen and then stored in an ultrafreezer set to maintain -80 °C prior to being transported to ( ), where they were received frozen in dry ice. All liver samples were stored in their containers as supplied at or below -70 °C prior to being homogenised.

### Study Design and Methods:

**Experimental dates:** Start: 25 November 2013, End: 21 February 2014

**Preparation of liver fractions:** Each liver sample was thawed and weighed. Whole homogenates of the individual liver samples were prepared in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4 using a Potter type, Teflon-glass, motor driven homogeniser (*Lake, 1987*). Liver whole homogenates were centrifuged at 10,000 g average for 20 minutes to obtain the postmitochondrial supernatants which were subsequently centrifuged at 105,000 g average for 60 minutes to separate the microsomal fraction from the cytosol. The microsomal fraction of each animal was re-suspended in fresh homogenising medium. Aliquots of liver whole homogenate, microsomal and cytosolic fractions from each animal were stored at -70°C or below.

**Assay of protein content:** Liver whole homogenate and microsomal protein content were determined by the general procedure of ( *et al. (1951)*), as described previously (*Lake, 1987*), employing bovine serum albumin as standard.

**Assay of UDPglucuronosyltransferase activity:** Hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate was determined in incubation mixtures containing 10 µM thyroxine and 5 mM UDPGA ( *et al. 2006*). The formation of thyroxine glucuronide was quantified by ultra performance liquid chromatography-mass spectrometry-mass spectrometry (UPLC-MS-MS), employing a Waters Acquity UPLC system coupled to a Waters Xevo TQ mass spectrometer.



In addition, the potential responsiveness of hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate determined in this study to an inducer of hepatic CYP enzymes was assessed with liver microsomes from vehicle treated (corn oil) and  $\beta$ -naphthoflavone (BNF) treated male Sprague-Dawley rats.

**Statistics:** Data were summarised in the form of mean and standard deviations (SDs) of the mean. Hepatic microsomal protein content and UDPglucuronosyltransferase enzyme activity data were tested for normality using the Kolmogorov-Smirnov test (level of significance determined to be at  $p < 0.05$ ) and heterogeneity using Bartlett's test (level of significance  $p < 0.01$ ). Control and SYN545974 treated groups were subjected to a one-way analysis of variance and comparisons between control and SYN545974 treated groups were made using two-sided Dunnett's tests. In all Dunnett's test comparisons a probability level of  $p < 0.05$  was taken to indicate statistical significance.

## RESULTS

**Assay of protein content:** The treatment of male rats with 1500 and 8000 ppm SYN545974 in the diet for 90 days significantly increased hepatic microsomal protein content to 114 and 122% of control, respectively.

**Table 6.8.2-2: Hepatic microsomal protein content**

Dose level of SYN545974 (ppm)	Microsomal protein (mg/g liver)
0 (control)	33.4 $\pm$ 3.82 (100)
250	35.8 $\pm$ 3.43 (107)
1500	38.0 $\pm$ 2.89** (114)
8000	40.8 $\pm$ 2.96** (122)

Values in parentheses are percentage of control levels

Significantly different from control: \*\* $p < 0.01$

**Assay of UDPglucuronosyltransferase activity:** The treatment of male rats with 250 ppm SYN545974 for 90 days significantly increased hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate expressed as specific activity and per gram of liver to 152 and 162% of control, respectively. Hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate expressed as specific activity, per gram of liver, per total liver and per relative liver weight was significantly increased to 171, 194, 224 and 239% of control, respectively, by treatment with 1500 ppm SYN545974 and to 288, 347, 421 and 486% of control, respectively, by treatment with 8000 ppm SYN545974.

**Table 6.8.2-3: Hepatic microsomal UDPglucuronosyltransferase activity towards thyroxine as substrate**

Dose level of SYN545974 (ppm)	UDPglucuronosyltransferase activity (substrate thyroxine)			
	pmol/min/mg protein	pmol/min/g liver	nmol/min/liver weight	nmol/min/liver weight/kg body weight

<b>0 (control)</b>	5.6±1.43 (100)	189±58.9 (100)	2.77±1.026 (100)	6.34±2.159 (100)
<b>250</b>	8.5±2.83* (152)	306±114.9* (162)	4.39±1.656 (158)	10.46±3.464 (165)
<b>1500</b>	9.6±2.71** (171)	366±117.0** (194)	6.20±1.568** (224)	15.17±4.447** (239)
<b>8000</b>	16.1±2.37** (288)	656±110.8** (347)	11.66±2.208** (421)	30.79±5.395** (486)

Values in parentheses are percentage of control levels

Significantly different from control: \*p<0.05, \*\*p<0.01

UDPglucuronosyltransferase activity towards thyroxine as substrate was induced to 1148% of control by BNF treatment, enzyme activity being 17.4 and 199.7 pmol/min/mg protein in corn oil and BNF-treated rat liver microsomes, respectively. The results of the assays with the reference rat liver microsomal preparations confirmed the potential responsiveness of the UDPglucuronosyltransferase activity determined to a known CYP inducer.

## CONCLUSION:

In a mechanistic GLP study, groups of 10 male rats were treated with pydiflumetofen at 0, 250, 1500 or 8000 ppm for 90 days. Liver samples were taken at the end of treatment and microsomal fractions obtained. These were analysed for protein content and for UDPglucuronosyltransferase (UDPGT) activity towards thyroxine.

Significant increases in hepatic microsomal UDPGT activity towards thyroxine (expressed as specific activity and per gram of liver tissue) were reported at all dose levels. Statistically significant increases in UDPGT activity towards thyroxine expressed as per total liver and per relative liver weight were additionally observed from the mid-dose upwards.

This mechanistic study was considered as supportive by the EU RMS and it was also considered as a line of evidence by EFSA in their weight-of-evidence (WoE) evaluation for T-mediated endocrine activity (line 18, see Table 6.8.3-2. in Section 6.8.3).

EFSA noted that the rat is generally recognized to have a low reserve of thyroid hormones, and is consequently sensitive to an increase in clearance of thyroid hormones by induction of liver enzymes. It was concluded by EFSA that the mechanistic relationship between liver toxicity and thyroid effect is only circumstantial. ECHA RAC (Risk Assessment Committee) did not identify the thyroid as a primary target organ of toxicity based on data from short-term and long-term toxicity studies. HSE agreed with RAC on this point as shown in the [GB MCL Technical Report](#).

HSE agrees with the conclusion of EFSA and the outcome of the EU peer-review process that phase II liver enzyme induction of UDPGT was observed in the rat.

## REFERENCES:

██████████ (██████████) Study No. 520843 (██████████ Report No. 33012). SYN545974 - A 13 week toxicity study of SYN545974 by oral (dietary) administration in rats.

Finch JM., Osimitz TG, Gabriel KL, Martin T, Henderson WJ, Capen CC, Butler WH, Lake BG, 2006. A mode of action for induction of thyroid gland tumors by Pyrethrins in the rat. Toxicol. Appl. Pharmacol. 214, 253-262.

Lake BG, 1987. Preparation and characterisation of microsomal fractions for studies of xenobiotic metabolism, In: Snell K., Mullock B. (Eds.), Biochemical Toxicology: A Practical Approach, IRL Press, Oxford, pp. 183–215.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

**GLP:** This study was not conducted according to Good Laboratory Practice Standards as defined by OECD. No claim of GLP compliance was made for this study.

## EXECUTIVE SUMMARY

Treatment with SYN545974 had no significant effect on rat thyroid peroxidase activity at any concentration tested. Treatment with PTU resulted in a 99.9% inhibition of thyroid peroxidase activity.

**SYN545974 is not an inhibitor of rat thyroid peroxidase activity *in vitro*.**

## MATERIALS AND METHODS

### Materials:

<b>Test Material:</b>	SYN545974
<b>Description:</b>	Technical material, off white powder
<b>Lot/Batch number:</b>	SMU2EP12007
<b>Purity:</b>	98.5% w/w
<b>CAS#:</b>	1228284-64-7
<b>Stability of test compound:</b>	Stable at <30°C. Recertification date end June 2016

**Vehicle and/or positive control:** Dimethyl sulfoxide (DMSO) / 6-propyl-2-thiouracil (PTU) (>99% purity).

<b>Test Animals:</b>	
<b>Species</b>	Rat
<b>Strain</b>	Wistar Han
<b>Age at termination</b>	67-74 days
<b>Source</b>	
<b>Housing</b>	Not reported
<b>Acclimatisation period</b>	Not reported
<b>Diet</b>	Laboratory animal diet <i>ad libitum</i> (no further details reported)
<b>Water</b>	Water <i>ad libitum</i>
<b>Environmental conditions</b>	Not reported

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**Study Design and Methods:**

**Experimental dates:** Start: 10 March 2014, End: 28 March 2014

**Preparation of thyroid gland microsomes:** Male rats were killed by exsanguination under ketamine and medetomidine anaesthesia (administered by intraperitoneal injection) and the thyroid glands attached to part of the trachea immediately removed and snap frozen in dry ice. The trachea/thyroid gland samples were stored at -70°C or below prior to preparation of thyroid gland microsomes. The trachea/thyroid glands of five rats (body weights 294, 300, 301, 302 and 306 g) were thawed and each thyroid gland dissected from the attached trachea. A whole homogenate of the pooled thyroid glands from five rats was prepared in 0.154 M KCl containing 50 mM Tris-HCl, pH 7.4 using a Potter type, Teflon-glass, motor driven homogeniser (*Lake, 1987*). The thyroid gland whole homogenate was centrifuged at 9,000 g average for 20 minutes to obtain the postmitochondrial supernatant which was subsequently centrifuged at 105,000 g average for 60 minutes to separate the microsomal fraction from the cytosol. The pooled thyroid gland microsomal fraction was resuspended in fresh homogenising medium. Aliquots of the pooled thyroid gland microsomal fraction were stored at -70°C or below and were thawed once only for the determination of thyroid peroxidase activity.

**Assay of protein content:** Thyroid gland microsomal protein content was determined by the general procedure of █████ *et al.* (1951), as described previously (*Lake, 1987*), employing bovine serum albumin as standard. The microsomal protein content of the pooled thyroid gland preparation was calculated to be 19.3 mg protein/g tissue.

**Assay of thyroid peroxidase activity:** Thyroid peroxidase activity was assayed by determining the monoiodination of L-tyrosine by a method based on studies by █████ *et al.* (1998) and *Freyberger and Ahr (2006)*. Incubations contained 500 µM L-tyrosine, 150 µM potassium iodide, 24 µg thyroid microsomal protein, either SYN545974 or PTU dissolved in dimethyl sulphoxide (2.5 µL/incubation) and 0.1 M phosphate buffer pH 7.4 in a total volume of 0.25 mL. The SYN545974 concentrations studied were 0 (control), 0.007, 0.1, 1.5 and 10 µM, whereas the PTU concentration studied was 10 µM. The low (0.007 µM) and intermediate (0.1 and 1.5 µM) concentrations of SYN545974 were selected following consideration of the available toxicokinetic and pharmacokinetic data. The high concentration (10 µM) was included in order to robustly assess the intrinsic potential of SYN545974 to affect thyroid peroxidase activity. After a 10 minute preincubation in a shaking water bath at 37°C, the reaction was initiated by the addition of 200 µM hydrogen peroxide. Blank incubations (to correct for non enzymatic formation of 3-iodo-L-tyrosine) contained all additions except for thyroid gland microsomes. After a 10 minute incubation in a shaking water bath at 37°C the reaction was terminated and levels of 3-iodo-L-tyrosine in deproteinised supernatants determined by ultra performance liquid chromatography-mass spectrometry-mass spectrometry (UPLC-MS-MS). Under these conditions the rate of formation of 3-iodo-L-tyrosine was linear with respect to both incubation time and protein concentration and the formation of 3,5-diiodo-L-tyrosine in control incubations was <2.5% of the formation of 3-iodo-L-tyrosine.

**Statistics:** Data were summarised in the form of mean and standard deviations (SDs) of the mean. Enzyme activity data were tested for normality using the Kolmogorov-Smirnov test (level of significance determined to be at  $p < 0.10$ ) and heterogeneity using Bartlett's test (level of significance  $p < 0.01$ ). Control and SYN545974 treated groups and control and PTU treated groups were subjected to a one-way analysis of variance. Comparisons between control and SYN545974 treated groups were made using two-sided Dunnett's tests and between control and PTU treated groups were made using a t-test. In all Dunnett's test and t-test comparisons a probability level of  $p < 0.05$  was taken to indicate statistical significance.

## RESULTS

**Assay of thyroid peroxidase activity:** Treatment with SYN545974 had no significant effect on rat thyroid peroxidase activity at any concentration tested. Treatment with PTU resulted in a 99.9% inhibition of thyroid peroxidase activity.

**Table 6.8.2-4: Effect of SYN545974 and PTU on rat thyroid peroxidase activity**

Addition	Thyroid peroxidase activity (nmol/min/mg protein)
Control (DMSO only)	7.07 ± 0.546 (100)
SYN545974 0.007 µM	6.81 ± 0.329 (96)
SYN545974 0.1 µM	6.72 ± 0.699 (95)
SYN545974 1.5 µM	6.76 ± 0.217 (96)
SYN545974 10 µM	7.05 ± 0.197 (100)
PTU 10 µM	0.007 ± 0.0015** (0.1)

Values in parentheses are percentage of control levels

Significantly different from control: \*\*p<0.01

## CONCLUSION:

Pydiflumetofen (tested at concentrations of 0, 0.007, 0.1, 1.5 and 10 µM) did not inhibit thyroid peroxidase (TPO) activity in a thyroid gland microsomal preparation pooled from five rats, whilst the positive control 6-propyl-2-thiouracil (PTU; 10 µM) showed the expected inhibition of TPO activity, thus demonstrating the validity of the assay.

This mechanistic study was considered as supportive by the EU RMS and it was also considered as a line of evidence by EFSA in their weight-of-evidence (WoE) evaluation for T-mediated endocrine activity (line 17, see Table 6.8.3-2 in Section 6.8.3).

HSE agrees with the conclusion of EFSA and the outcome of the EU peer-review process that pydiflumetofen does not cause an effect on TPO activity in the rat

## REFERENCES:

Doerge DL, Chang HC, Divi RL, Churchwell MI, 1998. Mechanism for inhibition of thyroid peroxidase by Leucomalachite Green. *Chemical Research in Toxicology* 11, 1098-1104.

Freyburger A, Ahr HJ, 2006. Studies on the goitrogenic mechanism of action of N,N,N',N'-tetramethylthiourea. *Toxicology*, 169-175.

Lake BG, 1987. Preparation and characterisation of microsomal fractions for studies of xenobiotic metabolism, In: Snell K., Mullock B. (Eds.), *Biochemical Toxicology: A Practical Approach*, IRL Press, Oxford, pp. 183–215.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ, 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265-275.

(██████, 2014)

### B.6.8.3. Studies on endocrine disruption

The new scientific criteria for the determination of endocrine disrupting properties, as laid down in Commission Regulation (EU) 2018/605<sup>14</sup>, are applicable to all substances for which a decision on approval or renewal of approval has been pending on 10 November 2018 according to Commission Implementing Regulation (EU) 2018/1659<sup>15</sup>. As the peer review on pydiflumetofen was already in an advanced stage or completed at the time of entry into force of the above mentioned Regulations, an assessment of the endocrine disrupting potential in line with the EFSA/ECHA (2018) guidance<sup>16</sup> was not available. Therefore, an assessment of the endocrine disrupting properties of the active substance pydiflumetofen in line with the EFSA/ECHA (2018) was conducted by EFSA and peer-reviewed by the EU MS in order to decide whether the information available in the application dossier was sufficient to conclude if the approval criteria on endocrine disruption potential are met.

#### Gather all relevant information

The source of information for all studies was the draft assessment report (DAR) prepared by HSE as well as the outcome of the peer review of the pesticide risk assessment conducted by EFSA in accordance with Article 12 of Regulation (EC) No 1107/2009.

Regarding the mammalian toxicology area, data were gathered from all repeated dose toxicity studies in mammals, including *in vivo* mechanistic data, as well as *in vitro* mechanistic studies that were included in the DAR.

The available literature review was done using the single concept approach and this therefore in line with the recommendations of the ECHA/EFSA Guidance (2018). No relevant studies were retrieved.

Data were populated in the Excel template (version published in December 2018) provided as Appendix E to the EFSA/ECHA guidance for the identification of endocrine disruptors (2018). According to this template each study was given a unique identification number (Study ID Matrix) that is important for its identification in the data-matrix and Lines of Evidence (LoE) spreadsheets of the Excel. A summary of all studies considered for the mammalian toxicology, including the Study ID Matrix is outlined in Table 6.8.3-1.

**Table 6.8.3-1. Outline of dataset considered for mammalian toxicology assessment**

Type of toxicity	Study type	Study ID Matrix
<b>Repeated dose toxicity studies in mammals</b>	Repeated dose 28-day oral toxicity study in rat	1
	Repeated dose 28-day oral toxicity study in mouse	2
	Repeated dose 90-day oral toxicity study in rat	3
	Repeated dose 90-day oral toxicity study in mouse	4
	Repeated dose 90-day oral toxicity study in dog	5
	1-year dog toxicity study	6
	Repeated dose 28-day dermal toxicity study in rat	7
	Two-generation reproduction toxicity test in rat	8
	Prenatal developmental toxicity study in rat	9
	Prenatal developmental toxicity study in rabbit	10

<sup>14</sup> Commission Regulation (EU) 2018/605 of 19 April 2018 amending Annex II to Regulation (EC) No 1107/2009 by setting out scientific criteria for the determination of endocrine disrupting properties. OJ L 101, 20.4.2018, p. 33–36.

<sup>15</sup> Commission Implementing Regulation (EU) 2018/1659 of 7 November 2018 amending Implementing Regulation (EU) No 844/2012 in view of the scientific criteria for the determination of endocrine disrupting properties introduced by Regulation (EU) 2018/605. OJ L 278, 8.11.2018, p. 3–6.

<sup>16</sup> ECHA (European Chemicals Agency) and EFSA (European Food Safety Authority) with the technical support of the Joint Research Centre (JRC), Andersson N, Arena M, Auteri D, Barmaz S, Grignard E, Kienzler A, Lepper P, Lostia AM, Munn S, Parra Morte JM, Pellizzato F, Tarazona J, Terron A and Van der Linden S, 2018. Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009. EFSA Journal 2018;16(6):5311, 135 pp. <https://doi.org/10.2903/j.efsa.2018.5311>. ECHA-18-G-01-EN

	Combined chronic toxicity/carcinogenicity studies in rat with interim sacrifice	11
	Carcinogenicity study in mouse	12
<b><i>In vivo mechanistic</i></b>	Repeated 28-day oral mechanistic study in mouse (hepatocellular proliferation and Phase I enzyme induction)	16
	Repeated 90-day oral mechanistic study in rat hepatic microsomes (Phase II enzyme induction)	18
	Repeated 28-day oral mechanistic study in mouse (Phase I enzyme induction)	19
<b><i>In vitro mechanistic</i></b>	In vitro mechanistic study for CAR activation	13
	In vitro mechanistic study in human hepatocytes (hepatocellular proliferation and Phase I enzyme induction)	14
	In vitro mechanistic study in mouse hepatocytes (hepatocellular proliferation and Phase I enzyme induction)	15
	In vitro assay for TPO activity in rat microsomes	17

**ED assessment for humans****ED assessment for T-modality**

	<b>Sufficiently investigated</b>
<b>T-mediated parameters</b>	Yes T-mediated parameters have been investigated in several oral toxicity studies in the rat (28 and 90-days, 24-months and two generation studies), mouse (90-days and 18-month studies), and dog (90-days, 1-year studies).

Table 6.8.3-2. Lines of evidence for adverse effects and endocrine activity related to T-modality

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
13	In vitro mechanistic	CAR nuclear receptor (in vitro)	Transfected COS-1 cells *	24	hr		1	µM	Increase	rat, mouse, human CAR3: ↑2.8 fold, ↑24 fold, ↑1.5 fold; Dose-dependent increase; Pydiflumetofen is a direct activator of CAR from mouse, rat and human and has high efficacy (i.e. maximal fold change compared to model activators) in all three species.	Direct CAR activator in rat, mouse, human. Phase I enzyme induction in mouse and human; Hepatocellular proliferation in mice (no effect in humans). No effect on TPO activity.	Sufficient evidence for CAR-mediated hepatocellular proliferation only in rodents; No TPO activation.	T
14		Cellular proliferation	Human	96	Hours	Uptake from the medium (in vitro)	35	µM	No effect	No effect on human hepatocytes proliferation			
15		Cellular proliferation	Mouse	96	Hours	Uptake from the medium (in vitro)	35	µM	Increase	Increased mouse hepatocytes proliferation			
14		Phase I enzyme induction (in vitro)	Human	96	Hours	Uptake from the medium (in vitro)	5	µM	Increase	Induction of alkoxyresorufin-O-dealkylase (PROD and BROD) enzyme activity at all doses; Increase less marked at higher doses (25 and 35 µM) in the presence of cytotoxicity (decreased ATP)			
15		Phase I enzyme induction (in vitro)	Mouse	96	Hours	Uptake from the medium (in vitro)	5	µM	Increase	Induction of alkoxyresorufin-O-dealkylase (PROD and BROD) enzyme activity at 5 and 15 µM; Not significant decrease at higher doses (25 and 35 µM) in the presence of cytotoxicity (decreased ATP)			
17		Thyroperoxidase activity (TPO) (in vitro)	Rat	10	Minutes	Uptake from the medium (in vitro)		µM	No effect	No effect on TPO activity in rat			
16	In vivo mechanistic	Phase I enzyme induction (in vivo)	Mouse	28	Days	Oral	10	mg/kg bw/day	Increase	Induction of PROD activity (1.9, 1.6 and 2.4 fold increase after 2, 7 and 28 days respectively; Not statistically significant)	Phase I enzyme induction and hepatocellular proliferation in mouse; Phase II		
16		Proliferation	Mouse	28	Days	Oral	10	mg/kg bw/day	Increase	Induction of hepatocellular proliferation (3.1, 2.3 and 5.6			



Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
										fold increase after 2, 7 and 28 days respectively)	enzyme induction in rat		
16		Phase I enzyme induction (in vivo)	Mouse	28	Days	Oral	324	mg/kg bw/day	Increase	Induction of PROD activity (28, 36 and 37 fold increase after 2, 7 and 28 days respectively); Induction of total P450 (2 fold increase at all time-points)			
16		Proliferation	Mouse	28	Days	Oral	324	mg/kg bw/day	Increase	Induction of hepatocellular proliferation (14, 5 and 6 fold increase after 2, 7 and 28 days respectively)			
19		Phase I enzyme induction (in vivo)	Mouse	28	Days	Oral	75	mg/kg bw/day	Increase	Induction of PROD activity (11.8 and 5.2 fold increase in males and females respectively); Induction of total P450 (1.5 and 1.2 fold increase in males and females respectively)Effect observed at all doses; Pydiflumetofen did not demonstrate the prototypical properties of peroxisome proliferators but exhibited characteristics in common with "phenobarbital-like" inducing agents			
19		Phase I enzyme induction (in vivo)	Mouse	28	Days	Oral	75	mg/kg bw/day	Increase	Induction of BQ (marker of Cyp3a activity) 1.5 fold increase in males. Effect at females observed from 600 mg/kg bw/day Effect observed at all doses; Pydiflumetofen did not demonstrate the prototypical properties of peroxisome proliferators but exhibited characteristics in common with "phenobarbital-like" inducing agents			
18		Phase II enzyme induction (in vivo)	Rat	96	Days	Oral	18.6	mg/kg bw/day	Increase	Increased hepatic microsomal UDP glucuronosyltransferase activity towards thyroxine as substrate			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
1	EATS-mediated	Thyroid histopathology	Rat	28	Days	Oral	1174	mg/kg bw/day	No effect		Thyroid histopathology in 90-day rat study and in 2-generation rat study (in adults) in the presence of liver toxicity. No thyroid histopathology in 28-d and 2-year rat studies, in 90-d and 1-yr dog studies and in all short and long-term mouse studies.	No evidence for thyroid adversity.	
1		Thyroid histopathology	Rat	28	Days	Oral	1322	mg/kg bw/day	No effect				
2		Thyroid histopathology	Mouse	28	Days	Oral	1115	mg/kg bw/day	No effect				
2		Thyroid histopathology	Mouse	28	Days	Oral	1312	mg/kg bw/day	No effect				
3		Thyroid histopathology	Rat	90	Days	Oral	111	mg/kg bw/day	Change	Minimal thyroid follicular cell hypertrophy (4/10 animals)			
3		Thyroid histopathology	Rat	90	Days	Oral	587	mg/kg bw/day	Change	Minimal/mild thyroid follicular cell hypertrophy (6/10 animals)			
3		Thyroid histopathology	Rat	90	Days	Oral	727	mg/kg bw/day	Change	Minimal/mild thyroid follicular cell hypertrophy (4/10 animals)			
3		Thyroid histopathology	Rat	90	Days	Oral	1187	mg/kg bw/day	Change	Minimal/mild thyroid follicular cell hypertrophy (7/10 animals)			
3		Thyroid histopathology	Rat	90	Days	Oral	1324	mg/kg bw/day	Change	Minimal/mild thyroid follicular cell hypertrophy (8/10 animals)			
4		Thyroid histopathology	Mouse	90	Days	Oral	1158	mg/kg bw/day	No effect				
4		Thyroid histopathology	Mouse	90	Days	Oral	1482.6	mg/kg bw/day	No effect				
5		Thyroid histopathology	Dog	90	Days	Oral	1000	mg/kg bw/day	No effect				
6		Thyroid histopathology	Dog	1	Years	Oral	300	mg/kg bw/day	No effect				
8		Thyroid histopathology	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Change	NOAEL parental Minimal thyroid follicular hypertrophy (7/24 for F0 and F1 vs 1/24 and 2/24 in control animals for F0 and F1 respectively)			
11		Thyroid histopathology	Rat	104	Weeks	Oral		mg/kg bw/day	No effect	NOAEL carcinogenicity			
11		Thyroid histopathology	Rat	52	Weeks	Oral		mg/kg bw/day	No effect	NOAEL systemic			
12		Thyroid histopathology	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	No effect				

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
12		Thyroid histopathology	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	No effect		Increased thyroid weight in 90-day and 1-y dog studies at $\geq$ MTD ( $\uparrow$ ALP more than 200%, increased liver weight more than 30%)		
1		Thyroid weight	Rat	28	Days	Oral	1174	mg/kg bw/day	No effect				
1		Thyroid weight	Rat	28	Days	Oral	1322	mg/kg bw/day	No effect				
5		Thyroid weight	Dog	90	Days	Oral	1000	mg/kg bw/day	Increase	Absolute (14% for both males and females) and relative (11% and 22% for males and females respectively)			
6		Thyroid weight	Dog	1	Years	Oral	300	mg/kg bw/day	Increase	Relative 50% in males and females; very close to the upper limit of historical control range.			
11		Thyroid weight	Rat	104	Weeks	Oral		mg/kg bw/day	No effect	NOAEL carcinogenicity			
11		Thyroid weight	Rat	52	Weeks	Oral		mg/kg bw/day	No effect	NOAEL systemic			
12		Thyroid weight	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	No effect				
12		Thyroid weight	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	No effect				
10		Fetal development	Rabbit	22	Days	Oral		mg/kg bw/day	Increase	NOAEL developmental Increase of interrupted costal cartilage (variation) above historical control range	Skeletal variation		
8	Sensitive to, but not diagnostic of, EATS	Reproduction	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	NOAEL reproductive	No effect on reproduction		
8		Reproduction	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	NOAEL reproductive			
1	Target organ toxicity	Liver histopathology	Rat	28	Days	Oral	322	mg/kg bw/day	Change	Minimal centrilobular hepatocellular hypertrophy (4/6 animals)	Sufficient evidence of liver histopathology in rat, mouse and dog	Overall sufficient evidence for liver toxicity	Overall evidence of systemic toxicity
3		Liver histopathology	Rat	90	Days	Oral	111	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (5/10 animals)			
3		Liver histopathology	Rat	90	Days	Oral	587	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (7/10 animals)			
3		Liver histopathology	Rat	90	Days	Oral	727	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (6/10 animals)			
3		Liver histopathology	Rat	90	Days	Oral	1187	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (10/10 animals)			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
3		Liver histopathology	Rat	90	Days	Oral	1324	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (9/10 animals)			
4		Liver histopathology	Mouse	90	Days	Oral	81.6	mg/kg bw/day	Change	NOAEL males Mild centrolobular hepatocyte hypertrophy (2/10 animals)			
4		Liver histopathology	Mouse	90	Days	Oral	846	mg/kg bw/day	Change	NOAEL females Mild centrolobular hepatocyte hypertrophy (6/10 animals)			
5		Liver histopathology	Dog	90	Days	Oral	1000	mg/kg bw/day	Change	Minimal hepatocellular hypertrophy in all animals			
8		Liver histopathology	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Change	NOAEL parental Slight hepatocellular hypertrophy (19/24 for F0 and 18/24 for F1 vs 0/24 in control animals)			
8		Liver histopathology	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Change	NOAEL parental Minimal hepatocellular hypertrophy (8/24 for F0 vs 0/24 in control animals)			
11		Liver histopathology	Rat	104	Weeks	Oral	51	mg/kg bw/day	Change	↑ hepatocyte hypertrophy 3/52 minimal, at higher dose: ↑ hepatocyte hypertrophy 39/52 minimal to moderate; ↑ eosinophilic inclusions hepatocytes 19/52 NOAEL systemic			
11		Liver histopathology	Rat	104	Weeks	Oral	102	mg/kg bw/day	Change	↑ hepatocyte hypertrophy 3/52 minimal-mild NOAEL systemic			
11		Liver histopathology	Rat	52	Weeks	Oral	51	mg/kg bw/day	Change	Hepatocellular hypertrophy (5/12); at higher dose: Hepatocellular hypertrophy (11/12). Also prominent liver lobular architecture in 3/12 males NOAEL systemic			
11		Liver histopathology	Rat	52	Weeks	Oral	102	mg/kg bw/day	Change	Hepatocellular hypertrophy (4/10) NOAEL systemic			
12		Liver histopathology	Mouse	80	Weeks	Oral	45.4	mg/kg bw/day	Change	↑ 6/49 hepatocellular hypertrophy; at higher dose (287.9 mkd): ↑ 18/50 hepatocellular hypertrophy, ↑ 10/50 eosinophilic foci of cellular alteration NOAEL systemic			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality		
12		Liver histopathology	Mouse	80	Weeks	Oral	45.4	mg/kg bw/day	Change	8.2% increase in hepatocellular carcinoma; at higher dose: 20% increase in hepatocellular carcinoma NOAEL carcinogenicity		Sufficient evidence of increased liver weight in rat, mouse and dog			
12		Liver histopathology	Mouse	80	Weeks	Oral	45.4	mg/kg bw/day	Change	18.4% increase in hepatocellular adenoma; at higher dose: 44% increase in hepatocellular adenoma NOAEL carcinogenicity					
16		Liver histopathology	Mouse	28	Days	Oral	324	mg/kg bw/day	Change	Increased mitotic cells (7/10 animals after 2 days); Centrilobular hypertrophy in 9/10, 9/10 and 10/10 after 2, 7 and 28 days respectively)					
1		Liver weight	Rat	28	Days	Oral	322	mg/kg bw/day	Increase	Absolute and covariate (19% and 22% respectively)					
1		Liver weight	Rat	28	Days	Oral	343	mg/kg bw/day	Increase	Absolute and covariate (20% and 28% respectively)					
2		Liver weight	Mouse	28	Days	Oral	96	mg/kg bw/day	Increase	Absolute and covariate (14% and 28% respectively)					
2		Liver weight	Mouse	28	Days	Oral	76	mg/kg bw/day	Increase	Absolute and covariate (9% and 17% respectively)					
3		Liver weight	Rat	90	Days	Oral	111	mg/kg bw/day	Increase	Absolute and covariate (21% and 29% respectively)					
3		Liver weight	Rat	90	Days	Oral	127	mg/kg bw/day	Increase	Absolute and covariate (16% and 18% respectively)					
3		Liver weight	Rat	90	Days	Oral	587	mg/kg bw/day	Increase	Absolute and covariate (23% and 44% respectively)					
3		Liver weight	Rat	90	Days	Oral	727	mg/kg bw/day	Increase	Absolute and covariate (36% and 41% respectively)					
3		Liver weight	Rat	90	Days	Oral	1187	mg/kg bw/day	Increase	Absolute and covariate (26% and 52% respectively)					
3		Liver weight	Rat	90	Days	Oral	1324	mg/kg bw/day	Increase	Absolute and covariate (40% and 43% respectively)					
4		Liver weight	Mouse	90	Days	Oral	81.6	mg/kg bw/day	Increase	NOAEL males Absolute and covariate (18% and 15% respectively)					
4		Liver weight	Mouse	90	Days	Oral	846	mg/kg bw/day	Increase	NOAEL females Absolute and covariate (60% and 62% respectively)					
5		Liver weight	Dog	90	Days	Oral	300	mg/kg bw/day	Increase	Absolute in males (34%) and covariate in males and					

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
										females (31% and 16% respectively)			
5		Liver weight	Dog	90	Days	Oral	1000	mg/kg bw/day	Increase	Absolute (44% and 38% for males and females respectively) and covariate (41% and 46% for males and females respectively)			
6		Liver weight	Dog	1	Years	Oral	300	mg/kg bw/day	Increase	Covariate (34% and 28% respectively)			
8		Liver weight	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Increase	NOAEL parental Relative (38% and 42% for F0 and F1 respectively)			
8		Liver weight	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Increase	NOAEL parental Relative (15% and 19% for F0 and F1 respectively)			
11		Liver weight	Rat	104	Weeks	Oral	51	mg/kg bw/day	Increase	↑ covariate weight (12%), at higher dose: ↑ covariate weight (24%)NOAEL systemic			
11		Liver weight	Rat	104	Weeks	Oral	102	mg/kg bw/day	Increase	↑ covariate weight (10%)NOAEL systemic			
11		Liver weight	Rat	52	Weeks	Oral	51	mg/kg bw/day	Increase	increased relative liver weight (16%). At higher dose (319 mkd): increased relative liver weight (38%)NOAEL systemic			
11		Liver weight	Rat	52	Weeks	Oral	31	mg/kg bw/day	Increase	increased relative liver weight (9%). At higher dose (102 mkd): increased relative liver weight (19%)NOAEL systemic			
12		Liver weight	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	Increase	↑52.3% covariate values NOAEL systemic			
12		Liver weight	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	Increase	↑17.1% covariate values NOAEL systemic			
16		Liver weight	Mouse	28	Days	Oral	324	mg/kg bw/day	Increase	Increased absolute (24% and 28% after 7 and 28 days respectively) and relative (21% and 28% after 7 and 28 days respectively)			
2	Systemic toxicity	Body weight	Mouse	28	Days	Oral	76	mg/kg bw/day	Decrease	Decreased bw (8%) and bw gain (55%)	Dose related decreased	Consistent effects in	

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
3		Body weight	Rat	90	Days	Oral	587	mg/kg bw/day	Decrease	Decreased bw (12%) and bw gain (27%)	body weight in rat, mouse and dog	body weight, food consumption and clinical chemistry in all species tested.	
3		Body weight	Rat	90	Days	Oral	727	mg/kg bw/day	Decrease	Decreased bw gain (21%)			
3		Body weight	Rat	90	Days	Oral	1187	mg/kg bw/day	Decrease	Decreased bw (15%) and bw gain (34%)			
3		Body weight	Rat	90	Days	Oral	1324	mg/kg bw/day	Decrease	Decreased bw gain (25%)			
5		Body weight	Dog	90	Days	Oral	300	mg/kg bw/day	Decrease	Decreased bw (50%) from day-1 to day-91			
5		Body weight	Dog	90	Days	Oral	1000	mg/kg bw/day	Decrease	Decreased bw in 2/4 males and all females during first week; decreased overall bw (6.9%; not statistically significant) and bw gain (more than 100%) in females			
8		Body weight	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Decrease	NOAEL parental Decreased bw gain (10% at weeks 0-17 for both F0 and F1 generations)			
8		Body weight	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Decrease	NOAEL developmental Decrease bw (10%) in males and females during lactation			
9		Body weight	Rat	14	Days	Oral	100	mg/kg bw/day	Decrease	NOAEL maternal Initial reduction in BWG between first days of dosing (day 6 to day 10)			
11		Body weight	Rat	104	Weeks	Oral	51	mg/kg bw/day	Decrease	↓BW 10.8% and ↓ BW gain 13% at week 104, at higher dose (319 mkd); ↓BW 4.4% at week 4, 18.2% week 104NOAEL systemic			
11		Body weight	Rat	104	Weeks	Oral	31	mg/kg bw/day	Decrease	↓BW 7.6% week 104, at higher dose (102 mkd): ↓BW 9.1% and BW gain (↓13%) at week 104NOAEL systemic			
11		Body weight	Rat	52	Weeks	Oral	51	mg/kg bw/day	Decrease	↓BW 6% throughout the 52-week period, at higher dose (319 mkd): ↓BW 13.0%NOAEL systemic			
11		Body weight	Rat	52	Weeks	Oral	31	mg/kg bw/day	Decrease	↓BW 7% throughout the 52-week period, at higher dose			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
										(319 mkd): ↓BW 10.0%NOAEL systemic			
12		Body weight	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	Decrease	↓ 11.6%NOAEL systemic			
12		Body weight	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	Decrease	↓ 6.9%NOAEL systemic			
1		Clinical chemistry and haematology	Rat	28	Days	Oral	343	mg/kg bw/day	Decrease	Decreased glutamate dehydrogenase	Clinical chemistry indicative of liver toxicity		
3		Clinical chemistry and haematology	Rat	90	Days	Oral	111	mg/kg bw/day	Decrease	Decreased ALP (30%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	127	mg/kg bw/day	Decrease	Decreased ALP (41%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	587	mg/kg bw/day	Decrease	Decreased ALP (31%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	727	mg/kg bw/day	Change	Decreased ALP (39%) and increased cholesterol (35%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	1187	mg/kg bw/day	Decrease	Decreased ALP (39%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	1324	mg/kg bw/day	Change	Decreased ALP (39%) and increased cholesterol (35%)			
4		Clinical chemistry and haematology	Mouse	90	Days	Oral	846	mg/kg bw/day	Increase	NOAEL females Increased cholesterol (29%)			
5		Clinical chemistry and haematology	Dog	90	Days	Oral	300	mg/kg bw/day	Decrease	Increased ALP (268% and 204% for males and females respectively) and increased triglycerides in males (68%)			
5		Clinical chemistry and haematology	Dog	90	Days	Oral	1000	mg/kg bw/day	Increase	Increased ALP (460% and 321% for males and females respectively) and increased triglycerides (144% and 37% for males and females respectively)			
6		Clinical chemistry and haematology	Dog	1	Years	Oral	300	mg/kg bw/day	Increase	Increased ALP (282% and 211% for males and females respectively)			



Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality		
7		Clinical chemistry and haematology	Rat	28	Days	Dermal	1000	mg/kg bw/day	Increase	Males: Increased globulin (7%) and total protein (3%); Females: Increased calcium (4%), phospholipids (25%), total cholesterol (28%)		Decreased food consumption in rat, mouse and dog			
11		Clinical chemistry and haematology	Rat	52	Weeks	Oral	319	mg/kg bw/day	Change	statistically significantly higher gamma glutamyl transferase on all occasions (weeks 14, 27, 52) and statistically significantly decreased levels of triglycerides at week 52					
16		Clinical chemistry and haematology	Mouse	28	Days	Oral	324	mg/kg bw/day	Decrease	Decreased AST (40% after 28 days)					
1		Food consumption	Rat	28	Days	Oral	343	mg/kg bw/day	Decrease	<42% for the first 1 to 2 days of treatment					
3		Food consumption	Rat	90	Days	Oral	587	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation					
3		Food consumption	Rat	90	Days	Oral	727	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation					
3		Food consumption	Rat	90	Days	Oral	1187	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation					
3		Food consumption	Rat	90	Days	Oral	1324	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation					
5		Food consumption	Dog	90	Days	Oral	1000	mg/kg bw/day	Decrease	6% in males and 17% in females					
11		Food consumption	Rat	104	Weeks	Oral	31	mg/kg bw/day	Decrease	slightly lower and achieved statistical significance on several occasions NOAEL systemic					
11		Food consumption	Rat	104	Weeks	Oral	51	mg/kg bw/day	Decrease	consistently slightly lower and achieved statistical significance on several occasions NOAEL systemic					
11		Food consumption	Rat	52	Weeks	Oral	51	mg/kg bw/day	Decrease	reduced at times throughout study NOAEL systemic					
11		Food consumption	Rat	52	Weeks	Oral	31	mg/kg bw/day	Decrease	reduced at times throughout study NOAEL systemic					

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
12		Food consumption	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	Decrease	slightly lower and achieved statistical significance on several occasions NOAEL systemic			
12		Food consumption	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	Decrease	slightly lower and achieved statistical significance on several occasions NOAEL systemic			

\* COS-1 cells transfected with cDNA expression vectors for CAR3 variants of mouse, rat or human along with cofactors and a CYP2B6 response element-luciferase reporter construct

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**Assessment of the integrated line of evidence and weight of evidence for T-mediated adversity and endocrine activity**

A more detailed evaluation of the Lines of Evidence (LoE) for T-mediated adversity is summarized in Table 6.8.3-3 where all doses tested in the data-set in the different species and studies are tabulated in order to transparently report time and dose-concordance of the observed effect(s). The weight of evidence for T-mediated adversity and for T-mediated endocrine activity is summarized in Table 6.8.3-4 and Table 6.8.3-5, respectively.

**Table 6.8.3-3. Evaluation of dose and time concordance for T-mediated adversity**

mg/kg bw/day	Species	1 month (28-days)	3 month (90-days)	2-generation reproduction (29 weeks)	12 months (1-year)	18 months (80-weeks)	24 months (104-weeks)
102	rat				Thyroid NO EFFECT  <b>Liver EFFECT</b> (hepatocellular hypertrophy, ↑ wt)		Thyroid NO EFFECT in females  <b>Liver EFFECT</b> (hepatocellular hypertrophy, ↑ wt)
111	rat		<b>Thyroid EFFECT</b> (minimal follicular cell hypertrophy in males) <b>Liver EFFECT</b> (hepatocellular hypertrophy, ↑ wt)				
277	rat			<b>Thyroid EFFECT</b> (minimal follicular cell hypertrophy) <b>Liver EFFECT</b> (hepatocellular hypertrophy)			
319	rat						Thyroid NO EFFECT in males  <b>Liver EFFECT</b> (hepatocellular hypertrophy, ↑ wt)
288	mouse					Thyroid NO EFFECT  <b>Liver EFFECT</b> (hepatocellular hypertrophy, carcinomas)	
300	dog				<b>Thyroid EFFECT</b> (↑ weight)  <b>Liver EFFECT</b> (↑ weight) ≥ MTD		

mg/kg bw/day	Species	1 month (28-days)	3 month (90-days)	2-generation reproduction (29 weeks)	12 months (1-year)	18 months (80-weeks)	24 months (104-weeks)
1000	dog		Thyroid EFFECT (↑ weight)  Liver EFFECT (↑ weight) > MTD				
1115	mouse	Thyroid NO EFFECT  Liver EFFECT (↑ weight)					
1158	mouse		Thyroid NO EFFECT  Liver EFFECT (hepatocellular hypertrophy)				
1174	rat	Thyroid NO EFFECT  Liver EFFECT (hepatocellular hypertrophy)					

**Table 6.8.3-4. WoE for T-mediated adversity**

<ul style="list-style-type: none"> <li>Thyroid weight was examined in multiple studies of different lengths at different doses and in multiple species (rat, mouse, dog). However, increase in thyroid weight was observed only in one species (i.e. dog) in the 90-day and 1-year toxicity studies (Study ID Matrix: 5 and 6). This effect was not considered adverse and it was observed at doses equal to or exceeding the MTD <ul style="list-style-type: none"> <li>Based on &gt; 30 % increase in liver weight and &gt; 200% increase in ALP.</li> </ul> </li> </ul>
<ul style="list-style-type: none"> <li>Thyroid histopathology was also examined thoroughly, and effects (namely minimal thyroid follicular cell hypertrophy observed in a proportion of animals, see next bullet-point) were observed only in one species (i.e. rat) in the 90-day (Study ID Matrix: 3) and 2-generation reproductive toxicity study (Study ID Matrix: 8).</li> </ul>
<ul style="list-style-type: none"> <li>Thyroid changes were always concomitant to liver adverse effects: <ul style="list-style-type: none"> <li>In the 90-day rat study, minimal thyroid follicular cell hypertrophy (in 4/10 male animals) was observed from the dose of 111 mg/kg bw/day where liver toxicity was evidenced as minimal hepatocyte hypertrophy and &gt; 20% increase in liver weight (the liver effect was considered adverse).</li> <li>In the 2-generation reproduction study in the rat, minimal thyroid follicular cell hypertrophy (in 7/24 male animals, F0 and F1 vs 1/24 and 2/24 in control animals in F0 and F1 respectively) was observed from the dose of 276 mg/kg bw/day where liver toxicity was evidenced as slight hepatocyte hypertrophy and &gt; 20% increase in liver weight, in both generations, which was considered adverse.</li> </ul> </li> </ul>
<ul style="list-style-type: none"> <li>The lowest effective dose for thyroid histopathology was 111 mg/kg bw/day observed in males in the 90-day rat study, which may be considered the overall LOAEL for the thyroid effect.</li> </ul>
<ul style="list-style-type: none"> <li>There were no thyroid effects in the 2-year rat study at 319/102 mg/kg bw/day in males/females (top dose of the study) where there was evidence of liver toxicity (hepatocyte hypertrophy, &gt;10% liver weight increase). At the expert meeting considerations were made that this lack of effect at higher doses in a longer study likely indicate that a biological adaptation exists with this effect when considering differences in age and consequently different physiological responses.</li> </ul>
<ul style="list-style-type: none"> <li>There were no histological changes in the thyroid at higher doses in the mouse (90-day: &gt; 1000 mg/kg bw/day, 80-week: &gt; 300 mg/kg bw/day) and dog (90-day: &gt; 1000 mg/kg bw/day, 1-year dog: 300 mg/kg bw/day – top dose).</li> </ul>

**Table 6.8.3-5. WoE for T-mediated endocrine activity**

<ul style="list-style-type: none"> <li><i>In vitro</i> mechanistic studies indicate that pydiflumetofen is a direct CAR activator in rat, mouse and human and Phase I liver enzyme induction is observed in mouse and human.</li> </ul>
<ul style="list-style-type: none"> <li>There was no effect on TPO activity in the rat (<i>in vitro</i> data).</li> </ul>
<ul style="list-style-type: none"> <li><i>In vivo/ex vivo</i> mechanistic data confirm Phase I liver enzyme induction and hepatocellular proliferation in mouse and indicate Phase II liver enzyme induction in the rat.</li> </ul>
<ul style="list-style-type: none"> <li>No data is available on thyroid hormone measurements.</li> </ul>

Detailed evaluation of available evidences for T mediated adversity are indicative that a minimal thyroid effect was only observed in rat in a proportion of animals in two short term studies. In one of them, the

same histopathological change was also observed in few control animals, indicating that the treatment related effect was consistent with an increase in incidence over control of minimal thyroid follicular cell hypertrophy which was not associated with a treatment related increase in severity. In addition, the available dataset showed relevant inconsistencies as the thyroid was not a target organ in the same species at higher doses and longer duration of treatment. Consistency across species was also lacking as not adverse effect on thyroid histopathology was observed in mouse and dog, again at higher doses and longer durations of treatment. Where present, thyroid effects were only observed concomitantly to liver effects. Liver effects were however observed also in studies of longer durations in the absence of thyroid effects. This is indicative that hepatotoxicity is the leading toxicity, but that the mechanistic relationship between liver toxicity and thyroid effect is only circumstantial. Mechanistic studies indicate that the liver effects are consequent to CAR activation and phase I and II enzyme induction; this is possibly explaining the inconsistency of the thyroid effect in the most sensitive species as it is generally recognized that the rat has a low reserve of thyroid hormones and is consequently sensitive to an increase in clearance of THs. Furthermore, TPO activity was not affected in rat. Based on the available evidence, pydiflumetofen is not showing a consistent pattern indicative of T mediated adversity.

#### Initial analysis of the evidence and identification of relevant scenario for the ED assessment of T-modality

Table 6.8.3-6. Selection of relevant scenario (refer to page 13 of the ED Guidance)

Adversity based on T-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected based on the assessed lines of evidence (indicated with an 'x')
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is no <b>"T-mediated"</b> adversity	X
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no <b>T-mediated endocrine activity</b> observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing <b>"EATS-mediated"</b> parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

#### Conclusion on ED assessment for T modality

EFSA reviewed the available evidence for T-mediated adversity in several studies with different doses and durations in multiple species. T-mediated parameters were investigated in oral toxicity studies in

the rat (28 and 90-days, 24-months and two generation studies), mouse (90-days and 18-month studies), and dog (90-days, 1-year studies).

EFSA noted that effects on the thyroid in the available dataset were not consistent across species in repeat dose toxicity studies. Increased incidence of thyroid histopathology (follicular cell hypertrophy) was observed in only one species (rat) in two short term studies (90-day repeat dose and a two-generation reproductive toxicity study). These changes were not associated with a treatment-related increase in severity, and were always concomitant with liver adverse effects (hepatocyte hypertrophy and >20% increase in liver weight).

In the two-generation reproductive toxicity study, ECHA RAC (Risk Assessment Committee) noted that top dose males showed statistically significant increases in liver and thyroid weights with associated hypertrophy in the P and F1 generations. RAC concluded that the effects on the thyroid observed in males, although not severe, were indicative of an adequately high dose of pydiflumetofen in the two-generation study. However, RAC did not identify the thyroid as a primary target organ of toxicity based on data from short-term and long-term toxicity studies. HSE agreed with RAC on this point as shown in the [GB MCL Technical Report](#).

EFSA observed that a longer-term study (2 years) in the rat did not show any treatment-related effects on the thyroid, where there was evidence of liver toxicity (hepatocyte hypertrophy, >10% liver weight increase). EFSA considered that the lack of effect on the thyroid at higher doses and with longer duration of treatment was likely to indicate a biological adaptation.

In relation to endocrine activity, an in vitro assay demonstrated that pydiflumetofen did not inhibit thyroid peroxidase (TPO) in the rat. However, pydiflumetofen caused induction of phase I and phase II enzymes in rat liver.

EFSA noted that increases in thyroid weight were also observed in the dog in short-term (90-days) and long-term (1-year) repeat dose toxicity studies. However, this effect was only observed at doses equal to or greater than the maximum tolerated dose (MTD) and it was not considered relevant.

Based on the available dataset, EFSA and the EU peer-review process concluded that there was no evidence for thyroid adversity.

Overall, HSE agrees with the assessment of EFSA and the EU peer-review process that pydiflumetofen does not show a consistent pattern indicative of thyroid adversity across short-term, long-term, carcinogenicity and reproductive toxicity studies. As there was no clear pattern of adversity, no further investigations into T-mediated activity were warranted.

Based on the overall weight of evidence, pydiflumetofen does not cause T-mediated adversity and this modality has been sufficiently investigated.

### ED assessment for EAS-modalities

	Sufficiently investigated
EAS-mediated parameters	Yes New version of OECD TG 416 (updated protocol from 2001).



Table 6.8.3-7. Lines of evidence for adverse effects and endocrine activity related to EAS-modalities

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
8	EATS-mediated	Age at balanopreputial separation	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Increase	NOAEL developmental; Delayed preputial separation; PND 45.9 vs 43 at control (statistically significant) consistently in the presence of reduced b.w. In the DAR delay in sexual maturation was secondary to reduced BW gain (10%).	Delays in sexual maturation was associated to decreased BW. No other EAS-mediated adverse effects were observed.	Low evidence for EAS-mediated adversity.	EAS
8		Age at Vaginal opening	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Increase	NOAEL developmental; Increased time for vaginal opening (33 days vs 30.3 days in control) in F1 generation; Not considered secondary to decreased bw in the DAR. In PRAS 182, it was concluded that the effect was associated with a decrease in BW.			
8		Ano-Genital distance	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Cervix histopathology	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 116.2 mg/kg bw/day			
8		Coagulating gland histopathology	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Coagulating gland weight	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Epididymis histopathology	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Epididymis weight	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Prostate histopathology (with seminal vesicles and coagulating glands)	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Prostate weight	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
8		Seminal vesicles weight	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Sperm morphology	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Sperm motility	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Sperm numbers	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Testis histopathology	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 276.6 mg/kg bw/day			
8		Uterus histopathology (with cervix)	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 116.2 mg/kg bw/day			
8		Uterus weight (with cervix)	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 116.2 mg/kg bw/day			
8		Vagina histopathology	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 116.2 mg/kg bw/day			
8		Estrus cyclicity	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 116.2 mg/kg bw/day			
8		Vaginal smears	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	No effect up to 116.2 mg/kg bw/day			
10	Sensitive to, but not diagnostic of, EATS	Fetal development	Rabbit	22	Days	Oral		mg/kg bw/day	Increase	NOAEL developmental Increase of interrupted costal cartilage (variation) above historical control range	Skeletal variation		
8		Reproduction	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	NOAEL reproductive	No effect on reproduction		
8		Reproduction	Rat	29	Weeks	Oral		mg/kg bw/day	No effect	NOAEL reproductive			
1	Target organ toxicity	Liver histopathology	Rat	28	Days	Oral	322	mg/kg bw/day	Change	Minimal centrilobular hepatocellular hypertrophy (4/6 animals)	Sufficient evidence of liver histopathology in rat, mouse and dog	Overall sufficient evidence for liver toxicity	Overall evidence of systemic toxicity
3		Liver histopathology	Rat	90	Days	Oral	111	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (5/10 animals)			
3		Liver histopathology	Rat	90	Days	Oral	587	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (7/10 animals)			
3		Liver histopathology	Rat	90	Days	Oral	727	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (6/10 animals)			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
3		Liver histopathology	Rat	90	Days	Oral	1187	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (10/10 animals)			
3		Liver histopathology	Rat	90	Days	Oral	1324	mg/kg bw/day	Change	Minimal hepatocyte hypertrophy (9/10 animals)			
4		Liver histopathology	Mouse	90	Days	Oral	81.6	mg/kg bw/day	Change	NOAEL males Mild centrilobular hepatocyte hypertrophy (2/10 animals)			
4		Liver histopathology	Mouse	90	Days	Oral	846	mg/kg bw/day	Change	NOAEL females Mild centrilobular hepatocyte hypertrophy (6/10 animals)			
5		Liver histopathology	Dog	90	Days	Oral	1000	mg/kg bw/day	Change	Minimal hepatocellular hypertrophy in all animals			
8		Liver histopathology	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Change	NOAEL parental Slight hepatocellular hypertrophy (19/24 for F0 and 18/24 for F1 vs 0/24 in control animals)			
8		Liver histopathology	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Change	NOAEL parental Minimal hepatocellular hypertrophy (8/24 for F0 vs 0/24 in control animals)			
11		Liver histopathology	Rat	104	Weeks	Oral	51	mg/kg bw/day	Change	↑ hepatocyte hypertrophy 3/52 minimal, at higher dose: ↑ hepatocyte hypertrophy 39/52 minimal to moderate; ↑ eosinophilic inclusions hepatocytes 19/52 NOAEL systemic			
11		Liver histopathology	Rat	104	Weeks	Oral	102	mg/kg bw/day	Change	↑ hepatocyte hypertrophy 3/52 minimal-mild NOAEL systemic			
11		Liver histopathology	Rat	52	Weeks	Oral	51	mg/kg bw/day	Change	Hepatocellular hypertrophy (5/12); at higher dose: Hepatocellular hypertrophy (11/12). Also prominent liver lobular			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
										architecture in 3/12 males NOAEL systemic			
11		Liver histopathology	Rat	52	Weeks	Oral	102	mg/kg bw/day	Change	Hepatocellular hypertrophy (4/10) NOAEL systemic			
12		Liver histopathology	Mouse	80	Weeks	Oral	45.4	mg/kg bw/day	Change	↑6/49 hepatocellular hypertrophy; at higher dose (287.9 mkd): ↑ 18/50 hepatocellular hypertrophy, ↑ 10/50 eosinophilic foci of cellular alteration NOAEL systemic			
12		Liver histopathology	Mouse	80	Weeks	Oral	45.4	mg/kg bw/day	Change	8.2% increase in hepatocellular carcinoma; at higher dose: 20% increase in hepatocellular carcinoma NOAEL carcinogenicity			
12		Liver histopathology	Mouse	80	Weeks	Oral	45.4	mg/kg bw/day	Change	18.4% increase in hepatocellular adenoma; at higher dose: 44% increase in hepatocellular adenoma NOAEL carcinogenicity			
16		Liver histopathology	Mouse	28	Days	Oral	324	mg/kg bw/day	Change	Increased mitotic cells (7/10 animals after 2 days); Centrilobular hypertrophy in 9/10, 9/10 and 10/10 after 2, 7 and 28 days respectively)			
1		Liver weight	Rat	28	Days	Oral	322	mg/kg bw/day	Increase	Absolute and covariate (19% and 22% respectively)	Sufficient evidence of increased liver weight in rat, mouse and dog		
1		Liver weight	Rat	28	Days	Oral	343	mg/kg bw/day	Increase	Absolute and covariate (20% and 28% respectively)			
2		Liver weight	Mouse	28	Days	Oral	96	mg/kg bw/day	Increase	Absolute and covariate (14% and 28% respectively)			
2		Liver weight	Mouse	28	Days	Oral	76	mg/kg bw/day	Increase	Absolute and covariate (9% and 17% respectively)			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
3		Liver weight	Rat	90	Days	Oral	111	mg/kg bw/day	Increase	Absolute and covariate (21% and 29% respectively)			
3		Liver weight	Rat	90	Days	Oral	127	mg/kg bw/day	Increase	Absolute and covariate (16% and 18% respectively)			
3		Liver weight	Rat	90	Days	Oral	587	mg/kg bw/day	Increase	Absolute and covariate (23% and 44% respectively)			
3		Liver weight	Rat	90	Days	Oral	727	mg/kg bw/day	Increase	Absolute and covariate (36% and 41% respectively)			
3		Liver weight	Rat	90	Days	Oral	1187	mg/kg bw/day	Increase	Absolute and covariate (26% and 52% respectively)			
3		Liver weight	Rat	90	Days	Oral	1324	mg/kg bw/day	Increase	Absolute and covariate (40% and 43% respectively)			
4		Liver weight	Mouse	90	Days	Oral	81.6	mg/kg bw/day	Increase	NOAEL males Absolute and covariate (18% and 15% respectively)			
4		Liver weight	Mouse	90	Days	Oral	846	mg/kg bw/day	Increase	NOAEL females Absolute and covariate (60% and 62% respectively)			
5		Liver weight	Dog	90	Days	Oral	300	mg/kg bw/day	Increase	Absolute in males (34%) and covariate in males and females (31% and 16% respectively)			
5		Liver weight	Dog	90	Days	Oral	1000	mg/kg bw/day	Increase	Absolute (44% and 38% for males and females respectively) and covariate (41% and 46% for males and females respectively)			
6		Liver weight	Dog	1	Years	Oral	300	mg/kg bw/day	Increase	Covariate (34% and 28% respectively)			
8		Liver weight	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Increase	NOAEL parental Relative (38% and 42% for F0 and F1 respectively)			
8		Liver weight	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Increase	NOAEL parental Relative (15% and 19% for F0 and F1 respectively)			
11		Liver weight	Rat	104	Weeks	Oral	51	mg/kg bw/day	Increase	↑ covariate weight (12%), at higher dose: ↑ covariate			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
										weight (24%) NOAEL systemic			
11		Liver weight	Rat	104	Weeks	Oral	102	mg/kg bw/day	Increase	↑ covariate weight (10%) NOAEL systemic			
11		Liver weight	Rat	52	Weeks	Oral	51	mg/kg bw/day	Increase	increased relative liver weight (16%). At higher dose (319 mkd): increased relative liver weight (38%) NOAEL systemic			
11		Liver weight	Rat	52	Weeks	Oral	31	mg/kg bw/day	Increase	increased relative liver weight (9%). At higher dose (102 mkd): increased relative liver weight (19%) NOAEL systemic			
12		Liver weight	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	Increase	↑52.3% covariate values NOAEL systemic			
12		Liver weight	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	Increase	↑17.1% covariate values NOAEL systemic			
16		Liver weight	Mouse	28	Days	Oral	324	mg/kg bw/day	Increase	Increased absolute (24% and 28% after 7 and 28 days respectively) and relative (21% and 28% after 7 and 28 days respectively)			
2	Systemic toxicity	Body weight	Mouse	28	Days	Oral	76	mg/kg bw/day	Decrease	Decreased bw (8%) and bw gain (55%)	Dose related decreased body weight in rat, mouse and dog	Consistent effects in body weight, food consumption and clinical chemistry in all species tested.	
3		Body weight	Rat	90	Days	Oral	587	mg/kg bw/day	Decrease	Decreased bw (12%) and bw gain (27%)			
3		Body weight	Rat	90	Days	Oral	727	mg/kg bw/day	Decrease	Decreased bw gain (21%)			
3		Body weight	Rat	90	Days	Oral	1187	mg/kg bw/day	Decrease	Decreased bw (15%) and bw gain (34%)			
3		Body weight	Rat	90	Days	Oral	1324	mg/kg bw/day	Decrease	Decreased bw gain (25%)			
5		Body weight	Dog	90	Days	Oral	300	mg/kg bw/day	Decrease	Decreased bw (50%) from day-1 to day-91			
5		Body weight	Dog	90	Days	Oral	1000	mg/kg bw/day	Decrease	Decreased bw in 2/4 males and all females during first week; decreased overall bw (6.9%; not statistically significant) and bw gain (more than 100%) in females			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
8		Body weight	Rat	29	Weeks	Oral	276.6	mg/kg bw/day	Decrease	NOAEL parental. Decreased bw gain (10% at weeks 0-17 for both F0 and F1 generations)			
8		Body weight	Rat	29	Weeks	Oral	116.2	mg/kg bw/day	Decrease	NOAEL developmental. Decrease bw (10%) in males and females during lactation			
9		Body weight	Rat	14	Days	Oral	100	mg/kg bw/day	Decrease	NOAEL maternal. Initial reduction in BWG between first days of dosing (day 6 to day 10)			
11		Body weight	Rat	104	Weeks	Oral	51	mg/kg bw/day	Decrease	↓BW 10.8% and ↓ BW gain 13% at week 104, at higher dose (319 mkd): ↓BW 4.4% at week 4, 18.2% week 104 NOAEL systemic			
11		Body weight	Rat	104	Weeks	Oral	31	mg/kg bw/day	Decrease	↓BW 7.6% week 104, at higher dose (102 mkd): ↓BW 9.1% and BW gain (↓13%) at week 104 NOAEL systemic			
11		Body weight	Rat	52	Weeks	Oral	51	mg/kg bw/day	Decrease	↓BW 6% throughout the 52-week period, at higher dose (319 mkd): ↓BW 13.0% NOAEL systemic			
11		Body weight	Rat	52	Weeks	Oral	31	mg/kg bw/day	Decrease	↓BW 7% throughout the 52-week period, at higher dose (319 mkd): ↓BW 10.0% NOAEL systemic			
12		Body weight	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	Decrease	↓ 11.6% NOAEL systemic			
12		Body weight	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	Decrease	↓ 6.9% NOAEL systemic			
1		Clinical chemistry and haematology	Rat	28	Days	Oral	343	mg/kg bw/day	Decrease	Decreased glutamate dehydrogenase	Clinical chemistry indicative of liver toxicity		
3		Clinical chemistry and haematology	Rat	90	Days	Oral	111	mg/kg bw/day	Decrease	Decreased ALP (30%)			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
3		Clinical chemistry and haematology	Rat	90	Days	Oral	127	mg/kg bw/day	Decrease	Decreased ALP (41%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	587	mg/kg bw/day	Decrease	Decreased ALP (31%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	727	mg/kg bw/day	Change	Decreased ALP (39%) and increased cholesterol (35%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	1187	mg/kg bw/day	Decrease	Decreased ALP (39%)			
3		Clinical chemistry and haematology	Rat	90	Days	Oral	1324	mg/kg bw/day	Change	Decreased ALP (39%) and increased cholesterol (35%)			
4		Clinical chemistry and haematology	Mouse	90	Days	Oral	846	mg/kg bw/day	Increase	NOAEL females. Increased cholesterol (29%)			
5		Clinical chemistry and haematology	Dog	90	Days	Oral	300	mg/kg bw/day	Decrease	Increased ALP (268% and 204% for males and females respectively) and increased triglycerides in males (68%)			
5		Clinical chemistry and haematology	Dog	90	Days	Oral	1000	mg/kg bw/day	Increase	Increased ALP (460% and 321% for males and females respectively) and increased triglycerides (144% and 37% for males and females respectively)			
6		Clinical chemistry and haematology	Dog	1	Years	Oral	300	mg/kg bw/day	Increase	Increased ALP (282% and 211% for males and females respectively)			
7		Clinical chemistry and haematology	Rat	28	Days	Dermal	1000	mg/kg bw/day	Increase	Males: Increased globulin (7%) and total protein (3%); Females: Increased calcium (4%), phospholipids (25%), total cholesterol (28%)			
11		Clinical chemistry and haematology	Rat	52	Weeks	Oral	319	mg/kg bw/day	Change	statistically significantly higher gamma glutamyl transferase on all occasions (weeks 14, 27, 52) and statistically			



Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
										significantly decreased levels of triglycerides at week 52			
16		Clinical chemistry and haematology	Mouse	28	Days	Oral	324	mg/kg bw/day	Decrease	Decreased AST (40% after 28 days)			
1		Food consumption	Rat	28	Days	Oral	343	mg/kg bw/day	Decrease	<42% for the first 1 to 2 days of treatment	Decreased food consumption in rat, mouse and dog		
3		Food consumption	Rat	90	Days	Oral	587	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation			
3		Food consumption	Rat	90	Days	Oral	727	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation			
3		Food consumption	Rat	90	Days	Oral	1187	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation			
3		Food consumption	Rat	90	Days	Oral	1324	mg/kg bw/day	Decrease	Decreased food consumption (approximately 50%) and food utilisation			
5		Food consumption	Dog	90	Days	Oral	1000	mg/kg bw/day	Decrease	6% in males and 17% in females			
11		Food consumption	Rat	104	Weeks	Oral	31	mg/kg bw/day	Decrease	slightly lower and achieved statistical significance on several occasions NOEL systemic			
11		Food consumption	Rat	104	Weeks	Oral	51	mg/kg bw/day	Decrease	consistently slightly lower and achieved statistical significance on several occasions NOEL systemic			
11		Food consumption	Rat	52	Weeks	Oral	51	mg/kg bw/day	Decrease	reduced at times throughout study. NOEL systemic			
11		Food consumption	Rat	52	Weeks	Oral	31	mg/kg bw/day	Decrease	reduced at times throughout study. NOEL systemic			

Study ID Matrix	Effect classification	Effect target	Species	Duration of exposure	Duration unit	Route of administration	Lowest Effect dose	Dose unit	Effect direction	Observed effect (positive and negative)	Assessment of each line of evidence	Assessment on the integrated line of evidence	Modality
12		Food consumption	Mouse	80	Weeks	Oral	306.2	mg/kg bw/day	Decrease	slightly lower and achieved statistical significance on several occasions. NOAEL systemic			
12		Food consumption	Mouse	80	Weeks	Oral	287.9	mg/kg bw/day	Decrease	slightly lower and achieved statistical significance on several occasions. NOAEL systemic			

### Assessment of the integrated line of evidence and weight of evidence for EAS-mediated adversity and endocrine activity

The weight of evidence for EAS-mediated adversity is summarized in Table 6.8.3-8.

**Table 6.8.3-8 WoE for EAS-mediated adversity**

<ul style="list-style-type: none"> <li>There was no evidence for EAS mediated adversity in several EAS-specific parameters measured in the 2-generation reproductive toxicity study in the rat, i.e. Ano-Genital distance, Cervix histopathology, Coagulating gland histopathology, Coagulating gland weight, Epididymis histopathology, Epididymis weight, Prostate histopathology (with seminal vesicles and coagulating glands), Prostate weight, Seminal vesicles weight, Sperm morphology, Sperm motility, Sperm numbers, Testis histopathology, Uterus histopathology (with cervix), Uterus weight (with cervix), Vagina histopathology, Estrus cyclicity, Vaginal smears. Moreover, no effects in reproduction were observed.</li> </ul>
<ul style="list-style-type: none"> <li>Delay in female sexual maturation evidenced as increased age at vaginal opening was observed at the top dose of 116.2 mg/kg bw/day and it was considered associated to decreased body weight (10% during lactation).</li> </ul>
<ul style="list-style-type: none"> <li>Delay in male sexual maturation evidenced as increased age at balanopreputial separation was observed at the top dose of 276.6 mg/kg bw/day and it was considered associated to decreased body weight (10% during lactation).</li> </ul>
<ul style="list-style-type: none"> <li>There was no delay in sexual maturation in males and females at lower doses (i.e. 46.1 and 36.1 mg/kg bw/day, in males and females, respectively) where body weight was not affected.</li> </ul>

### Initial analysis of the evidence and identification of relevant scenario for the ED assessment of EAS-modalities

**Table 6.8.3-9. Selection of relevant scenario (refer to page 13 of the ED Guidance)**

Adversity based on EAS-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected based on the assessed lines of evidence (indicated with an 'x')
No (sufficiently investigated)	Yes/No	1a	Conclude: ED criteria not met because there is no “ <b>EAS-mediated</b> ” adversity	<b>X</b> (endocrine activity not assessed since EAS-mediated adversity is negative)
Yes (sufficiently investigated)	Yes/No	1b	Perform MoA analysis	
No (not sufficiently investigated)	Yes	2a (i)	Perform MoA analysis (additional information may be needed for the analysis)	
No (not sufficiently investigated)	No (sufficiently investigated)	2a (ii)	Conclude: ED criteria not met because no <b>EAS-mediated endocrine activity</b> observed	
No (not sufficiently investigated)	No (not sufficiently investigated)	2a (iii)	Generate missing level 2 and 3 information. Alternatively, generate missing “EATS-	

Adversity based on EAS-mediated parameters	Positive mechanistic OECD CF level 2/3 Test	Scenario	Next step of the assessment	Scenario selected based on the assessed lines of evidence (indicated with an 'x')
			mediated" parameters. Depending on the outcome move to corresponding scenario	
Yes (not sufficiently investigated)	Yes/No	2b	Perform MoA analysis	

#### Overall assessment for EAS-modalities

HSE agrees with EFSA and the EU peer-review process that EAS-mediated parameters have been sufficiently investigated as a modern, GLP and OECD test guideline compliant rat two-generation reproductive toxicity study (OECD TG No. 416; test protocol according to latest version of January 2001) is available.

Delays in sexual maturation in both sexes of the F1 generation were observed at the top dose in the rat 2-generation reproductive toxicity study. Pup body weights were statistically significantly reduced at the top dose compared with controls in both sexes of the F1 generation during lactation. EFSA concluded that the delay in sexual maturation observed in both sexes of the F1 generation at the top dose was associated with decreased pup body weight (EFSA, 2019).

In contrast to the conclusions of EFSA, RAC deemed that the decrease in pup body weight during lactation was unconvincing, and that the delay in sexual maturation could not be considered as the secondary unspecific consequence of the reduced pup body weight development. RAC concluded that the delay in puberty onset seen in both sexes of the F1 generation was clear (ie statistically significant and outside time-relevant HCD) and specific (ie independent of reductions in pup body weight development), despite the absence of endocrine effects and effects on other developmental landmarks, ano-genital distance and other reproductive parameters and organs. On this basis RAC classified pydiflumetofen with Repr 2; H361f.

Based on the available data, HSE agreed with RAC (see [GB MCL TR](#)) that a relation to treatment of the statistically significant delay in sexual maturation observed in both sexes of the F1 generation at the top dose could not be excluded. HSE noted that pup body weights were statistically significantly and consistently reduced at the top dose compared with controls in both sexes of the F1 generation from day 7 to day 21 of lactation (by 10-12%). However, the reduction was within the range of extended (2008-2014) HCD and, more importantly, was not replicated in the F2 generation. Therefore, HSE agreed with RAC that the apparent decrease in pup body weight of the F1 generation was most likely a spurious effect. HSE also noted that there were no functional consequences of the delay in sexual maturation in the F1 generation (ie mating performance and fertility were unaffected).

Overall, HSE agrees with the assessment of EFSA and the EU peer-review process that in the absence of other EAS-mediated endocrine effects, changes in other developmental landmarks, ano-genital distance and other reproductive parameters and organs, the delay in sexual maturation in F1 generation pups alone is not sufficient evidence to support a direct effect of the test substance on the endocrine system. As there was no clear pattern of EAS-mediated adversity and no other effects on reproductive organs in either repeat-dose toxicity or two-generation reproductive toxicity studies, no further investigations into EAS-mediated activity were warranted.

Based on the overall weight of evidence, pydiflumetofen does not cause EAS-mediated adversity. The ED criteria are not met because there is no “**EAS-mediated**” adversity. In addition, this modality has been sufficiently investigated.

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**Overall conclusion on the ED assessment for humans**

Pydiflumetofen does not show a consistent pattern indicative of thyroid adversity across short-term and long-term studies in multiple species (rat, mouse and dog) indicative of T-mediated adversity. Thyroid effects were only observed concomitantly to liver effects. As there was insufficient evidence of thyroid-mediated adverse effects, HSE concludes that pydiflumetofen does not cause T-mediated adversity and this modality has been sufficiently investigated.

Pydiflumetofen did not cause specific effects on endocrine or reproductive organs in either repeat-dose toxicity or two-generation reproductive toxicity studies. In the two-generation study in rats, both sexes of the F1 generation showed a delay in sexual maturation. However, there were no functional consequences of this delay (ie mating performance and fertility were unaffected), and no changes were observed in other developmental landmarks and reproductive parameters. HSE agrees with the assessment of EFSA and the EU peer-review process that in the absence of other endocrine effects, changes in other developmental landmarks, ano-genital distance and other reproductive parameters and organs, the delay in sexual maturation in F1 pups alone is not sufficient evidence to support a direct effect of the test substance on the endocrine system. HSE concludes that pydiflumetofen does not cause a pattern of EAS-mediated adversity and this modality has been sufficiently investigated.

Overall, HSE agrees with the conclusion of EFSA that based on the available evidence, the EATS modalities are considered sufficiently investigated and pydiflumetofen does not cause endocrine-mediated adverse effects. HSE therefore concludes that in accordance with point 3.6.5 of Annex II to Regulation 1107/2009, as amended by Regulation 2018/605, pydiflumetofen is not an endocrine disruptor in humans.

**B.6.9. MEDICAL DATA AND INFORMATION****B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies**

SYN545974 is a succinate dehydrogenase inhibitor (SDHI) fungicide. It has only been manufactured at pre-production scale at a Syngenta plant at Münchwilen, Switzerland since 2011. Formulation activities have taken place at Syngenta R&D sites at Jealott's Hill, UK, Greensboro, US and Münchwilen, Switzerland. Field trials have also taken place globally including EU, South-Africa, Australia, New Zealand, China, Japan, Korea, Taiwan, USA, Canada, Argentina, Chile, Brazil, Mexico. The key products include suspension concentrate (SC) and emulsifiable concentrate (EC) formulations.

The Occupational Health group of Syngenta has maintained a data base of incidents involving chemical exposure of workers since 1983. From 1994 data has been collected from all our manufacturing, formulation and packing sites around the world. A query of the Syngenta internal database in June 2015 for SYN545974 produced zero records of adverse health effects reported during AI manufacture, subsequent formulation and field trials.

**B.6.9.2. Data collected on humans**

As SYN545974 is still under development, no commercial sales have been made.

In accordance with Regulation (EU) 283/2013, validated analytical methods need to be provided for body fluids (blood and urine) in order to support the risk assessment for humans. Analytical methods for body fluids (blood and milk) have been assessed and validated (see DAR vol. 3 B.5.1.2). No method for the analysis SYN545974 and 2,4,6-trichlorophenol residues in urine is available.

**B.6.9.3. Direct observation**

SYN545974 has not yet been introduced into the market. There is no information on record.

**B.6.9.4. Epidemiological studies**

No epidemiological studies have been conducted with SYN545974.

**B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test****Specific signs of poisoning:**

SYN545974 is of low acute toxicity. Intoxication is only likely if large quantities are ingested. In animal studies, minor clinical signs of toxicity were evident at 5000 mg/kg bw. The same would be expected to occur in humans if similar dose levels were consumed. However, no cases of intoxication with SYN545974 have yet been observed.

**Clinical tests:**

No specific monitoring programs have been performed in humans.

**B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment**

**General advice:** Have the product container, label or Material Safety Data Sheet with you when calling the Syngenta emergency number, a poison control center or physician, or going for treatment.

**Inhalation:** Move the victim to fresh air. If breathing is irregular or stopped, administer artificial respiration. Keep patient warm and at rest. Call a physician or poison control centre immediately.

**Skin contact:** Take off all contaminated clothing immediately. Wash off immediately with plenty of water. If skin irritation persists, call a physician. Wash contaminated clothing before re-use.

**Eye contact:** Rinse immediately with plenty of water, also under the eyelids, for at least 15 minutes. Remove contact lenses. Immediate medical attention is required.

**Ingestion:** If swallowed, seek medical advice immediately and show this container or label. Do NOT induce vomiting.

**B.6.10. REFERENCES RELIED ON****REVIEW OF SCIENTIFIC OPEN LITERATURE**

A summary of the methodology employed regarding literature review for SYN545974 and metabolites/impurities is given below:

1. A very broad search was conducted in 18 scientific source databases for SYN545974 and metabolites using the search terms listed. This was performed for a period of 10 years up to 2015, when the dossier was first submitted to the EU.

2. Duplicates titles from between the data bases were automatically removed from the output.
3. A rapid assessment of the titles was conducted to remove any additional duplicates and any obviously irrelevant titles (where enough information was available from the title alone).
4. A further rapid assessment was conducted using summary abstracts and any clearly irrelevant titles were removed.
5. A detailed assessment of the full-text documents for the remaining titles was conducted using the criteria developed for study relevance
6. Any relevant papers were highlighted and assessed for reliability.

The literature review was carried out for pydiflumetofen (Adepidyn™) and its impurities and metabolites according to the requirements of Article 8(5) of Regulation (EC) No. 1107/2009 and in accordance with the EFSA Guidance document (EFSA Journal 2011; 9(2):2092). The list of criteria for relevance for each data requirement are listed in Table 6.10-1 and 6.10-2 (equivalent to Table 1 in the guidance).

**Table 6.10-1: List of Criteria for relevance for toxicological and toxicokinetic studies**

<b>Data requirements(s) (indicated by the correspondent CA data point (s))</b>	<b>Criteria for relevance</b>
*CA 6.1 ADME studies	<ol style="list-style-type: none"> <li>1. Well identified test material including purity and impurity profile</li> <li>2. Relevant test species e.g. rodent – rat/mouse – non-rodent – dog</li> <li>3. Relevant endpoint e.g. ADME measurement or metabolite identification</li> <li>4. Well described condition of the test and quantitative assessment of results to substantiate and evaluate whether the study conclusions and endpoints are robust</li> </ol>
*CA 6.2 Acute toxicity	<ol style="list-style-type: none"> <li>1. Well identified test material including purity and impurity profile</li> <li>2. Test species likely to be relevant to mammalian toxicology assessment – rats and mice, rabbit, guinea pig</li> <li>3. Relevant route of administration for risk assessment</li> <li>4. Describe observations, examinations, analyses performed or necropsy</li> <li>5. Different outcome to those studies currently reported</li> </ol>
*CA 6.4 Genotoxicity	<ol style="list-style-type: none"> <li>1. Well identified test material including purity and impurity profile</li> <li>2. Relevant cell line or species used</li> <li>3. “validated” or widely used test method</li> <li>4. In vitro observation not addressed by in vivo data (including tissue specific effects)</li> <li>5. In vivo effect in somatic or germs cells in relevant species</li> <li>6. Relevant route of exposure to test substance</li> <li>7. Contradicts submitted studies, impacts WoE.</li> <li>8. Recognised methods for scoring studies outcomes used where applicable</li> </ol>
*CA 6.3, 6.5, 6.6, 6.7, 6.8.1 Short term, chronic, reproductive and neurotoxicity, studies on metabolites	<ol style="list-style-type: none"> <li>1. Well identified test material including purity and impurity profile</li> <li>2. Test species likely to be relevant to mammalian toxicology assessment – rodents rats and mice, non- rodent dog is preferred</li> <li>3. Sufficient number of animals per group to establish statistical significance</li> <li>4. Test several dose levels (minimum 3)</li> <li>5. Relevant route of administration for risk assessment</li> <li>6. Include negative control (preferable)</li> <li>7. Establish dose response</li> <li>8. Describe observations, examinations, analyses performed or necropsy</li> <li>9. Contradicts submitted studies and/or changes key endpoints</li> </ol>
CA 6.8.2 Supplementary studies on the active substance	<ol style="list-style-type: none"> <li>1. Identified test material</li> <li>2. Unusual routes of exposure acceptable as they may introduce important information on other possible toxicological effects</li> <li>3. Regulatory use usually limited to addressing species sensitivity /safety factors etc.</li> <li>4. Examples of studies <ol style="list-style-type: none"> <li>a. Effects of combined exposures</li> <li>b. Hormonal effects (if not guideline studies or included in 5.8.3)</li> <li>c. Hypersensitivity of specific sub-populations</li> <li>d. Gender and age variation in susceptibility (if not included in 5.6 Reproductive studies)</li> <li>e. Mode of action investigations</li> </ol> </li> </ol>

Data requirements(s) (indicated by the correspondent CA data point (s))	Criteria for relevance
CA 6.8.3 Endocrine disrupting properties	<ol style="list-style-type: none"> <li>1. Identified test material</li> <li>2. All studies considered relevant at this stage – need to be checked for reliability</li> </ol>
CA 6.9 Medical data (including epidemiology) CP 6.2 to 6.4	<ol style="list-style-type: none"> <li>1. Identified test material</li> <li>2. All records considered relevant at this stage - need to be checked for reliability</li> </ol>

\* Recommended protocols under each data point include but are not limited to those listed in the Commission Communications 2013/C 95/01 and 2013/C 95/02

**Table 6.10-2: List of Criteria for relevance for operator exposure information/studies**

Data requirements(s) (indicated by the correspondent CP data point (s))	Criteria for relevance
General criteria CP 7.2 all sections	<ol style="list-style-type: none"> <li>5. Sufficient replicates must be included in the study to demonstrate statistical robustness</li> <li>6. Agronomic practices must be relevant to scenario in submission, including: crop type ,application method and parameters (e.g. boom height), application rate</li> <li>7. Leaf type and plant growth stage must be relevant to scenario in submission</li> <li>8. Climactic/meteorological conditions of study must be relevant to scenario in submission, including rainfall, wind speed and temperature</li> <li>9. Raw data must be available for analysis</li> <li>10. Statistical analysis must be robust and relevant</li> <li>11. Assessment of outliers/extreme values must be robust and relevant</li> </ol>
Operator/worker exposure studies CP 7.2.1.2 and CP 7.2.3.2	<ol style="list-style-type: none"> <li>1. Studies should follow accepted OECD protocol</li> <li>2. Studies performed to GLP are preferred</li> <li>3. Replicates should be minimum of 10</li> </ol>
Biomonitoring studies CP 7.2.1.2, CP 7.2.2.2 and CP 7.2.3.2	<ol style="list-style-type: none"> <li>1. Internal exposures must be clearly related to specific external doses</li> <li>2. Replicates should be minimum of 10</li> </ol>
Air monitoring studies CP 7.2.2.2	<ol style="list-style-type: none"> <li>10. Monitoring parameters must be relevant to bystander/resident exposures, including monitoring distance, height and duration:</li> <li>11. Accurate logs of relevant local activity must be available (e.g. crop spraying)</li> <li>12. Accurate logs of local climactic/meteorological conditions must be available for the duration of the monitoring period, including rainfall, wind speed, wind direction, temperature and humidity</li> </ol>
Dislodgeable foliar residue studies CP 7.2.3.2	<ol style="list-style-type: none"> <li>1. Study must have been conducted on a similar formulation</li> <li>2. Application number and interval must be relevant</li> <li>3. Replicates must be minimum of 40</li> </ol>
Foliar decline studies CP 7.2.3.2	<ol style="list-style-type: none"> <li>3. Data must demonstrate minimum of two clear half lives</li> <li>4. Sufficient data points must be provided to demonstrate decline curves between repeat applications</li> <li>5. Studies with significant rainfall in first 48 hours should be discounted</li> <li>6. Replicates must be minimum of 10</li> </ol>

\* Recommended protocols under each data point include but are not limited to those listed in the Commission Communications 2013/C 95/01 and 2013/C 95/02

Any documents deemed relevant will be checked for reliability according to the criteria described by Klimisch *et al* (1997)<sup>17</sup> using the ToxRTool ([http://ihcp.jrc.ec.europa.eu/our\\_labs/eurl-ecvam/archivepublications/toxrtool](http://ihcp.jrc.ec.europa.eu/our_labs/eurl-ecvam/archivepublications/toxrtool)).

The results are summarised in Table 6.10-3 as specified in the Guidance (Table 3). Out of a total of 4091 records 56 were identified for further discussion in DAR vol. 3 B.6.8, all of them related to specific metabolites of pydiflumetofen.

<sup>17</sup> Klimisch H-J, Andreae M and Tillmann U (1997) A Systematic Approach for Evaluating the Quality of Experimental Toxicological and Ecotoxicological Data. Reg Tox Pharmacol 25, 1-5



**Table 6.10-3: Results of study selection process**

<b>Data requirement(s) captured in the search</b>	<b>Number SYN545974 (Initial Search)</b>	<b>Number SYN545974 (Top-Up Search)</b>	<b>Number Specific Metabolite Search</b>	<b>Number Common SDHI Metabolites Search</b>	<b>Total</b>
Total number of summary records retrieved after all* searches of peer-reviewed literature (excluding duplicates)	1	17	4072	1	4091
Number of summary records excluded from the search results after rapid assessment for relevance**	1	17	3836	1	3854
Total number of full-text documents assessed in Detail*	0	0	236	0	236
Number of studies excluded from further consideration after detailed assessment for relevance	0	0	180	0	180
Number of studies not excluded for relevance after detailed assessment (i.e. relevant studies and studies of unclear relevance)	0	0	56	0	56

\*both from bibliographic databases and other sources of peer-reviewed literature

\*\* aligned with EFSA Journal 2011; 9(2) 2092: rapid assessment means exclusion of “obviously irrelevant records” based on titles.

No external research has been published on the parent molecule SYN545974 or the common SDHI metabolites (NOA449410 and SYN508272) found as metabolites in livestock. The literature review showed no relevant publications for all the specified impurities. The SYN545974 specific metabolite search which returned many thousands of hits did not contain any of the SYN545974 metabolites, with the exception of 2,4,6-trichlorophenol (2,4,6-TCP) (a metabolite of SYN545974 identified in rats, mice and livestock commodities). However, rather many of the hits were for the common class of chemistry e.g. trichlorophenols and the research often included trichlorophenols other than 2,4,6-TCP. Only trichlorophenol research specifically on 2,4,6-TCP was considered potentially relevant for this submission and other trichlorophenol data was not assessed. Following exclusion of references from the rapid assessment, the full text was assessed from the remaining 236 titles which were identified as potentially relevant or unclear on the basis of their titles and/or abstracts identified 59 of the studies as potentially relevant for this submission of SYN545974.

The following paper was not identified in this literature search, but it was included as it is an important and reliable publication:

Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The Comet Assay with Multiple Mouse Organs: Comparison of Comet Assay Results and Carcinogenicity with 208 Chemicals Selected from the IARC Monographs and U.S. NTP Carcinogenicity Database"Critical Reviews in Toxicology. (2000 Nov) 30:629-799. Journal code: CRT. ISSN: 1040-8444.

Details of why each paper has been excluded for relevance or reliability are given in Table 6.10-4.

Table 6.10-4: List of references excluded following detailed review listed by data point number

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
<b>2,4,6-TCP Initial search</b>					
CA 5.8.1	Abe A; Urano K	1994	Influence of chemicals commonly found in a water environment on the Salmonella mutagenicity test.	Science of the Total Environment, (1994 Aug 15) 153 (1-2) 169-75. Journal code: UJ0. ISSN: 0048-9697.	2,4,6-TCP is tested as part of a mix. The effect cannot be attributed to 2,4,6-TCP exclusively.
CA 5.8.1	Ahn, Joo-Myung; Hwang, Ee Taek; Youn, Chul-Hee; Banu, Danusia L.; Kim, Byoung Chan; Niazi, Javed H.; Gu, Man Bock	2009	Prediction and classification of the modes of genotoxic actions using bacterial biosensors specific for DNA damages	Biosensors & Bioelectronics, (2009) Vol. 25, No. 4, pp. 767-772. CODEN: BBIOE4. ISSN: 0956-5663.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Ahlborg U G; Thunberg T M	1980	Chlorinated phenols: occurrence, toxicity, metabolism, and environmental impact.	Critical reviews in toxicology, (1980 Jul) Vol. 7, No. 1, pp. 1-35. Ref: 214 Journal code: 8914275. ISSN: 1040-8444. L-ISSN: 1040-8444. Report No.: NASA-81002918.	Review. 2,46-TCP is not discussed.
CA 5.8.1	Ahotupa, M; Hietanen, E; Nienstedt, W	1981	Drug-metabolizing enzyme activities in rats exposed to chlorinated phenols	Adv. Physiol. Sci., Proc. Int. Congr., 28th, (1981) Vol. 29, No. Gastrointest. Def. Mech., pp. 421-31. CODEN: 45TGAW.	Conference abstract. No access to study details and data.
CA 5.8.1	Alexandersson R; Hedenstierna G	1982	Pulmonary function after long-term exposure to trichlorophenol.	International archives of occupational and environmental health, (1982 Feb) Vol. 49, No. 3-4, pp. 275-80. Journal code: 7512134. ISSN: 0340-0131. L-ISSN: 0340-0131.	It is not specified which trichlorophenol the workers examined were exposed to, the effects described cannot specifically be attributed to 2,4,6-TCP exposure
CA 5.8.1	Angerer, J.	2001	Chlorophenols (2,4-dichlorophenol, 2,5-dichlorophenol, 2,6-dichlorophenol, 2,3,4-trichlorophenol, 2,4,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol)	Analyses of Hazardous Substances in Biological Materials, (2001) Vol. 7, pp. 143-169. CODEN: AHSMEB. ISSN: 0179-7247.	Development of gas chromatographic method to detect chlorophenols. Not relevant to assess toxicity.
CA 5.8.1	Anonymous	1979	2,4,5- and 2,4,6-trichlorophenols.	IARC monographs on the evaluation of the carcinogenic risk of chemicals to humans, (1979 Oct) Vol. 20, pp. 349-67. Journal code: 7902489. ISSN: 0250-9555. L-ISSN: 0250-9555.	Review. The IARC monograph is superseded by a more recent monograph on Polychlorophenols which includes 2,4,6-TCP. No new data presented.
CA 5.8.1	Anonymous	1980	Annual research report 1974 and 1975.	Annual research report 1974 and 1975. (1980), 141 p.	2,4,6-TCP was not included in this review.
CA 5.8.1	Anonymous	1981	81st Annual report, 1981.	81st Annual report, 1981. (1981), 74 p.	2,4,6-TCP was not included in this review.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Anonymous	1981 (a)	Information Profiles on Potential Occupational Hazards: Chlorophenols	19810800	Review. ATSDR CDC risk evaluation is superseded by a more recent risk evaluation in 2,4,6-TCP, which was included.
CA 5.8.1	Anonymous	1984	Toxicology card. 196. 2,4,5-Trichlorophenol and 2,4,6-trichlorophenol	Cahiers de Notes Documentaires, (1984) Vol. 114, pp. 131-4. CODEN: CNDIBJ. ISSN: 0007-9952.	Safety evaluation by the French Government. Other relevant safety evaluations were already considered in this literature review.
CA 5.8.1	Anonymous	1987	Chlorophenols (Group 2B)	IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans, (1987) Suppl. 7 154-6.	IARC safety evaluation. A more recent IARC safety on Polychlorophenols was already considered in this review.
CA 5.8.1	Anonymous	2002	2,4,6-Trichlorophenol.	Report on carcinogens : carcinogen profiles / U.S. Dept. of Health and Human Services, Public Health Service, National Toxicology Program, (2002) Vol. 10, pp. 247-8. Journal code: 101157309. ISSN: 1551-8272. L-ISSN: 1551-8272.	Review. Evaluation of potential exposure risk. No new data presented.
CA 5.8.1	Anonymous	2004	2,4,6-Trichlorophenol.	Report on carcinogens : carcinogen profiles / U.S. Dept. of Health and Human Services, Public Health Service, National Toxicology Program, (2004) Vol. 11, pp. III263-4. Journal code: 101157309. ISSN: 1551-8272. L-ISSN: 1551-8272.	Review. Evaluation of potential exposure risk. No new data presented.
CA 5.8.1	Anonymous	2011	2,4,6-Trichlorophenol.	Report on carcinogens : carcinogen profiles / U.S. Dept. of Health and Human Services, Public Health Service, National Toxicology Program, (2011) Vol. 12, pp. 424-6. Journal code: 101157309. E-ISSN: 1551-8280. L-ISSN: 1551-8272.	Review. Evaluation of potential exposure risk. No new data presented.
CA 5.8.1	Aschmann C; Stork T; Wassermann O	1989	Short-Term Effects of Chlorophenols on the Function and Viability of Primary Cultured Rat Hepatocytes.	Arch.Toxicol. (63, No. 2, 121-26, 1989) 4 Fig. 2 Tab. 31 Ref. (RW) CODEN: ARTODN	2,4,6-TCP was not tested in this study.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Ashby, J; Tennant, R W.	1988	Chemical structure, Salmonella mutagenicity and extent of carcinogenicity as indicators of genotoxic carcinogenesis among 222 chemicals tested in rodents by the U.S. NCI/NTP	Mutation Research, Genetic Toxicology Testing, (1988) Vol. 204, No. 1, pp. 17-115. CODEN: MRGTE4. ISSN: 0165-1218.	Evaluation of correlation between chemical structure and extent of carcinogenicity as indicators of genotoxic carcinogenesis. 2,4,6-TCP is not discussed in detail. No new data presented.
CA 5.8.1	Ashby, J; Tennant, R W.	1991	Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP	Mutation Research, Reviews in Genetic Toxicology, (1991) Vol. 257, No. 3, pp. 229-306. CODEN: MRRTEP. ISSN: 0165-1110.	Evaluation of correlations among chemical structure, mutagenicity to Salmonella, and carcinogenicity to rats and mice. No new data presented.
CA 5.8.1	Ashby, J; Tennant, R W.	1994	Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. [Erratum to document cited in CA115(19):200827e]	Mutation Research, Reviews in Genetic Toxicology, (1994) Vol. 317, No. 2, pp. 175. CODEN: MRRTEP. ISSN: 0165-1110.	Erratum for paper excluded.
CA 5.8.1	Beard JD, Hoppin JA; Richards M; Alavanja MCR; Blair A; Sandler DP; Kamel F	2013	Pesticide exposure and self-reported incident depression among wives in the Agricultural Health Study.	Environmental research, (2013 Oct) Vol. 126, pp. 31-42. Electronic Publication Date: 2 Aug 2013 Journal code: 0147621. E-ISSN: 1096-0953. L-ISSN: 0013-9351. Report No.: NLM-NIHMS495075; NLM-PMC3805780.	Evaluation does not include exposure to 2,4,6-TCP.
CA 5.8.1	Ben Israel O; Ben Israel H; Ulitzur S	1998	Identification and quantification of toxic chemicals by use of Escherichia coli carrying lux genes fused to stress promoters.	Appl.Environ.Microbiol. (64, No. 11, 4346-52, 1998) 4 Fig. 2 Tab. 29 Ref. CODEN: AEMIDF	Method development. 2,4,6-TCP was used to validate the assay.
CA 5.8.1	Benoit-Guyod, J. L.; Andre, C.; Clavel, K.	1984	Chlorophenols: degradation and toxicity	Journal Francais d'Hydrologie (1984), 15(3), 249-63 CODEN: JFHYD8; ISSN: 0335-9581	2,4,6-TCP was tested in Guppy and Artemia fish. Test system not relevant for mammalian toxicity evaluation.
CA 5.8.1	Berger, F D.; Manderville, R A.; Sturla, S J.	2014	Synthesis and properties of duplex DNA containing oxygen-linked deoxyguanosine adducts derived from phenolic carcinogens	Abstracts of Papers, 248th ACS National Meeting & Exposition, San Francisco, CA, United States, August 10-14, 2014, (2014) pp. TOXI-55. CODEN: 69SZG4.	Conference abstract. Details on the study and primary data not accessible. Not clear if study tested 2,4,6-TCP.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Bueno de Mesquita H B Doornbos G; Van der Kuip D A; Kogevinas M; Winkelmann R	1993	Occupational exposure to phenoxy herbicides and chlorophenols and cancer mortality in The Netherlands.	American journal of industrial medicine, (1993 Feb) Vol. 23, No. 2, pp. 289-300. Journal code: 8101110. ISSN: 0271-3586. L-ISSN: 0271-3586.	Exposure to 2,4,6-TCP was not analysed.
CA 5.8.1	Bukowska, B. [Reprint Author]; Marczak, A.; Michalowicz, J.; Wisniewska, K.	2009	Effects of Phenol, Catechol, Chloro- and Metylphenol on Human Erythrocyte Membrane (in vitro).	Polish Journal of Environmental Studies, (2009) Vol. 18, No. 4, pp. 569-577. ISSN: 1230-1485.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Chadwick, R. W.; George, S. E.; Chang, J.; Kohan, M. J.; Dekker, J. P.; Allison, J. C.; Long, J. E.; Duffy, M. C.; Forehand, L. R.	1993	Effects of age, species difference, antibiotics and toxicants on intestinal enzyme activity and genotoxicity.	Environmental Toxicology and Chemistry (1993), Volume 12, Number 8, pp. 1339-1352, 49 refs. ISSN: 0730-7268 DOI: 10.1897/1552-8618(1993)12[1339:EOAS DA]2.0.CO;2	2,4,6-TCP was not evaluated in this study.
CA 5.8.1	Chen J Jiang J; Zhang F; Yu H; Zhang J	2004	Cytotoxic effects of environmentally relevant chlorophenols on L929 cells and their mechanisms.	Cell biology and toxicology, (2004 May) Vol. 20, No. 3, pp. 183-96. Journal code: 8506639. ISSN: 0742-2091. L-ISSN: 0742-2091.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Chen, Y; Zhou, P- J; Liu, Q-Y; Zhao, X-H	2011	IN VITRO STUDY OF THE INTERACTION BETWEEN 2,4,6-TRICHLOROPHENOL AND BOVINE SERUM ALBUMIN	Fresenius Environmental Bulletin [Fresenius Environ. Bull.]. Vol. 20, no. 6A, pp. 1513-1519. 2011. ISSN: 1018-4619	Biochemical assay.
CA 5.8.1	Chen X Chen M; Xu B; Tang R; Han X; Qin Y; Xu B; Hang B; Mao Z; Huo W; Xia Y; Xu Z; Wang X	2013	Parental phenols exposure and spontaneous abortion in Chinese population residing in the middle and lower reaches of the Yangtze River.	Chemosphere, (2013 Sep) Vol. 93, No. 2, pp. 217-22. Electronic Publication Date: 25 May 2013 Journal code: 0320657. E-ISSN: 1879-1298. L-ISSN: 0045-6535.	2,4,6-TCP was not evaluated in this study.
CA 5.8.1	Chernoff, N; Kavlock, R J.	1982	An in vivo teratology screen utilizing pregnant mice	Journal of Toxicology and Environmental Health, (1982) Vol. 10, No. 4-5, pp. 541-50. CODEN: JTEHD6. ISSN: 0098-4108.	2,4,6-TCP was not tested in this study.
CA 5.8.1	CHERNOFF N; KAVLOCK R J	1983	A TERATOLOGY TEST SYSTEM WHICH UTILIZES POSTNATAL GROWTH AND VIABILITY IN THE MOUSE	ENVIRON SCI RES, (1983) (27) 417-427. CODEN: EVSRB.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Chernoff N Setzer R W; Miller D B; Rosen M B; Rogers J M	1990	Effects of chemically induced maternal toxicity on prenatal development in the rat.	Teratology, (1990 Dec) Vol. 42, No. 6, pp. 651-8. Journal code: 0153257. ISSN: 0040-3709. L-ISSN: 0040-3709.	2,4,6-TCP was not tested in this study.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Chung, YS [Reprint author]; Ichikawa, K [Reprint author]; Seko, Y; Imura, N; Utsumi, H [Reprint author]	1996	Analysis of chlorophenols toxicity using transgenic cells.	Journal of Toxicological Sciences, (1996) Vol. 21, No. 5, pp. 345. Meeting Info.: 23rd Annual Meeting of the Japanese Society of Toxicological Sciences. Fukuoka, Japan. July 24-26, 1996. CODEN: JTSCDR. ISSN: 0388-1350.	Conference abstract. Details on the study and primary data not accessible.
CA 5.8.1	CICUREL L; SCHMID B P	1988 (a)	POST-IMPLANTATION EMBRYO CULTURE: VALIDATION WITH SELECTED COMPOUNDS FOR TERATOGENICITY TESTING	Xenobiotica, (1988) (18) 617-624. CODEN: XENOB.	2,4,6-TCP was not tested in this study.
CA 5.8.1	CICUREL L; SCHMID B P	1988	POSTIMPLANTATION EMBRYO CULTURE FOR THE ASSESSMENT OF THE TERATOGENIC POTENTIAL AND POTENCY OF COMPOUNDS	Experientia, (1988) (44) 833-840. CODEN: EXPEA.	2,4,6-TCP was not tested in this study.
CA 5.8.1	COGGON D; ACHESON E D	1982	DO PHENOXY HERBICIDES CAUSE CANCER IN MAN.	LANCET (/ , NO.8280, 1057-59, 1982)	Exposure to 2,4,6-TCP was not analysed.
CA 5.8.1	Combes R D; Willington S E; Zajac W; Toraason M; Bohrman J S; Krieg E; Langenbach R	1992	Evaluation of the V79 cell metabolic co-operation assay as a screen in vitro for developmental toxicants.	Toxicology In Vitro, (1992) 6 (2) 165-74. ISSN: 0887-2333.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Cook RR	1980	Analysis of the EPA's Alsea II study.	Proceedings of the 33rd Annual Meeting of the Southern Weed Science Society. (1980), pp. 172-176, 1 refs. Conference: Proceedings of the 33rd Annual Meeting of the Southern Weed Science Society.	2,4,6-TCP was not included in the analysis.
CA 5.8.1	Dai J; Lantero D R; Sloat A L; Adams M; Akman S A; Manderville R A	2003	DNA adduction and in vitro mutagenicity of chlorophenols by peroxidase activation: role of ambient (O- vs C-) phenoxy radicals.	Abstr.Pap.Am.Chem.Soc. (226 Meet., Pt. 1, TOXI061, 2003) CODEN: ACSRAL	Conference abstract. Details on the study and primary data not accessible.
CA 5.8.1	Davies T S; Lynch B S; Monro A M; Munro I C; Nestmann E R	2000	Rodent carcinogenicity tests need be no longer than 18 months: an analysis based on 210 chemicals in the IARC monographs.	Food Chem.Toxicol. (38, No. 2-3, 219-35, 2000) 7 Tab. 54 Ref. CODEN: FCTOD7	Review. 2,4,6-TCP was not discussed in detail. No new data.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Degirmenci, E.; Ono, Y.; Kawara, O.; Utsumi, H.	2000	Genotoxicity analysis and hazardousness prioritization of a group of chemicals	Water Science and Technology, (2000) Vol. 42, No. 7-8, Hazard Assessment and Control of Environmental Contaminants (Eco hazard 99), pp. 125-131. CODEN: WSTED4. ISSN: 0273-1223.	2,4,6-TCP was not included in the evaluation.
CA 5.8.1	Denomme M A; Leece B; Gyorkos J; Homonko K; Safe S	1983	Polychlorinated benzene and phenol congeners as inducers of rat hepatic drug-metabolizing enzymes in immature male Wistar rats.	Canadian journal of physiology and pharmacology, (1983 Sep) Vol. 61, No. 9, pp. 1063-70. Journal code: 0372712. ISSN: 0008-4212. L-ISSN: 0008-4212.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Duri S, Tran CD	2013	Supramolecular composite materials from cellulose, chitosan, and cyclodextrin: facile preparation and their selective inclusion complex formation with endocrine disruptors.	Langmuir : the ACS journal of surfaces and colloids, (2013 Apr 23) Vol. 29, No. 16, pp. 5037-49. Electronic Publication Date: 8 Apr 2013 Journal code: 9882736. E-ISSN: 1520-5827. L-ISSN: 0743-7463. Report No.: NLM-NIHMS461304; NLM-PMC3640277.	2,4,6-TCP was not tested in this study.
CA 5.8.1	EPA working group	1982	An exposure and risk assessment for chlorinated phenols	Environmental Protection Agency, (1982) EPA-440/4-85-007 118 p.	Review. More recent risk evaluations for 2,4,6-TCP by the EPA available. No new data.
CA 5.8.1	Exon, J H; Koller, L D	1986	Toxicity of 2-chlorophenol, 2,4-dichlorophenol, and 2,4,6-trichlorophenol	Water Chlorination: Chem., Environ. Impact Health Eff., Proc. Conf., 5th, (1985) pp. 307-30. CODEN: 54RLA7.	Conference abstract. Details on the study and primary data not accessible.
CA 5.8.1	Fang, Yan-jun; Gao, Xian-jun; Rao, Kai-feng; Chen, Xiang; Gao, Zhi-xian; Chao, Fu-huan	2010	Determination of estrogen effect of typical EDCs	Jiefangjun Yufang Yixue Zazhi, (2010) Vol. 28, No. 2, pp. 113-115. CODEN: JYYZEQ. ISSN: 1001-5248.	Authors do not state with stereoisomer of TCP was used. Not clear if 2,4,6-TCP was tested.
CA 5.8.1	Farrar RF	1978	Annual Report 1978.	Annual Report 1978. (1979), 65 p.	Review. 2,4,6-TCP was not discussed.
CA 5.8.1	FAUSTMAN E M	1988	SHORT-TERM TESTS FOR TERATOGENS	Mutation Research, (1988) (205) 355-384. CODEN: MUREA.	Review. 2,4,6-TCP was not discussed.
CA 5.8.1	Fishbein, L.	1975	Identification of carcinogenic, mutagenic and teratogenic substances in the environment.	Environmental Quality and Safety (1975), Volume 4, pp. 200-225, 219 refs.	Review of techniques to identify toxic substances in the environment.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	FISKESJOE G; LASSEN C; RENNBERG L	1981	CHLORINATED PHENOXYACETIC ACIDS AND CHLOROPHENOLS IN THE MODIFIED ALLIUM TEST.	CHEM.-BIOL.INTERACTIONS (34, NO.3, 333-44, 1981)	2,4,6-TCP was not tested in this study.
CA 5.8.1	Fitzloff J F; Portig J; Stein K	1982	Lindane metabolism by human and rat liver microsomes.	Xenobiotica; the fate of foreign compounds in biological systems, (1982 Mar) Vol. 12, No. 3, pp. 197-202. Journal code: 1306665. ISSN: 0049-8254. L-ISSN: 0049-8254.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Galloway S M; Sofuni T; Shelby M D; Thilagar A; Kumaroo V; Kaur P; Gulati D; Putman D L; Murli H; Marshall R; Tanaka N; Anderson B; Zeiger E; Ishidate M Jr	1997	Multilaboratory comparison of in vitro tests for chromosome aberrations in CHO and CHL cells tested under the same protocols.	Environmental and Molecular Mutagenesis, (1997) 29 (2) 189-207. Journal code: EMM. ISSN: 0893-6692.	Review. No new data.
CA 5.8.1	Geike, F.	1976	Jahresbericht 1975.	Jahresbericht 1975. (1976), 137 p.	Review did not include 2,4,6-TCP.
CA 5.8.1	Gold, LS; Slone, T H.; Stern, B R.; Bernstein, L	1993	Comparison of target organs of carcinogenicity for mutagenic and non-mutagenic chemicals	Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis, (1993) Vol. 286, No. 1, pp. 75-100. CODEN: MUREAV. ISSN: 0027-5107.	No specific conclusion is made on 2,4,6-TCP in this review. No new data.
CA 5.8.1	Goldner WS, Sandler DP; Yu F; Shostrom V; Hoppin JA; Kamel F; LeVan TD	2013	Hypothyroidism and pesticide use among male private pesticide applicators in the agricultural health study.	Journal of occupational and environmental medicine / American College of Occupational and Environmental Medicine, (2013 Oct) Vol. 55, No. 10, pp. 1171-8. Journal code: 9504688. E-ISSN: 1536-5948. L-ISSN: 1076-2752. Report No.: NLM-NIHMS491361; NLM-PMC3795845.	2,4,6-TCP exposure was not evaluated in this study.
CA 5.8.1	GRAY LE J R; KAVLOCK R J; OSTBY J; FERRELL J	1983	ASSESSMENT OF THE UTILITY OF POSTNATAL TESTING FOLLOWING PRENATAL EXPOSURE TO FORTY CHEMICALS	PROG CLIN BIOL RES, (1983) (140) 39-62. CODEN: PCBRD.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Gray L E Jr; Kavlock R J	1984	An Extended Evaluation of an In Vivo Teratology Screen Utilizing Postnatal Growth and Viability in the Mouse.	Teratog.Carcinog.Mutagen . (4, No. 5, 403-26, 1984) 5 Fig. 6 Tab. 19 Ref. CODEN: TCMUD8	Exposure to 2,4,6-TCP was not analysed.



CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Guelden M; Moerchel S; Tahan S; Seibert H	2002	Impact of protein binding on the availability and cytotoxic potency of organochlorine pesticides and chlorophenols in vitro.	Toxicology (175, No. 1-3, 201-13, 2002) CODEN: TXCYAC	2,4,6-TCP was not tested in this study.
CA 5.8.1	Gulden, Michael; Seibert, Hasso	2003	In vitro-in vivo extrapolation: estimation of human serum concentrations of chemicals equivalent to cytotoxic concentrations in vitro	Toxicology, (2003) Vol. 189, No. 3, pp. 211-222. CODEN: TXCYAC. ISSN: 0300-483X.	Method development. 2,4,6-TCP was used to verify the assay.
CA 5.8.1	HALAPPA GOWDA T. P.; LOCK J. D.; KURTZ R. G.	1985	A comprehensive study of risk assessment for a hazardous compound of public health concern	Water, Air, and Soil Pollution, (1985), 24(2), 189-206, refs. 2 p. ISSN: 0049-6979	Review. Risk assessment for 2,4,6-TCP by evaluating environmental exposure risk. No new data relevant to mammalian toxicology.
CA 5.8.1	Hardell L; Eriksson M	1981	Soft-tissue sarcomas, phenoxy herbicides, and chlorinated phenols.	Lancet (London, England), (1981 Aug 1) Vol. 2, No. 8240, pp. 250. Journal code: 2985213R. ISSN: 0140-6736. L-ISSN: 0140-6736.	2,4,6-TCP is not discussed.
CA 5.8.1	Hardell, L.; Eriksson, M.	1981	Phenoxy acids, chlorophenols and cancer.	Lakartidningen, (1981) Vol. 78, No. 34, pp. 2862-2863. ISSN: 0023-7205 CODEN: LAKAA3	No abstract or full text were available for evaluation, but this publication from the title seems to be a review and has the same authors as the citation above. The other article by Hardell did not discuss 2,4,6-TCP and was excluded.
CA 5.8.1	Hardell L; Axelson O	1982	Soft-tissue sarcoma, malignant lymphoma, and exposure to phenoxyacids or chlorophenols	Lancet 19, (June 1982) 1 (8286) 1408-1409. Ref: 10.	2,4,6-TCP exposure was not evaluated.
CA 5.8.1	Haseman J K; Lockhart A	1994	The relationship between use of the maximum tolerated dose and study sensitivity for detecting rodent carcinogenicity.	Fundam.Appl.Toxicol. (22, No. 3, 382-91, 1994) 2 Tab. 36 Ref. CODEN: FAATDF	Data evaluation does not conclude specifically on 2,4,6-TCP, no new data generated.
CA 5.8.1	Hay A	1982	Phenoxy herbicides, trichlorophenols, and soft-tissue sarcomas.	Lancet (London, England), (1982 May 29) Vol. 1, No. 8283, pp. 1240. Journal code: 2985213R. ISSN: 0140-6736. L-ISSN: 0140-6736.	2,4,6-TCP is not discussed.
CA 5.8.1	Heil, J; Hantge, E; Lindemann, G; Zahn, R K	1988	The recognition of genotoxic effects applying the DNA-synthesis-inhibition-test (DIT)	GWF, Wasser/Abwasser, (1988) Vol. 129, No. 5, pp. 398-402. CODEN: GWWAAQ. ISSN: 0016-3651.	Study uses 2,4,6-TCP to validate a new <i>in vitro</i> method. Method development, not relevant.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Henry B; Grant S G; Klopman G; Rosenkranz H S	1998	Induction of forward mutations at the thymidine kinase locus of mouse lymphoma cells: evidence for electrophilic and non-electrophilic mechanisms.	Mutation Research, (1998 Feb 2) 397 (2) 313-35. Journal code: NNA. ISSN: 0027-5107.	Authors establish an <i>in silico</i> prediction model on the basis of the NTP mouse lymphoma mutation database. No new data.
CA 5.8.1	Henschler D	1994	Toxicity of chlorinated organic compounds: effects of the introduction of chlorine in organic molecules.	Angew.Chem.Int.Ed.Engl. (33, No. 19, 1920-35, 1994) 8 Tab. 61 Ref. CODEN: ACIEAY	Review. No new data. Conclusions on toxicity of 2,4,6-TCP not different to risk evaluation of governmental agencies included in this literature search.
CA 5.8.1	Honchar, P	1982	Health Hazard Evaluation Report Number HETA 80-039-1179, Long Island Railroad, New York, New York	19820500	Review. 2,4,6-TCP is not discussed.
CA 5.8.1	Huang Q; Wang X; Liao Y; Kong L; Han S; Wang L	1995	Discriminant analysis of the relationship between genotoxicity and molecular structure of organochlorine compounds.	Bull.Environ.Contam.Toxi col. (55, No. 6, 796-801, 1995) 1 Tab. 3 Ref. CODEN: BECTA6	Authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Huff J E; Haseman J K	1991	Exposure to Certain Pesticides May Pose Real Carcinogenic Risk.	Chem.Eng.News (69, No. 1, 33-37, 1991) CODEN: CENEAR	Review. 2,4,6-TCP is not discussed in detail. No new data.
CA 5.8.1	Huijbregts, M A. J.; Rombouts, L J. A.; Ragas, A M. J.; van de Meent, Dik	2005	Human toxicological effect and damage factors of carcinogenic and noncarcinogenic chemicals for life cycle impact assessment	Integrated Environmental Assessment and Management, (2005) Vol. 1, No. 3, pp. 181-244. CODEN: IEAMCK. ISSN: 1551-3777.	2,4,6-TCP was not included in this study.
CA 5.8.1	Hughes, J [Reprint author]; Courage, C [Reprint author]; Capleton, A [Reprint author]; Duarte-Davidson, R[Reprint author]	2001	Prioritising environmental chemicals for human health risk assessment.	Toxicology, (November 1st, 2001) Vol. 168, No. 1, pp. 73. print. Meeting Info.: British Toxicology Society Annual Congress. Keele, Staffordshire, UK. June 10-13, 2001. British Toxicology Society. CODEN: TXCYAC. ISSN: 0300-483X.	Conference abstract. Details on the study and primary data not accessible.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	HULTH L; HOGLUND L; BERGMAN A; MOLLER L	1978	CONVULSIVE PROPERTIES OF LINDANE, LINDANE METABOLITES, AND THE LINDANE ISOMER ALPHA-HEXACHLOROCYCLOHEXANE. EFFECTS ON THE CONVULSIVE THRESHOLD FOR PENTYLENETETRAZOL AND THE BRAIN CONTENT OF GAMMA-AMINOBUTYRIC ACID /GABA/ IN THE MOUSE.	TOXICOL.APPL.PHARMACOL. (46, NO.1, 101-08, 1978)	Pentylentetrazol was tested in combination with 2,4,6-TCP. Because animals were exposed to a mixture, effects cannot be specifically attributed to 2,4,6-TCP.
CA 5.8.1	IKEDA Y [Reprint author]; KAMATA E; AIDA Y; NAITO K; SUZUKI Y; TOBE M	1985	ACUTE TOXICITY OF CHEMICALS IN HOUSEHOLD GOODS II.	Bulletin of National Institute of Hygienic Sciences (Tokyo), (1985) No. 103, pp. 37-50. CODEN: ESKHA5. ISSN: 0077-4715.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Jackson M A; Stack H F; Waters M D	1993	The genetic toxicology of putative nongenotoxic carcinogens.	Mutation Research, (1993 Mar) 296 (3) 241-77. Ref: 435. Journal code: NNA. ISSN: 0027-5107.	Review. Authors discuss genotoxic studies on 2,4,6-TCP. No new data and no different conclusions than in other publications already considered for this review.
CA 5.8.1	Jacobi, H.; Krieger, G.; Witte, I. Jacobi, H.	1995	Characterization and applicability of a cytotoxicity assay determining growth inhibition after a 1-hour treatment with xenobiotics in human cell culture.	Toxicology in Vitro, (1995) Vol. 9, No. 5, pp. 751-756. ISSN: 0887-2333 CODEN: TIVIEQ	Method development. 2,4,6-TCP was used to validate the assay.
CA 5.8.1	Janik F Wolf H U	1992	The Ca(2+)-transport-ATPase of human erythrocytes as an in vitro toxicity test system--acute effects of some chlorinated compounds.	Journal of applied toxicology : JAT, (1992 Oct) Vol. 12, No. 5, pp. 351-8. Journal code: 8109495. ISSN: 0260-437X. L-ISSN: 0260-437X.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Jaworska, JS.; Schultz, T. W	1994	Mechanism-based comparisons of acute toxicities elicited by industrial organic chemicals in procaryotic and eucaryotic systems	Ecotoxicology and Environmental Safety, (1994) Vol. 29, No. 2, pp. 200-13. CODEN: EESADV. ISSN: 0147-6513.	Study compares toxicity of bioreactive chemicals between the eucaryotic systems Pimephales promelas and Tetrahymena pyriformis and the procaryotic systems Escherichia coli and Photobacterium phosphoreum. Test system is not relevant to predict mammalian toxicity.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Johnson, C. C.; Feingold, M.; Tilley, B.	1990	A meta-analysis of exposure to phenoxy acid herbicides and chlorophenols in relation to risk of soft tissue sarcoma.	International Archives of Occupational and Environmental Health (1990), Volume 62, Number 7, pp. 513-520, 39 refs. ISSN: 0340-0131 DOI: 10.1007/BF00381182	Meta-analysis does not evaluate exposure to 2,4,6-TCP.
CA 5.8.1	Johnson E S	1990	Association Between Soft Tissue Sarcomas, Malignant Lymphomas, and Phenoxy Herbicides / Chlorophenols: Evidence from Occupational Cohort Studies.	Fundam.Appl.Toxicol. (14, No. 2, 219-34, 1990) 4 Tab. 126 Ref. CODEN: FAATDF	Evaluation of cohort studies. 2,4,6-TCP is not discussed in detail. No new data.
CA 5.8.1	Jung, J; Ishida, K; Nishihara, T	2004	Anti-estrogenic activity of fifty chemicals evaluated by in vitro assays	Life Sciences, (2004) Vol. 74, No. 25, pp. 3065-3074. CODEN: LIFSAK. ISSN: 0024-3205.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Kar, S; Roy, K	2012	First report on development of quantitative interspecies structure-carcinogenicity relationship models and exploring discriminatory features for rodent carcinogenicity of diverse organic chemicals using OECD guidelines	Chemosphere, (2012) Vol. 87, No. 4, pp. 339-355. CODEN: CSMHAF. ISSN: 0045-6535.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Kawano, S; Hirose, K; Iguchi, S; Hiraga, K	1979	Toxicological studies of polychlorinated aromatic compounds in female rats	Tokyo-toritsu Eisei Kenkyusho Kenkyu Nenpo, (1979) No. 30-2, pp. 116-23. CODEN: TRENAP. ISSN: 0493-4490.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Kim, SH [Reprint author]; Ichikawa, K; Koshiishi, I; Utsumi, H	2001	Application of in vitro micronucleus assay to 255 chemicals for evaluation of environmental genotoxicity.	Mutation Research, (20 October, 2001) Vol. 483, No. Suppl. 1, pp. S161. print. Meeting Info.: 8th International Conference on Environmental Mutagens. Shizuoka, Japan. October 21-26, 2001. CODEN: MUREAV. ISSN: 0027-5107.	Conference abstract. Details on the study and primary data not accessible.
CA 5.8.1	KIMBROUGH R	1972	TOXICITY OF CHLORINATED HYDROCARBONS AND RELATED COMPOUNDS.	ARCH.ENVIRON.HEALTH (25, NO.2, 125-31, 1972)	2,4,6-TCP was not evaluated in this study.
CA 5.8.1	Kirkland, David; Aardema, Marilyn; Henderson, Leigh; Mueller, Lutz	2005 a	Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity	Mutation Research, Genetic Toxicology and Environmental Mutagenesis, (2005) Vol. 584, No. 1-2, pp. 1-256. CODEN: MRGMFI. ISSN: 1383-5718.	2,4,6-TCP was not included in the evaluation.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Kirkland, David; Aardema, Marilyn; Henderson, Leigh; Mueller, Lutz	2005	Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity. [Erratum to document cited in CA143:243161]	Mutation Research, Genetic Toxicology and Environmental Mutagenesis, (2005) Vol. 588, No. 1, pp. 70. CODEN: MRGMFL. ISSN: 1383-5718.	Erratum to a publication that was excluded above.
CA 5.8.1	Kirkland D[Reprint Author]; Aardema, M; Mueller, L; Hayashi, M	2006	Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens II. Further analysis of mammalian cell results, relative predictivity and tumour profiles.	Mutation Research, (SEP 19 2006) Vol. 608, No. 1, pp. 29-42. ISSN: 1383-5718.	Review. Authors discuss genotoxic studies on 2,4,6-TCP. No new data and no different conclusions than in other publications already considered for this review. No new data.
CA 5.8.1	Kirkland, D; Reeve, L; Gatehouse, D; Vanparys, P	2011	A core in vitro genotoxicity battery comprising the Ames test plus the in vitro micronucleus test is sufficient to detect rodent carcinogens and in vivo genotoxins	Mutation Research, Genetic Toxicology and Environmental Mutagenesis, (2011) Vol. 721, No. 1, pp. 27-73. CODEN: MRGMFL. ISSN: 1383-5718.	Review. Authors discuss genotoxic studies on 2,4,6-TCP. No new data and no different conclusions other than in other publications already considered for this review. No new data.
CA 5.8.1	Kitchin K T; Brown J L	1994	Dose-response relationship for rat liver DNA damage caused by 49 rodent carcinogens.	Toxicology, (1994 Mar 11) 88 (1-3) 31-49. Journal code: VWR. ISSN: 0300-483X.	This publication is the same data set as published in Kitchin <i>et al</i> (1988). No new data and 2,4,6-TCP is not discussed in detail.
CA 5.8.1	Klein, W; Kocsis, F; Wottawa, A	1976	DNA-repair and environmental contaminants	Ber. Oesterr. Studienges. Atomenerg., (1976) No. SGAE BER. No. 2613, pp. 8 pp.. CODEN: BOAEBM.	Review. Full text was not available for review, no copyright available.
CA 5.8.1	Klopman, G; Frierson, M R; Rosenkranz, H S	1990	The structural basis of the mutagenicity of chemicals in Salmonella typhimurium: The Gene-Tox data base	Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis, (1990) Vol. 228, No. 1, pp. 1-50. CODEN: MUREAV. ISSN: 0027-5107.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Kogevinas M; Saracci R; Bertazzi P A; Bueno Mesquita B H de; Coggon D; Green L M	1992	Cancer Mortality from Soft-Tissue Sarcoma and Malignant Lymphomas in an International Cohort of Workers Exposed to Chlorophenoxy Herbicides and Chlorophenols.	Chemosphere (25, No. 7-10, 1071-76, 1992) 2 Tab. 8 Ref. CODEN: CMSHAF	2,4,6-TCP exposure was not evaluated.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Kogevinas M.; Kauppinen T.; Winkelmann, R.; Becher, H.; Bertazzi, P. A.; Bueno-de-Mesquita, H. B.; Coggon, D.; Green, L.; Johnson, E.; Littorin, M.; Lynge, E.; Marlow, D. A.; Mathews, J. D.; Neuberger, M.; Benn, T.; Pannett, B.; Pearce, N.; Saracci, R.	1995	Soft tissue sarcoma and non-Hodgkin's lymphoma in workers exposed to phenoxy herbicides, chlorophenols, and dioxins: two nested case-control studies.	Epidemiology (1995), Volume 6, Number 4, pp. 396-402, 36 refs. ISSN: 1044-3983 DOI: 10.1097/00001648-199507000-00012	Study does not analyse exposure to 2,4,6-TCP.
CA 5.8.1	Kong, Zhiming; Zhang, Guodong; Sun, Liwei	2002	Study on DNA damage of spermary cell in mice induced by environmental endocrine disrupting chemicals	Huanjing Wuran Yu Fangzhi, (2002) Vol. 24, No. 2, pp. 76-78. CODEN: HWYFEW. ISSN: 1001-3865.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Koshiishi, I; Wakamatsu, S; Murahashi, T; Han, Y-H; Takeshita, K; Utsumi, H	2003	Evaluation of endocrine-disrupting activity of multiple contaminated samples using E-screen test	Mizu Kankyo Gakkaishi, (2003) Vol. 26, No. 11, pp. 769-773. CODEN: MKGAEY. ISSN: 0916-8958.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Kubo, T; Urano, K; Utsumi, H	2002	Mutagenicity characteristics of 255 environmental chemicals	Journal of Health Science, (2002) Vol. 48, No. 6, pp. 545-554. CODEN: JHSCFD. ISSN: 1344-9702.	Method development. 2,4,6-TCP was used to validate the assay. Of the 160 mutagens identified in the literature, the authors were only able to identify 48 in their optimized Ames assay.
CA 5.8.1	Laignelet L Riviere J L; Lhuguenot J C	1990	Contribution to the toxicological evaluation of an imidazole fungicide (Prochloraz). Study of its interactions with cytochrome P-450 and its metabolism in the rat.	(1990), 192 refs. 192 p. Dissertation Information: Dijon, Th. doct. : Toxicol., 90 DIJO S050	Exposure and toxicity of 2,4,6-TCP were not tested in this study.
CA 5.8.1	Laignelet L Riviere J L; Lhuguenot J C	1992	Metabolism of an imidazole fungicide (prochloraz) in the rat after oral administration.	Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, (1992 Jul) Vol. 30, No. 7, pp. 575-83. Journal code: 8207483. ISSN: 0278-6915. L-ISSN: 0278-6915.	Exposure and toxicity of 2,4,6-TCP were not tested in this study. 2,4,6-TCP is only identified as minor metabolite in the urine.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Lampi, P [Reprint author]; Vohlonen, I; Tuomisto, J; Heinonen, OP	2000	Increase of specific symptoms after long-term use of chlorophenol polluted drinking water in a community.	European Journal of Epidemiology, (March, 2000) Vol. 16, No. 3, pp. 245-251. print. CODEN: EJEPE8. ISSN: 0393-2990.	Effects cannot be pinpointed to 2,4,6-TCP itself.
CA 5.8.1	LAWLOR T; HAWORTH S R; VOYTEK P	1979	EVALUATION OF THE GENETIC ACTIVITY OF NINE CHLORINATED PHENOLS, SEVEN CHLORINATED BENZENES AND THREE CHLORINATED HEXANES	ENVIRON MUTAGEN, (1979) (1) 143. CODEN: ENMUD.	Abstract only. Details on the study and primary data not accessible.
CA 5.8.1	Legault, R.; Blaise, C.; Rokosh, D.; Chong-Kit, R.	1994	Comparative assessment of the SOS Chromotest kit and the Mutatox test with the Salmonella plate incorporation (Ames test) and fluctuation tests for screening genotoxic agents	Environmental Toxicology and Water Quality, (1994) Vol. 9, No. 1, pp. 45-57. CODEN: ETWQEZ. ISSN: 1053-4725.	Method development. 2,4,6-TCP is used to validate the assay.
CA 5.8.1	Lemaire, G.; Mnif, W.; Mauvais, P.; Balaguer, P.; Rahmani, R.	2006	Activation of $\alpha$ - and $\beta$ -estrogen receptors by persistent pesticides in reporter cell lines.	Life Sciences (2006), Volume 79, Number 12, pp. 1160-1169 ISSN: 0024-3205 DOI: 10.1016/j.lfs.2006.03.023 Published by: Elsevier, New York URL (Availability): <a href="http://www.sciencedirect.com/science/journal/00243205">http://www.sciencedirect.com/science/journal/00243205</a>	2,4,6-TCP was not tested in this study.
CA 5.8.1	Limaye A Kashyap RS; Kapley A; Galande S; Purohit HJ; Dagainawala HF; Taori GM	2008	Modulation of signal transduction pathways in lymphocytes due to sub-lethal toxicity of chlorinated phenol.	Toxicology letters, (2008 Jun 10) Vol. 179, No. 1, pp. 23-8. Electronic Publication Date: 8 Apr 2008 Journal code: 7709027. ISSN: 0378-4274. L-ISSN: 0378-4274.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Liu QiongYu; Zhou PeiJiang; Chen Yan; Liu, Q. Y.; Zhou, P. J.; Chen, Y.	2011	Kinetic studies of the toxicological effect of 2,4-dichlorophenol and 2,4,6-trichlorophenol on alkaline phosphatase activity in vitro.	Fresenius Environmental Bulletin (2011), Volume 20, Number 9a, pp. 2460-2464, 24 refs. ISSN: 1018-4619 Published by: Parlar Scientific Publications, Freising URL (Availability): <a href="http://www.psp-parlar.de">http://www.psp-parlar.de</a>	Biochemical assay.
CA 5.8.1	MATSUOKA A; SAWADA M; SOFUNI T; ISHIDATE M J R	1988	CHROMOSOME ABERRATION TEST ON 25 CHEMICALS: COOPERATIVE STUDY WITH NTP OF U.S.A.	Mutation Research, (1988) (203) 377-378. CODEN: MUREA.	Abstract only. Details on the study and primary data not accessible. Not clear if 2,4,6-TCP was tested from abstract.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Mayura, K; Smith, E E.; Clement, BA.; Phillips, TD.	1991	Evaluation of the developmental toxicity of chlorinated phenols utilizing Hydra attenuata and postimplantation rat embryos in culture	Toxicology and Applied Pharmacology, (1991) Vol. 108, No. 2, pp. 253-66. CODEN: TXAPA9. ISSN: 0041-008X.	2,4,6-TCP was tested negative in Hydra attenuata and not further investigated. Non-relevant test system to predict mammalian toxicity.
CA 5.8.1	McElroy, Nathan R.; Thompson, E. D.; Jurs, Peter C.	2003	Classification of Diverse Organic Compounds That Induce Chromosomal Aberrations in Chinese Hamster Cells	Journal of Chemical Information and Computer Sciences, (2003) Vol. 43, No. 6, pp. 2111-2119. CODEN: JCISD8. ISSN: 0095-2338.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Michalowicz J Sicinska P	2009	Chlorophenols and chlorocatechols induce apoptosis in human lymphocytes (in vitro).	Toxicology letters, (2009 Dec 15) Vol. 191, No. 2-3, pp. 246-52. Electronic Publication Date: 18 Sep 2009 Journal code: 7709027. E-ISSN: 1879-3169. L-ISSN: 0378-4274.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Michalowicz J Majsterek I	2010	Chlorophenols, chlorocatechols and chloroguaiacols induce DNA base oxidation in human lymphocytes (in vitro).	Toxicology, (2010 Feb 9) Vol. 268, No. 3, pp. 171-5. Electronic Publication Date: 16 Dec 2009 Journal code: 0361055. E-ISSN: 1879-3185. L-ISSN: 0300-483X.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Miura, H; Ohmori, S; Yamakawa, M	1978	Are chlorinated phenols capable of hepatic porphyria induction?	Sangyo Igaku (1978), 20(3), 162-73 CODEN: SAIGBL; ISSN: 0047-1879	2,4,6-TCP was not tested in this study.
CA 5.8.1	Mombelli, E.	2012	Evaluation of the OECD (Q)SAR Application Toolbox for the profiling of estrogen receptor binding affinities	SAR and QSAR in Environmental Research, (2012) Vol. 23, No. 1-2, pp. 37-57. CODEN: SQERED. ISSN: 1026-776X.	Evaluation of (Q)SAR Application Toolbox for profiling of chemicals. according to their ER binding propensities. The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	MOSES M; SELIKOFF I J	1981	SOFT TISSUE SARCOMAS, PHENOXY HERBICIDES, AND CHLORINATED PHENOLS.	LANCET (/ , NO.8234, 1370, 1981)	Exposure to 2,4,6-TCP was not evaluated.
CA 5.8.1	Naber, H.	1977	Annual report 1977. Jaarboek 1977.	Jaarboek 1977. (1978), 136 p.	2,4,6-TCP was not included in this review.
CA 5.8.1	Narasimhan T R; Mayura K; Clement B A; Safe S H; Phillips T	1992	Effects of Chlorinated Phenols on Rat Embryonic and Hepatic Mitochondrial Oxidative Phosphorylation.	Environ.Toxicol.Chem. (11, No. 6, 805-14, 1992) 4 Fig. 4 Tab. 33 Ref. CODEN: ETOCDK	2,4,6-TCP was not tested in this study.



CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Needham D Creedy C L; Dawson J R	1992	The profile of rat liver enzyme induction produced by prochloraz and its major metabolites.	Xenobiotica; the fate of foreign compounds in biological systems, (1992 Mar) Vol. 22, No. 3, pp. 283-91. Journal code: 1306665. ISSN: 0049-8254. L-ISSN: 0049-8254.	Exposure to 2,4,6-TCP was not tested in this study.
CA 5.8.1	Nendza, M.; Wenzel, A.; Wienen, G.	1992	Classification of contaminants by mode of action based on in vitro assays	SAR and QSAR in Environmental Research, (1995) Vol. 4, No. 1, pp. 39-50. CODEN: SQERED. ISSN: 1062-936X.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	NESTMANN E R; LEE E H; MATULA T I; DOUGLAS G R; MUELLER J C	1980	MUTAGENICITY OF CONSTITUENTS IDENTIFIED IN PULP AND PAPER MILL EFFLUENTS USING THE SALMONELLA/MAMMALIAN-MICROSOME ASSAY	Mutation Research, (1980) (79) 203-212. CODEN: MUREA.	2,4,6-TCP was not tested in this study.
CA 5.8.1	NESTMANN E R; LEE E H	1983	MUTAGENICITY OF CONSTITUENTS OF PULP AND PAPER MILL EFFLUENT IN GROWING CELLS OF SACCHAROMYCES CEREVISIAE	Mutation Research, (1983) (119) 273-280. CODEN: MUREA.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Nishihara, T; Nishikawa, J; Kanayama, T; Dakeyama, F; Saito, K; Imagawa, M; Takatori, S; Kitagawa, Y; Hori, S; Utsumi, H	2000	Estrogenic activities of 517 chemicals by yeast two-hybrid assay	Journal of Health Science, (2000) Vol. 46, No. 4, pp. 282-298. CODEN: JHSCFD. ISSN: 1344-9702.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Nitcheva, D K.; Piegorsch, W W.; West, R. W	2007	On use of the multistage dose-response model for assessing laboratory animal carcinogenicity	Regulatory Toxicology and Pharmacology (2007), 48(2), 135-147 CODEN: RTOPDW; ISSN: 0273-2300	Publication does not evaluate 2,4,6-TCP in detail. No new data.
CA 5.8.1	Oeberg, L. G.; Paul, K. G.	1985	The transformation of chlorophenols by lactoperoxidase.	Biochimica et Biophysica Acta, G (General Subjects) (1985), Volume 842, Number 1, pp. 30-38, 63 refs.	Biochemical assay.
CA 5.8.1	Oenfelt, A	1987	Spindle disturbances in mammalian cells. III. Toxicity, c-mitosis and aneuploidy with 22 different compounds. Specific and unspecific mechanisms	Mutation Research, Environmental Mutagenesis and Related Subjects, (1987) Vol. 182, No. 3, pp. 135-54. CODEN: MEMSE8.	2,4,6-TCP was not tested in this study.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Ohnishi, T.; Yoshida, T.; Igarashi, A.; Muroi, M.; Tanamoto, K.	2008	Effects of possible endocrine disruptors on MyD88-independent TLR4 signaling.	FEMS Immunology and Medical Microbiology (2008), Volume 52, Number 2, pp. 293-295, 8 refs. ISSN: 0928-8244 DOI: 10.1111/j.1574-695X.2007.00355.x Published by: Blackwell Publishing, Oxford URL (Availability): <a href="http://www.blackwell-synergy.com/doi/ref/10.1111/j.1574-695X.2007.00355.x">http://www.blackwell-synergy.com/doi/ref/10.1111/j.1574-695X.2007.00355.x</a>	2,4,6-TCP was not tested in this study.
CA 5.8.1	Onodera, S; Yoshimatsu, K; Yonaha, M	1997	Transformation of aqueous phenolic compounds in the presence of natural organics and chlorine into Ames mutagenic chloro-o-benzoquinones	Kankyo Kagaku, (1997) Vol. 7, No. 1, pp. 31-37. CODEN: KKAGEY. ISSN: 0917-2408.	Study analyses the production of mutagenic o-benzoquinone (BQ) and chloro-o-BQ by chlorine treatment of wastewater contaminated with phenolic compounds. The mutagenicity of chlorophenols themselves is not assessed, only refers back to other publications.
CA 5.8.1	Ousaa, A.; Elidrissi, B.; Ghamali, M.; Chtita, S.; Bouachrine, M.; Lakhelifi, T.	2014	Acute toxicity of halogenated phenols: combining DFT and QSAR studies	Journal of Computational Methods in Molecular Design, (2014) Vol. 4, No. 3, pp. 10-18, 9 pp.. CODEN: JCMMDA. ISSN: 2231-3176.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Packham E D; Thompson J E; Mayfield C I; Inniss W E; Kruuv J	1981	Perturbation of lipid membranes by organic pollutants.	Archives of environmental contamination and toxicology, (1981) Vol. 10, No. 3, pp. 347-56. Journal code: 0357245. ISSN: 0090-4341. L-ISSN: 0090-4341.	Finding is not relevant for toxicological assessment.
CA 5.8.1	Parent-Massin, D.; Thouvenot, D.	1993	In vitro study of pesticide hematotoxicity in human and rat progenitors.	Journal of Pharmacological and Toxicological Methods (1993), Volume 30, Number 4, pp. 203-207, 23 refs. ISSN: 1056-8719 DOI: 10.1016/1056-8719(93)90018-A	2,4,6-TCP was not tested in this study.
CA 5.8.1	Pearce N E; Smith A H; Howard J K; Sheppard R A; Giles H J; Teague C A	1986	Non-Hodgkin's lymphoma and exposure to phenoxyherbicides, chlorophenols, fencing work, and meat works employment: a case-control study.	British journal of industrial medicine, (1986 Feb) Vol. 43, No. 2, pp. 75-83. Journal code: 0370637. ISSN: 0007-1072. L-ISSN: 0007-1072. Report No.: NLM-PMC1007611.	Due to the limited number of subjects, it was not possible to conclude to a link.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Phillips T D; Narasimhan T R; Mayura K; Clement B A; Safe S	1991	Effect of chlorinated phenols on rat embryonic and hepatic mitochondrial oxidative phosphorylation.	Toxicologist, (1991 Feb) 11 (1) 71. ISSN: 0731-9193.	Conference abstract. 2,4,6-TCP was not tested in this study.
CA 5.8.1	Pohl, H R.; Holler, J S. Luukinen, B	2005	Health effects classification and its role in the derivation of minimal risk levels: Reproductive and endocrine effects.	Regulatory Toxicology and Pharmacology, (Jul 2005) Vol. 42, No. 2, pp. 209-217. Refs: 80 ISSN: 0273-2300 CODEN: RTOPOW	Review about MRLs based on reproductive and endocrine effects and about the guidance provided categorization in the ATSDR's Guidance for Developing Toxicological Profiles. No new data.
CA 5.8.1	Roberts, M. S.; Anderson, R. A.; Swarbrick, J.; Moore, D. E	1978	The percutaneous absorption of phenolic compounds: the mechanism of diffusion across the stratum corneum	Journal of Pharmacy and Pharmacology (1978), 30(8), 486-90 CODEN: JPPMAB; ISSN: 0022-3573	Dermal absorption is not a relevant route of exposure for the metabolite.
CA 5.8.1	Roberts, D W.; Aptula, A O.; Patlewicz, G	2007	Electrophilic Chemistry Related to Skin Sensitization. Reaction Mechanistic Applicability Domain Classification for a Published Data Set of 106 Chemicals Tested in the Mouse Local Lymph Node Assay	Chemical Research in Toxicology, (2007) Vol. 20, No. 1, pp. 44-60. CODEN: CRTOEC. ISSN: 0893-228X.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Rosen M B; Rogers R M; Miller D B; Mattschek C; Chernoff N	1988	Effects of Chemical-Induced Maternal Toxicity on the Sprague-Dawley (CD) Rat.	Teratology (37, No. 5, 486, 1988) CODEN: TJADAB	Conference abstract. 2,4,6-TCP does not appear to have been tested in this study.
CA 5.8.1	Rosenkranz, H S.; Ennever, F K.; Dimayuga, M; Klopman, G	1990 a	Significant differences in the structural basis of the induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells	Environmental and Molecular Mutagenesis, (1990) Vol. 16, No. 3, pp. 149-77. CODEN: EMMUEG. ISSN: 0893-6692.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Rosenkranz, H S.; Ennever, F K.; Klopman, G	1990	Relationship between carcinogenicity in rodents and the induction of sister chromatid exchanges and chromosomal aberrations in Chinese hamster ovary cells	Mutagenesis, (1990) Vol. 5, No. 6, pp. 559-71. CODEN: MUTAEX. ISSN: 0267-8357.	Study evaluates the correlation between chromosomal damage in <i>in vitro</i> assays and carcinogenicity in rodents. No specific conclusion was made on 2,4,6-TCP itself. No new data.
CA 5.8.1	Rosenkranz, Herbert S.; Klopman, Gilles	1990	The structural basis of the mutagenicity of chemicals in Salmonella typhimurium: The National Toxicology Program data base	Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis, (1990) Vol. 228, No. 1, pp. 51-80. CODEN: MUREAV. ISSN: 0027-5107.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Rosenkranz, H S.; Klopman, G	1990	Structural basis of carcinogenicity in rodents of genotoxicants and non-genotoxicants	Mutation Research, Fundamental and Molecular Mechanisms of Mutagenesis, (1990) Vol. 228, No. 2, pp. 105-24. CODEN: MUREAV. ISSN: 0027-5107.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Rosenkranz H S; Zhang Y P; Klopman G	1998	Studies on the potential for genotoxic carcinogenicity of fragrances and other chemicals.	Food Chem.Toxicol. (36, No. 8, 687-96, 1998) 1 Fig. 9 Tab. 48 Ref. CODEN: FCTOD7	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Rotroff, DM.; Martin, MT.; Dix, DJ.; Filer, DL.; Houck, KA.; Knudsen, TB.; Sipes, NS.; Reif, DM.; Xia, M; et al	2014	Predictive Endocrine Testing in the 21st Century Using in Vitro Assays of Estrogen Receptor Signaling Responses	Environmental Science & Technology, (2014) Vol. 48, No. 15, pp. 8706-8716. CODEN: ESTHAG. ISSN: 0013-936X.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Russell, L.B.; Selby, P.B.; Von Halle, E.; et. al.	1981	Use of the mouse spot test in chemical mutagenesis: Interpretation of past data and recommendations for future work.	Mutation Research, (1981) Vol. 86, No. 3, pp. 355-379. CODEN: MUREAV	Review. No new data.
CA 5.8.1	Sadik O A; Witt D M	1999	Monitoring endocrine-disrupting chemicals.	Environ.Sci.Technol. (33, No. 17, 368A-74A, 1999) 2 Fig. 2 Tab. 14 Ref. CODEN: ESTHAG	Review. No new data.
CA 5.8.1	Saracci, R.; Kogevinas, M.; Bertazzi, P. A.; Bueno de Mesquita, B. H.; Coggon, D.; Green, L. M.; Kauppinen, T.; L'Abbe, K. A.; Littorin, M.; Lynge, E.; Mathews, J. D.; Neuberger, M.; Osman, J.; Pearce, N.; Winkelmann, R	1991	Cancer mortality in workers exposed to chlorophenoxy herbicides and chlorophenols.	Lancet (British edition) (1991), Volume 338, Number 8774, pp. 1027-1032, 35 refs. ISSN: 0140-6736 DOI: 10.1016/0140-6736(91)91898-5	Epidemiological study does not conclude specifically on 2,4,6-TCP exposure.
CA 5.8.1	Schardein J L	1993	Pesticides.	Chemically Induced Birth Defects, (1993) 2 675-721. Ref: 407. ISBN: 0-8247-8775-7	Book chapter. Reference was not available for evaluation because of copyright.
CA 5.8.1	Schuurmann, G; Segner, H; Jung, K	1997	Multivariate mode-of-action analysis of acute toxicity of phenols	Aquatic Toxicology, (1997) Vol. 38, No. 4, pp. 277-296. CODEN: AQTOGD. ISSN: 0166-445X.	Non-relevant test system: The test battery consists of nine different species: three fish, one waterflea, one ciliate, one marine bacterium in two different assays, two fish cell lines and one plant pollen.

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CA 5.8.1	Serafimova, R.; Todorov, M.; Pavlov, T.; Kotov, S.; Jacob, E.; Aptula, A.; Mekenyan, O.	2007	Identification of the Structural Requirements for Mutagenicity, by Incorporating Molecular Flexibility and Metabolic Activation of Chemicals. II. General Ames Mutagenicity Model. [Erratum to document cited in CA146:516278]	Chemical Research in Toxicology, (2007) Vol. 20, No. 8, pp. 1225. CODEN: CRTOEC. ISSN: 0893-228X.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Serafimova, R.; Todorov, M.; Pavlov, T.; Kotov, S.; Jacob, E.; Aptula, A.; Mekenyan, O	2007 a	Identification of the Structural Requirements for Mutagenicity, by Incorporating Molecular Flexibility and Metabolic Activation of Chemicals. II. General Ames Mutagenicity Model	Chemical Research in Toxicology (2007), 20(4), 662-676 CODEN: CRTOEC; ISSN: 0893-228X	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Sipes, NS.; Martin, MT.; Kothiya, P; Reif, D M.; Judson, R S.; Richard, A M.; Houck, K A.; Dix, D J.; Kavlock, R J.; Knudsen, T B.	2013	Profiling 976 ToxCast Chemicals across 331 Enzymatic and Receptor Signaling Assays	Chemical Research in Toxicology, (2013) Vol. 26, No. 6, pp. 878-895. CODEN: CRTOEC. ISSN: 0893-228X.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Shehu, R A.; Al-Hamidi, A A.A.; Rabbani, N; Duhaime, A S.	1998	Inhibition of camel lens $\zeta$ s-crystallin/NADPH : Quinone oxidoreductase activity by chlorophenols	Journal of Enzyme Inhibition (1998) Volume 13, Number 3, pp. 229-236, 23 refs. CODEN: ENINEG ISSN: 8755-5093	2,4,6-TCP was not tested in this study.
CA 5.8.1	Shoja, R.; Laskowski, W.; Roth, R.; Lochmann, E. R.	1992	Repair deficient Saccharomyces strains as test organisms for the toxicity of chemicals in the environment	Umweltwissenschaften und Schadstoff-Forschung (1992), 4(5), 261-4 CODEN: USZOE9; ISSN: 0934-3504	2,4,6-TCP was not discussed in this review.
CA 5.8.1	Shoji, R; Sakai, Y; Sakoda, A; Suzuki, M	2000	Preservation of microplate-attached human hepatoma cells and their use in cytotoxicity tests	Cytotechnology, (2000) Vol. 32, No. 2, pp. 147-155. CODEN: CYTOER. ISSN: 0920-9069.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Smith A H; Pearce N E; Fisher D O; Giles H J; Teague C A; Howard J K	1984	Soft tissue sarcoma and exposure to phenoxyherbicides and chlorophenols in New Zealand.	Journal of the National Cancer Institute, (1984 Nov) Vol. 73, No. 5, pp. 1111-7. Journal code: 7503089. ISSN: 0027-8874. L-ISSN: 0027-8874.	Exposure to 2,4,6-TCP was not evaluated.
CA 5.8.1	Smith M K; Zenick H; George E L	1986	Reproductive toxicology of disinfection by-products.	Environmental health perspectives, (1986 Nov) Vol. 69, pp. 177-82. Ref: 32 Journal code: 0330411. ISSN: 0091-6765. L-ISSN: 0091-6765. Report No.: NLM-PMC1474316.	Review. No new data.

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CA 5.8.1	SOFUNI T; YAMAZAKI N; MATSUOKA A; SUZUKI T; HAYASHI M	1991	EFFECT OF EXPERIMENTAL PROTOCOLS ON DETECTION OF CHROMOSOMAL ABERRATIONS IN TWO CHINESE HAMSTER CELL LINES (CHL AND CHO).	MUTAT RES, (1991 DEC) 253 (3) 276-7. CODEN: MUREA. ISSN: 0165-7992.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Sofuni, T; Matsuoka, A; Sawada, M; Ishidate, M, Jr.; Zeiger, E; Shelby, M D.	1990	A comparison of chromosome aberration induction by 25 compounds tested by two Chinese hamster cell (CHL and CHO) systems in culture	Mutation Research, Genetic Toxicology Testing, (1990) Vol. 241, No. 2, pp. 175-213. CODEN: MRGTE4. ISSN: 0165-1218.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Tarasov, V. A.; Velibekov, R. M.; Lyubimova, I. K.; Aslanyan, M. M.	2001	Low efficiency of short-term tests in the assessment of the potential mutagenic hazard of chemical compounds to mammals	Russian Journal of Genetics (Translation of Genetika (Moscow, Russian Federation)), (2001) Vol. 37, No. 7, pp. 838-846. CODEN: RJGEEQ. ISSN: 1022-7954.	Evaluation does not include 2,4,6-TCP.
CA 5.8.1	Tarasov, V. A.; Abilev, S. K.; Velibekov, R. M.; Aslanyan, M. M.	2003	Efficiency of Batteries of Tests for Estimating Potential Mutagenicity of Chemicals	Russian Journal of Genetics (Translation of Genetika (Moscow, Russian Federation)), (2003) Vol. 39, No. 10, pp. 1191-1200. CODEN: RJGEEQ. ISSN: 1022-7954.	Evaluation does not include 2,4,6-TCP.
CA 5.8.1	Thomas, J; Spencer, P J. Haseman, J K. Goodman, J I. Ward, J M. Loughran Jr., T P.	2007	A review of large granular lymphocytic leukemia in Fischer 344 rats as an initial step toward evaluating the implication of the endpoint to human cancer risk assessment.	Toxicological Sciences, (Sep 2007) Vol. 99, No. 1, pp. 3-19. Refs: 85 ISSN: 1096-6080; E-ISSN: 1096-0929 CODEN: TOSCF2	Review. No new data.
CA 5.8.1	Tsuchiya, T; Matuoka, A; Sekita, S; Hisano, T; Takahashi, A; Ishidate, M, Jr.	1989	Human embryonic cell growth assay for teratogens with or without metabolic activation system using microplate	Teratogenesis, Carcinogenesis, and Mutagenesis, (1988) Vol. 8, No. 5, pp. 265-72. CODEN: TCMUD8. ISSN: 0270-3211.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Vizethum W; Goerz G	1979	Induction of the hepatic microsomal and nuclear cytochrome P-450 system by hexachlorobenzene, pentachlorophenol and trichlorophenol.	Chemico-biological interactions, (1979 Dec) Vol. 28, No. 2-3, pp. 291-9. Journal code: 0227276. ISSN: 0009-2797. L-ISSN: 0009-2797.	2,4,6-TCP was not tested in this study.

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CA 5.8.1	Vollrath, V; Iruretagoyena, M; Chianale, J	2004	Constitutive and inducible expression of mrp2 (ABCC2) gene is regulated by the cellular detoxification pathway ARE-Nrf2 in the mouse liver.	Gastroenterology, (APR 2004) Vol. 126, No. 4, Suppl. 2, pp. A766. Meeting Info.: Digestive Disease Week/105th Annual Meeting of the American-Gastroenterological-Association. New Orleans, LA, USA. May 16 -20, 2004. Amer Gastroenterol Assoc. CODEN: GASTAB. ISSN: 0016-5085.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Wang, YQ; Tan, CY; Zhuang, SL; Zhai, P-Z; Cui, Y; Zhou, QH; Zhang, H; Fei, Z	2014	In vitro and in silico investigations of binding interactions between chlorophenols and trypsin	Journal of Hazardous Materials, (2014) Vol. 278, pp. 55-65. CODEN: JHMAD9. ISSN: 0304-3894.	Biochemical assay.
CA 5.8.1	Waters, M. D.; Stack, H. F.; Jackson, M. A.; Bridges, B. A.	1993	Hazard identification: efficiency of short-term tests in identifying germ cell mutagens and putative nongenotoxic carcinogens	Environmental Health Perspectives, (1993) Vol. 101, No. Suppl. 3, pp. 61-72. CODEN: EVHPAZ. ISSN: 0091-6765.	Review. No new data. No specific conclusion on 2,4,6-TCP is made.
CA 5.8.1	Wiles M C; Barhoumi R; Phillips T D; Burghardt R C	2001	Measurement of cytotoxicity in rat liver cells exposed to polychlorinated phenols.	I.Sci. (60, No. 1, Suppl., 235, 2001) CODEN: ; Toxi	Conference abstract. Details on the study and primary data not accessible.
CA 5.8.1	Witte I; Jacobi H; Juhl Strauss U	1995	Correlation of synergistic cytotoxic effects of environmental chemicals in human fibroblasts with their lipophilicity.	Chemosphere (31, No. 9, 4041-49, 1995) 1 Fig. 2 Tab. 26 Ref. CODEN: CMSHAF	2,4,6-TCP was tested as part of a mixture, no specific effects can be attributed to 2,4,6-TCP.
CA 5.8.1	Yamamoto, K.; Yamamoto, Y.; Ueda, M.	1980	A fatal case of 2,4,6-trichlorophenol (2,4,6-TCP) poisoning.	Japanese Journal of Legal Medicine, (1980) Vol. 34, No. 6, pp. 631-636. ISSN: 0047-1887 CODEN: NHOZAX	The victim was exposed to a mixture of chlorophenols. No specific effect can be attributed to 2,4,6-TCP.
CA 5.8.1	Yang, L; Xu, J; Liu, Z	2003	Study on the mutagenicity of phenolic compounds using the Ames test	Dongbei Shida Xuebao, Ziran Kexueban, (2003) Vol. 35, No. 1, pp. 82-85. CODEN: DSZKEE. ISSN: 1000-1832.	The publication was not available for review due to copyright. It is not clear from the abstract if 2,4,6-TCP was studied.
CA 5.8.1	Xu Xiaohui Nembhard Wendy N; Kan Haidong; Kearney Greg; Zhang Zhi-Jiang; Talbot Evelyn O	2011	Urinary trichlorophenol levels and increased risk of attention deficit hyperactivity disorder among US school-aged children.	Occupational and environmental medicine, (2011 Aug) Vol. 68, No. 8, pp. 557-61. Electronic Publication Date: 3 May 2011 Journal code: 9422759. E-ISSN: 1470-7926. L-ISSN: 1351-0711. Report No.: NLM-PMC3131187.	Although 2,4,6-TCP was detected in the urine of children, the source of exposure or potential metabolism cannot be excluded.

CA data point number	Author(s)	Year	Title	Source	Reason(s) for not including the study in the dossier
CA 5.8.1	Zeiger E	1987	Carcinogenicity of Mutagens: Predictive Capability of the Salmonella Mutagenesis Assay for Rodent Carcinogenicity.	Cancer Res. (47, No. 5, 1287-96, 1987) 8 Tab. 31 Ref. CODEN: CNREA8	Review. 2,4,6-TCP is not discussed in detail. No new data.
CA 5.8.1	Zeiger E; Anderson B; Haworth S; Lawlor T; Mortelmans K	1992	Salmonella mutagenicity tests: V. Results from the testing of 311 chemicals.	Environmental and Molecular Mutagenesis, (1992) 19 (Suppl 21) 2-141. Journal code: EMM. ISSN: 0893-6692.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Zenick, H; Blackburn, K; Hope, E; Oudiz, D; Goeden, H	1984	Evaluating male reproduction toxicity in rodents: a new animal model	Teratogenesis, Carcinogenesis, and Mutagenesis, (1984) Vol. 4, No. 1, pp. 109-28. CODEN: TCMUD8. ISSN: 0270-3211.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Zhao, Li; Wang, Ming-shi; Liu, Zheng-tao; Feng, Liu; Kong, Zhi-ming	2006	Phenols-induced DNA toxicity study and quantitative structure-activity relationship with murine spleen cells	Anquan Yu Huanjing Xuebao, (2006) Vol. 6, No. 5, pp. 27-30. CODEN: AYHXA2. ISSN: 1009-6094.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Zhao F Mayura K; Hutchinson R W; Lewis R P; Burghardt R C; Phillips T D	1995	Developmental toxicity and structure-activity relationships of chlorophenols using human embryonic palatal mesenchymal cells.	Toxicology letters, (1995 Jun) Vol. 78, No. 1, pp. 35-42. Journal code: 7709027. ISSN: 0378-4274. L-ISSN: 0378-4274.	2,4,6-TCP was not tested in this study.
CA 5.8.1	Zhong, M; Nie, X; Yan, A; Yuan, Q	2013	Carcinogenicity Prediction of Noncongeneric Chemicals by a Support Vector Machine	Chemical Research in Toxicology, (2013) Vol. 26, No. 5, pp. 741-749. CODEN: CRTOEC. ISSN: 0893-228X.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Zhou, W; Huang, C; Li, Yan; D, Jinyou; W, Y; Yang, L	2013	A systematic identification of multiple toxin-target interactions based on chemical, genomic and toxicological data	Toxicology, (2013) Vol. 304, pp. 173-184. CODEN: TXCYAC. ISSN: 0300-483X.	The authors use existing data to develop an <i>in silico</i> prediction method. No new data presented.
CA 5.8.1	Zilkah S; Osband M E; McCaffrey R	1981	Effect of inhibitors of plant cell division on mammalian tumor cells in vitro.	Cancer research, (1981 May) Vol. 41, No. 5, pp. 1879-83. Journal code: 2984705R. ISSN: 0008-5472. L-ISSN: 0008-5472.	2,4,6-TCP was not tested in this study.

All documents listed in Table 6.10-3 and not excluded (i.e. not listed in Table 6.10-4) have been summarised in the relevant sections of the B6 where an assessment of reliability has been conducted and the conclusions documented.

A top-up search of the literature from 2015 up to August 2022 was performed by the applicant. No publications of significance to the regulatory outcome of the assessment were identified in the toxicology area.



Data Point	Author (s)	Year	Title Compagny Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA1 5.1.1	██████ ██████	04/06/2015	SYN545974 - The Absorption and Excretion of [Phenyl U 14C] and [Pyrazole 5 14C] SYN545974 Following Single Oral Administration in the Rat Report No. 34214 Document No. VV-413069 , SYN545974_10248 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	██████ ██████	04/06/2015	SYN545974 – Tissue Depletion of [Phenyl-U-14C] and [Pyrazole-5-14C]-SYN545974 Following Single Oral Administration in the Rat Report No. 34340 Document No. VV-413071 , SYN545974_10252 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	██████ ██████ ██████ ██████	04/06/2015	SYN545974 – Pharmacokinetics of [Phenyl-U-14C] and [Pyrazole-5-14C] SYN545974 Following Single Oral and Intravenous Administration in the Rat Report No. 34107 Document No. VV-413070 , SYN545974_10250 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	██████ ██████ ██████ ██████ ██████	05/08/2015	SYN545974 - Biotransformation of [14C]-SYN545974 in Rat Report No. 34216 Document No. VV-413262 , SYN545974_10259 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.1.1	■■■■	15/09/2015	SYN545974 - Oral (Gavage) Toxicokinetic Study in the Pregnant Rabbit Report No. BFI0126 Document No. VV-410782 , SYN545974_10125 Test Facility ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	■■■■	19/08/2014	SYN545974 – Pharmacokinetics of SYN545974 in the Mouse Following Multiple Oral and Single Intravenous Administration Report No. 33408 Document No. VV-410696 , SYN545974_10124 Test Facility ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	■■■■	16/06/2014	SYN545974 – Pharmacokinetics of SYN545974 in the Rat Following Multiple Oral and Single Intravenous Administration Report No. 33409 Document No. VV-407624 , SYN545974_10104 Test Facility ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	■■■■	03/04/2017	Pydiflumetofen – In Vitro Comparative Metabolism of [Phenyl-U-14C]Pydiflumetofen and [Pyrazole-5- 14C]Pydiflumetofen in Human and Rat Liver Microsomes Report No. 20160346 Document No. VV-467499 , SYN545974_10511 Test Facility Innovative Environmental Services GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.1.1	██████ ██████ ██████ ██ ██████ ██ ██████ ██	03/08/2015	SYN545974 – The Excretion and Biotransformation of [Phenyl-U-14C] and [Pyrazole-5-14C]-SYN545974 Following Single Oral Administration in the Mouse Report No. 35415 Document No. VV-413261 , SYN545974_10257 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.1.1	██████ ██████ ██████ ██████	20/05/2015	SYN545974 - A Preliminary Study of Pharmacokinetics, Absorption, Metabolism and Excretion in Rats Following Single Oral and Intravenous Administration of 14C-SYN545974 Report No. SGA/64 Document No. VV-412533 , SYN545974_10188 Test Facility Quotient Bioresearch Limited GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.2.1	██████ ██████ ██████ ██	20/12/2012	SYN545974 - Acute Oral Toxicity Study in the Rat (Up and Down Procedure) Report No. 12/344-001P Document No. VV-403476 , SYN545974_10043 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.2.2	██████ ██████ ██████ ██	14/01/2013	SYN545974 – Acute Dermal Toxicity Study in Rats Report No. 12/344-002P Document No. VV-403625 , SYN545974_10046 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.2.3	██████ ██████ ██	29/01/2013	SYN545974 – Acute Inhalation Toxicity Study (Nose-Only) in the Rat Report No. 12/334-004P Document No. VV-403817 , SYN545974_10051 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.2.4	██████ ██████ ██████	26/11/2012	SYN545974 - Primary Skin Irritation Study in Rabbits Report No. 12/344-006N Document No. VV-403217 , SYN545974_10035 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.2.5	██████ ██████ ██████	26/11/2012	SYN545974 - Acute Eye Irritation Study in Rabbits Report No. 12/344-005N Document No. VV-403216 , SYN545974_10033 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.2.6	██████ ██████	31/01/2013	SYN545974 – Local Lymph Node Assay in the Mouse Report No. 12/344-037E Document No. VV-403659 , SYN545974_10052 Test Facility ██████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.2.7	██████ ██████	02/09/2015	SYN545974 - In Vitro 3T3 NRU Phototoxicity Test Report No. 151200 Document No. VV-414016 , SYN545974_10297 Test Facility ██████████ ██████████ GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.3.1	██████ ██████	20/12/2012	SYN545974, SYN546022 - 28 Day Dietary Toxicity Study in Mice Report No. 32153 Document No. VV-403466 , SYN545974_10042 Test Facility ██████████ ██████████ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N

KCA1 5.3.1	■■■■ ■■■■	27/07/2017	SYN545974, SYN546022 - 28 Day Dietary Toxicity Study in Rat Report No. 32168 Incl Amendment 1 Document No. VV-403515 , SYN545974_10044 Test Facility ■■■■■ ■■■■■ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N
KCA1 5.3.2	■■■■ ■■■■	19/05/2015	SYN545974 - 90 Day Oral (Capsule) Study in the Dog Report No. ■■■■■ Document No. VV-412679 , SYN545974_10207 Test Facility ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.3.2	■■■■ ■■■■	19/05/2015	SYN545974 - 52 Week Oral (Capsule) Toxicity Study in the Dog Report No. ■■■■■ Document No. VV-412677 , SYN545974_10205 Test Facility ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.3.2	■■■■ ■■■■	17/04/2018	SYN545974 - Rebuttal for Increase in ALP in SYN545974 52 Week Dog Study Report No. TK0103660 Document No. VV-265256 , SYN545974_10622 Test Facility N/A Not GLP Unpublished	N/A	N	N/A	SYN	N
KCA1 5.3.2	■■■■ ■■■■ ■■■■ ■■■■	28/07/2015	SYN545974 - A 13 Week Toxicity Study of SYN545974 by Oral (Dietary) Administration in Rats Report No. 33012 Document No. VV-412756 , SYN545974_10210 Test Facility ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.3.2	████ ███ ████	31/08/2016	SYN545974 - Historical Control Data for SYN545974_10210 and SYN545974_10211 Report No. SYN545974_10210 SYN54597 4_10211 Document No. VV-134274 , SYN545974_10448 Test Facility █████ ███ Not GLP Unpublished	N	N	N/A		N
KCA1 5.3.2	████ ███	28/07/2015	SYN545974 - A 13 Week Toxicity Study of SYN545974 by Oral (Dietary) Administration in Mice Report No. 33011 Document No. VV-412757 , SYN545974_10211 Test Facility █████ █████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.3.3	████ ███	14/01/2013	SYN545974 – 28-Day Dermal Toxicity Study in the Wistar Rat Report No. D62072 Document No. VV-403629 , SYN545974_10047 Test Facility █████ █████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.4.1	████ ████ ███ ████ ████ ████	15/01/2013 (amended 06/12/2016)	SYN545974 - Chromosome Aberration Test in Human Lymphocytes In Vitro Report No. 1498902 Document No. VV-403748 , SYN545974_10048 Test Facility Envigo CRS GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.4.1	████ ████ ███	22/10/2012	SYN545974 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay Report No. 1498901 Document No. VV-402587 , SYN545974_10018 Test Facility Harlan Cytotest Cell Research GmbH (Harlan CCR) GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.4.1	■■■■ ■■■■ ■■■■	03/12/2014	SYN545974 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay Report No. 1648701 Document No. VV-410893 , SYN545974_10127 Test Facility Harlan Cytotest Cell Research GmbH (Harlan CCR) GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.4.1	■■■■ ■■■■ ■■■■	24/01/2013	SYN545974 – Cell Mutation Assay at the Thymidine Kinase Locus (TK +/-) in Mouse Lymphoma L5178Y Cells Report No. 1498903 Document No. VV-403728 , SYN545974_10049 Test Facility Harlan Cytotest Cell Research GmbH (Harlan CCR) GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.4.2	■■■■ ■■■■ ■■■■	12/12/2014	SYN545974 – Micronucleus Assay in Bone Marrow Cells of the Mouse Report No. 1648702 Document No. VV-411006, SYN545974_10141 Test Facility ■■■■■ ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.4.2	■■■■ ■■■■ ■■■■	26/05/2017	SYN545974 - Rat Bone Marrow Chromosome Aberration Assay Report No. 8359217 Document No. VV-467608 , SYN545974_10513 Test Facility ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.4.2	■■■■ ■■■■ ■■■■	20/12/2012	SYN545974 – Micronucleus Assay in Bone Marrow Cells of the Mouse Report No. 1498904 Document No. VV-403487 , SYN545974_10045 Test Facility ■■■■■ ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.5	■■■■	01/09/2015	SYN545974 - Human Relevance Framework Assessment of Liver Tumour Induction in CD-1 Mice Report No. TK0258437 Document No. VV-111156 , SYN545974_10290 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.5	■■■■	24/08/2015	SYN545974 - A 28-Day Dietary Liver Mode of Action Study in Male CD-1 Mice Report No. ■■■■ Document No. VV-414071 , SYN545974_10267 Test Facility ■■■■ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N
KCA1 5.5	■■■■	24/01/2012	Ex-Vivo Enzyme Analysis of Liver Samples Taken at Termination of a 28 Day Dietary Study of SYN545974 and SYN546022 in the Mouse Report No. ■■■■ Document No. VV-412755 , SYN545974_10209 Test Facility ■■■■ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N
KCA1 5.5	■■■■	21/08/2015	SYN545974 – In Vitro Hepatocyte Proliferation Index And Enzyme Activity Measurements In Male CD-1 Mouse Hepatocyte Cultures Report No. CXR1503 Document No. VV-414087 , SYN545974_10272 Test Facility CXR Biosciences Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N



KCA1 5.5	■■■■■ ■■	21/08/2015	SYN545974 – In Vitro Hepatocyte Proliferation Index And Enzyme Activity Measurements In Male Human Hepatocyte Cultures Report No. CXR1504 Document No. VV-414085 , SYN545974_10270 Test Facility CXR Biosciences Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.5	■■■■■ ■■■ ■■	03/10/2014	SYN545974 - CAR3 Transactivation Assay with Mouse, Rat and Human CAR Report No. TK0219831 Document No. VV-414596 , SYN545974_50044 Test Facility Dept of Vet & Biomedical Sciences Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.5	■■■■■ ■■■■■	2015 (amendment 01/03/2016)	SYN545974 - 104 Week Rat Dietary Carcinogenicity Study with a Combined 52 Week Toxicity Study Report No. 36248 Document No. VV-413432 , SYN545974_10245 Test Facility ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.5	■■■■■ ■■■■■	30/07/2016	SYN545974 - Historical Control Data for SYN545974_10245 Report No. 36248 SYN545974_10245 Document No. VV-134505 , SYN545974_10464 Test Facility ■■■■■ ■■■■■ Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.5	■■■■■ ■■■■■	2015 (amendment 16/01/2017)	SYN545974 - 80 Week Mouse Dietary Carcinogenicity Study Report No. 35914 + Amendment 1 (2015) + Amendment 2 (2016) + Amendment 3 (2017) Document No. VV-412943 , SYN545974_10237 Test Facility ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.6.1	██████ ██████	30/07/2015	SYN545974 - Oral (Dietary) Two-Generation Reproduction Toxicity Study in the Rat Report No. ██████ Document No. VV-413436 , SYN545974_10246 Test Facility ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.6.2	██████ ██████	30/07/2015	SYN545974 – Additional Historical Control Data to Support Developmental Toxicity Studies in Rats Report No. TK0103655 Document No. VV-29008 , SYN545974_10244 Test Facility ██████ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.6.2	██████ ██████	02/09/2016	SYN545974 - Historical Control Data for SYN545974_10190 Report No. SYN545974_10190 Document No. VV-134276 , SYN545974_10453 Test Facility ██████ Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.6.2	██████ ██████	2015 (amendment 13/02/2018)	SYN545974 - Oral (Gavage) Prenatal Developmental Toxicity Study in the Rat Report No. ██████ + Amendment 1 (2018) Document No. VV-412267 , SYN545974_10190 Test Facility ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.6.2	██████ ██████	26/10/2011	SYN545974 & SYN546022 – Preliminary Oral (Gavage) Prenatal Developmental Toxicity Dose Range Finding Study in the Rat Report No. ██████ Document No. VV-398209 , SYN545974_10003 Test Facility ██████ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N

KCA1 5.6.2	■■■■	05/09/2016	SYN545974 - Historical Control Data for SYN545974_10003 Report No. SYN545974_10003 Document No. VV-134282 , SYN545974_10454 Test Facility ■■■■ Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.6.2	■■■■	09/02/2018	Pydiflumetofen - Additional Historical Control Data to Support Prenatal Developmental Toxicity Studies in the Rabbit Report No. ■■■■ Document No. VV-264239 , SYN545974_10597 Test Facility ■■■■ Not GLP Unpublished	Y	N	N/A	SYN	N
KCA1 5.6.2	■■■■	23/02/2018	Pydiflumetofen - Additional Historical Control Data and Assessment to Support the Prenatal Developmental Toxicity Study in the Rabbit Report No. TK0103653 Document No. VV-264133 , SYN545974_10599 Test Facility ■■■■ Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.6.2	■■■■	23/04/2018	Pydiflumetofen - Additional Historical Control Data to Support the Prenatal Developmental Toxicity Study in the Rat Report No. TK0103655 Document No. VV-264361 , SYN545974_10623 Test Facility ■■■■ Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.6.2	■■■■	14/05/2015	SYN545974 - Oral (Gavage) Prenatal Developmental Toxicity Study in the Rabbit Report No. ■■■■ Document No. VV-412446 , SYN545974_10177 Test Facility ■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.6.2	■■■■	08/06/2015	SYN545974 - Preliminary Oral (Gavage) Prenatal Developmental Toxicity Study in the Rabbit Report No. ■■■■■ Document No. VV-412592 , SYN545974_10192 Test Facility ■■■■■ Not GLP Unpublished This is CONFIDENTIAL INFORMATION	Y	N	N/A	SYN	N
KCA1 5.7.1	■■■■	15/09/2015	SYN545974 – Acute Oral (Gavage) Neurotoxicity Study in the Wistar Rat Report No. ■■■■■ Document No. VV-412490 , SYN545974_10198 Test Facility ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.7.1	■■■■	15/09/2015	SYN545974 – An Abbreviated Acute Oral (Gavage) Neurotoxicity Study in the Female Wistar Rat Report No. ■■■■■ Document No. VV-412489 , SYN545974_10197 Test Facility ■■■■■ ■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Armstrong, M.	31/12/1993	2,4,6-Trichlorophenol (TCP) induces chromosome breakage and aneuploidy in vitro Report No. N/A Document No. VV-137989 , NA_13759 Test Facility N/A Mutation Research, 303 (1993) 101-108 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Arrhenius, E.	31/12/1977	Disturbance of microsomal detoxication mechanisms in Liver by chlorophenol pesticides Report No. N/A Document No. VV-137994 , NA_13764 Test Facility N/A Chem.-Biol. Interactions, 18 (1977) 35-46 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	Bahig, M.	31/12/1981	Excretion and metabolism of 2,4,6-trichlorophenol-14c in rats Report No. N/A Document No. VV-137986 , NA_13756 Test Facility N/A Chemosphere, Vol. 10, No, 3, pp 323 - 327, 1981 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Baliko va, M.	31/12/1988	Process of excretion and toxicity of lindane and its metabolite 2,4,6-trichlorophenol in rats after acute oral dosage Report No. N/A Document No. VV-138070 , NA_13791 Test Facility N/A Biochem Clin. Bohemoslov. 17. 1988. 3. s. 193-196 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	■■■■■ ■■■■■	30/08/2016	SYN545974 vs SYN545547 MULTI-(Q)SAR genotoxicity assessment (DPD) Report No. N/A Document No. VV-175977 , SYN545974_10433 Test Facility N/A Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.8.1	■■■■■ ■■■■■ ■■■■■ ■■■■■	19/04/2018	NOA449410 - Additional Historical Control Data to Support the Prenatal Developmental Toxicity Study in the Rabbit Report No. 2009/1072507 Document No. VV-264300 , NOA449410_10013 Test Facility ■■■■■ ■■■■■ Not GLP Unpublished	Y	N	N/A	SYN	N
KCA1 5.8.1	Bercz, J.	31/12/1990	Subchronic Toxicity Studies of 2,4,6-Trichlorophenol in Sprague-Dawley Rats Report No. N/A Document No. VV-137992 , NA_13762 Test Facility N/A Journal of the American College of Toxicology Volume 9, Number 5, 1990 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	Blackburn, K.	31/12/1986	Evaluation of the Reproductive Toxicology of 2,4,6-Trichlorophenol in Male and Female Rats Report No. N/A Document No. VV-137991 , NA_13761 Test Facility N/A Fundamental and Applied Toxicology 6, 233- 239 (1986) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	██████ ██████ ██████	26/11/2009	CSAA798670 - Chromosome Aberration Test in Human Lymphocytes In Vitro Report No. 1266902 Document No. VV-385866 , NOA449410_10001 Test Facility RCC Cytotest Cell Research GmbH GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████ ██████	18/12/2013	SYN508272 - In Vitro Chromosome Aberration Test in Human Lymphocytes Report No. 1555902 Document No. VV-406535 , SYN508272_10904 Test Facility Harlan Cytotest Cell Research GmbH (Harlan CCR) GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████	18/04/2018	SYN547897 - Chemical Read-Across for Genotoxicity Potential Using a Multi-(Q)SAR Approach Report No. TK0210177 Document No. VV-264363 , SYN545974_10625 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.8.1	██████ ██████	09/04/2021	CA6519 - 14-Day In Vivo Dose Range Finder Assay in Rats Report No. AG23LM.DRF000R.BTL Document No. VV-898118 Test Facility ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.8.1	■■■■	09/04/2021	In Vivo Mutation Assay of CA6519 at the cII Locus in Big Blue® Transgenic F344 Rats Report No. AG23LM.171.BTL Document No. VV-898121 Test Facility ■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Butt, C.	31/12/2011	Halogenated Phenolic Contaminants Inhibit the In Vitro Activity of the Thyroid-Regulating Deiodinases in Human Liver Report No. N/A Document No. VV-138063 , NA_13784 Test Facility N/A Toxicological Sciences 124(2), 339–347 (2011) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Carlson, G.	31/12/1978	Effect of trichlorophenols on xenobiotic metabolism in the rat Report No. N/A Document No. VV-138157 , NA_13803 Test Facility N/A Toxicology, 11 (1978) 145-151 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Cascorbi, I.	31/12/1989	Correlation between the lipophilicity of substituted phenols and their inhibition of the Na <sup>+</sup> /K <sup>+</sup> -ATPase of Chinese hamster ovary cells Report No. N/A Document No. VV-138156 , NA_13802 Test Facility N/A Toxicology, 58 (1989) 197-210 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	■■■■	26/10/2020	CA6519 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay Report No. 2113900 Document No. VV-877637 Test Facility ICCR-Roßdorf GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.8.1	██████ ██	02/10/2009	NOT FOR SUBMISSION Reg.No. 5621781 (Metabolite of BAS 700 F) Acute oral toxicity study in rats Report No. 2009/1084176 Document No. VV-404052 , SYN508272_10903   R958945_11278 Test Facility ██████████ ████████████████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██	24/06/2014	SYN508272 - Micronucleus Assay in Bone Marrow Cells of the Rat Report No. 1602600 Document No. VV-407604 , SYN508272_10910 Test Facility ██████████ ████████████████████ ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████ ██████	13/11/2015	SYN508272 - A 28 Day Dietary Toxicity Study in Rats Report No. 35015 Document No. VV-414642 , SYN508272_10919 Test Facility ██████████ ████████████████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Ekwall , B.	31/12/1987	Toxicity of Chlorophenols to HeLa Cells as Measured in the MIT-24 System Report No. N/A Document No. VV-138137 , NA_13767 Test Facility N/A Alternatives to laboratory animals: ATLA 14(3): 178-181 Not GLP Published	N/A	N	N/A	N/A	N



KCA1 5.8.1	Exon, J.	31/12/1985	Toxicity of 2-Chlorophenol, 2,4-Dichlorophenol, and 2,4,6-Trichlorophenol Report No. N/A Document No. VV-138158 , NA_13804 Test Facility N/A (1985) pp. 307-330. Jolley, R. L. et al. (ED.). Water Chlorination, VOL. 5. Chemistry, Environmental Impact And Health Effects. Fifth Conference On Water Chlorination: Environmental Impact And Health Effects, Lewis Publishers, INC. Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Fahrig, R.	31/12/1978	Genetic Activity of Chlorophenols and Chlorophenol Impurities Report No. N/A Document No. VV-137995 , NA_13765 Test Facility N/A Pentachlorophenol Volume 12 of the series Environmental Science Research pp 325-338 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Galloway, S.	31/12/1987	Chromosome Aberrations and Sister Chromatid Exchanges in Chinese Hamster Ovary Cells: Evaluations of 108 Chemicals Report No. N/A Document No. VV-138062 , NA_13783 Test Facility N/A Environmental and Molecular Mutagenesis Volume 10, Supplement 10:1-175 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	■■■■■ ■■■■■	15/02/2017	SYN545547 - Bacterial Reverse Mutation Test Report No. MK43KP Document No. VV-466959 , SYN545547_10005 Test Facility Envigo CRS Limited GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.8.1	■■■■	01/03/2017	SYN545547 - In Vitro Mutation Test using Mouse Lymphoma L5178Y Cells Report No. XS02YK Document No. VV-467049 , SYN545547_10007 Test Facility Envigo CRS Limited GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	■■■■	28/03/2017	SYN545547 - In Vitro Micronucleus Test in Human Lymphocytes Report No. NH83HP Document No. VV-467413 , SYN545547_10009 Test Facility Envigo CRS Limited GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Gotz, R.	31/12/1980	Effects of Pentaachlorophenol and 2,4,6-Triehtlorophenol on the Disposition of Sulfobromophthalein and Respiration of Isolated Liver Cells Report No. N/A Document No. VV-138152 , NA_13798 Test Facility N/A Arch. Toxicol. 44, 147-155 (1980) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	■■■■	04/03/2021	CA6519 - 14 Day Oral (Gavage) Dose Range-Finding Study in the Rat Report No. ■■■■ Document No. VV-894153 Test Facility ■■■■ Not GLP Unpublished	Y	N	N/A	SYN	N
KCA1 5.8.1	Hattula , M.	31/12/1985	Mutagenesis Of Mammalian Cells In Culture By Chlorophenols, Chlorocatechols And Chloroguaiacols Report No. N/A Document No. VV-138136 , NA_13766 Test Facility N/A Chemosphere, Vol.14, No.10, pp 1617-1625, 1985 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	Haworth, S.	31/12/1983	Salmonella Mutagenicity Test Results for 250 Chemicals Report No. N/A Document No. VV-138075 , NA_13796 Test Facility N/A Environmental Mutagenesis Supplement 1:3-142 (1983) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Heil, J.	31/12/1992	Detection of mammalian carcinogens with an immunological DNA synthesis- inhibition test Report No. N/A Document No. VV-138064 , NA_13785 Test Facility N/A Carcinogenesis vol.13 no.12 pp.2389-2394, 1992 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Henschler, R.	31/12/2001	Proliferation and differentiation of murine haemopoietic progenitor cells in stroma-free culture in the presence of metabolites of chlorinated pesticides Report No. N/A Document No. VV-138150 , NA_13780 Test Facility N/A Toxicology in Vitro 15 (2001) 31-37 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Huff, J.	31/12/2012	Long-term toxicology and carcinogenicity of 2,4,6- trichlorophenol Report No. N/A Document No. VV-138169 , NA_13807 Test Facility N/A Chemosphere 89 (2012) 521– 525 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Janssón, K.	31/12/1986	Inability of chlorophenols to induce 6-thioguanine-resistant mutants in V79 Chinese hamster cells Report No. N/A Document No. VV-138139 , NA_13769 Test Facility N/A Mutation Research, 171 (1986) 165-168 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	Jansso n, K.	31/12/1992	Genotoxicity of 2,4,6- trichlorophenol in V79 Chinese hamster cells Report No. N/A Document No. VV-137988 , NA_13758 Test Facility N/A Mutation Research, 280 (1992) 175-179 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Jansso n, K.	31/12/1993	The toxicity of chlorophenols in V79 Chinese hamster cells Report No. N/A Document No. VV-138141 , NA_13771 Test Facility N/A Toxicology Letters, 69 (1993) 289-294 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Jia, R- W	30/04/2010	Cytotoxic Characteristics and Plasma Membrane Component Analysis in Vero Cells Exposed to 2,4,6-trichlorophenol Report No. N/A Document No. VV-138168 , NA_13806 Test Facility N/A J Environ Health, April 2010, Vol. 270, No. 4 pp302-305 Not GLP Published	N/A	N	N/A	SYN	N
KCA1 5.8.1	Judis, J.	31/12/1982	Binding of Selected Phenol Derivatives to Human Serum Proteins Report No. N/A Document No. VV-138073 , NA_13794 Test Facility N/A Journal of Pharmaceutical Sciences Vol. 71, No. 10, October 1982 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Juhl, U.	31/12/1989	The in vitro metabolites of 2,4,6-trichlorophenol and their dna strand breaking properties Report No. N/A Document No. VV-137987 , NA_13757 Test Facility N/A Chem-BioL Interactions, 69 (1989) 333- 344 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	██████ ██████	08/10/2009	Repeated dose 90-day oral toxicity study in Wistar rats; Administration in the diet Report No. 2009/1072503 Document No. VV-404251 , R958945_11273 Test Facility ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Kitchin , K. Brown, J.	31/12/1988	Biochemical effects of three chlorinated phenols in rat liver Report No. N/A Document No. VV-138060 , NA_13781 Test Facility N/A Toxicological and Environmental Chemistry, Vol. 16 (3), pp. 165-172 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Kitchin , K. Brown, J.	31/12/1994	Dose-response relationship for rat liver DNA damage caused by 49 rodent carcinogens Report No. N/A Document No. VV-137973 , NA_13808 Test Facility N/A Toxicology 88 (1994) 31-49 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Ma, Y.	31/12/2011	Modulation of steroidogenic gene expression and hormone synthesis in H295R cells exposed to PCP and TCP Report No. N/A Document No. VV-138151 , NA_13797 Test Facility N/A Toxicology 282 (2011) 146–153 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Matsuo ka, A.	31/12/1998	In vitro clastogenicity of 19 organic chemicals found in contaminated water and 7 structurally related chemicals Report No. N/A Document No. VV-138068 , NA_13789 Test Facility N/A Environ. Mutagen Res., 20 : 159 - 165(1998) Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	McGregor, D.	31/12/1988	Responses of the L5178Y tk+/tk- Mouse Lymphoma Cell Forward Mutation Assay: 111. 72 Coded Chemicals Report No. N/A Document No. VV-138154 , NA_13800 Test Facility N/A Environmental and Molecular Mutagenesis U:SS-154 (1988) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Miyagawa, M.	31/12/1995	The in vivo-in vitro replicative DNA synthesis (RDS) test with hepatocytes prepared from male B6C3F1 mice as an early prediction assay for putative nongenotoxic (Ames-negative) mouse hepatocarcinogens Report No. N/A Document No. VV-138066 , NA_13787 Test Facility N/A Mutation Research 343 (1995) 157-183 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Murayama, J.	31/08/1990	Comparative Acute Cytotoxicities of 37 Xenobiotics Detected in Drinking Water to Rat Hepatocyte Primary Culture Report No. N/A Document No. VV-138144 , NA_13774 Test Facility N/A Japanese Journal of Toxicology and Environmental Health 36(4), 267-276, 1990-08-31 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	National Cancer Institute	31/12/1979	Bioassay of 2, 4, 6-trichlorophenol for possible carcinogenicity Report No. N/A Document No. VV-137983 , NA_13753 Test Facility N/A National Cancer Institute, Carcinogenesis, Technical Report Series NO. 155 1979 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	■■■■	25/05/2018	SYN548263 - Micronucleus Test in Human Lymphocytes In Vitro Report No. 1880200 Document No. VV-469574 , SYN548263_10003 Test Facility Envigo CRS GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	■■■■	27/10/2020	CA6519 - Micronucleus Test in Human Lymphocytes In Vitro Report No. 2114000 Document No. VV-877675 Test Facility ICCR-Roßdorf GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Ono, Y.	31/12/1996	Evaluation of DNA damage by active oxygen induced by organochlorine compounds and nitroarenes Report No. N/A Document No. VV-138071 , NA_13792 Test Facility N/A Journal of Japan Society on Water Environment Vol. 19 (1996) No. 11 P 871-877 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Onoder a, S.	31/12/1998	Behaviour of Mutagenic Formation from Phenolic Compounds in Water Disinfection with Chlorine and their mutagenic potential formation Report No. N/A Document No. VV-138072 , NA_13793 Test Facility N/A Jpn. J. Toxicol. Environ. Health Hygienic chemistry 44(4) 289-299 (1998) Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	Ozaki, A.	31/12/2004	Chemical analysis and genotoxicological safety assessment of paper and paperboard used for food packaging Report No. N/A Document No. VV-138147 , NA_13777 Test Facility N/A Food and Chemical Toxicology 42 (2004) 1323–1337 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	██████████ ██████████ ██████████ ██████████ ██████████	26/02/2021	Pydiflumetofen - Multi-(Q)SAR and Read across Genotoxicity Assessment of Pydiflumetofen and Definition of Residue Metabolites Report No. Pydiflumetofen QSAR-1 Document No. VV-893259 Test Facility Syngenta Ltd Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.8.1	██████████ ██████████ ██████████ ██████████ ██████████	26/02/2021	Pydiflumetofen - QSAR Report for dietary metabolites Report No. N/A Document No. VV-893581 Test Facility Syngenta Ltd Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.8.1	Pekari, K.	31/12/1986	Kinetics of 2,4,6-trichlorophenol in different organs of the rat Report No. N/A Document No. VV-137990 , NA_13760 Test Facility N/A Arch Toxicol (1986) 59: 41-44 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Pereira , M.	31/12/1982	Initiation/promotion bioassay in rat liver: use of gamma glutamyltranspeptidasepositive foci to indicate carcinogenic activity Report No. N/A Document No. VV-138146 , NA_13776 Test Facility N/A Toxicologic Pathology ISSN: 0192-6233 Vol. 10, No. 2, 1982 Not GLP Published	N/A	N	N/A	N/A	N



KCA1 5.8.1	██████ ██	21/01/2011	DF-Pyrazole acid (CA4312):Screening Acute Oral Toxicity Study In The Rat Report No. ██████ 2364/0169 Document No. VV-380723 , CA4312/0003 Test Facility ██████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████.	31/12/2014	In situ electrochemical assessment of cytotoxicity of chlorophenols in MCF-7 and HeLa cells Report No. N/A Document No. VV-138138 , NA_13768 Test Facility N/A Analytical Biochemistry 462 (2014) 60–66 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Ransan en, L.	31/12/1977	The Mutagenicity of MCPA and Its Soil Metabolites, Chlorinated Phenols, Catechols and Some Widely Used Slimicides in Finland Report No. N/A Document No. VV-138074 , NA_13795 Test Facility N/A Bulletin of Environmental Contamination & Toxicology Vol. 18, No. 5 1977 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Russell , L. Selby, P. Sherid an, W. Valcov ic, L. Von Halle, E.	31/12/1981	Use of the mouse spot test in chemical mutagenesis: interpretation of past data and recommendations for future work Report No. N/A Document No. VV-138148 , NA_13778 Test Facility N/A Mutation Research, 86 (1981) 355-379 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	Sakazaki, H.	31/12/2001	Immunotoxicological Evaluation of Environmental Chemicals Utilizing Mouse Lymphocyte Mitogenesis Test Report No. N/A Document No. VV-138142 , NA_13772 Test Facility N/A Journal of Health Science, 47(3) 258-271 (2001) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Sasaki, Y.	31/12/2000	The Comet Assay with Multiple Mouse Organs: Comparison of Comet Assay Results and Carcinogenicity with 208 Chemicals Selected from the IARC Monographs and U.S. NTP Carcinogenicity Database Report No. N/A Document No. VV-137993 , NA_13763 Test Facility N/A Critical Reviews in Toxicology, 30(6):629-799 (2000) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	■■■■■	16/03/2018	SYN548263 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay Report No. 1880400 Document No. VV-469506 , SYN548263_10002 Test Facility Envigo CRS GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Shannon, R.	31/12/1991	Mitochondrial response to chlorophenols as a short-term toxicity assay Report No. N/A Document No. VV-138140 , NA_13770 Test Facility N/A Environmental Toxicology and Chemistry, Vol. 10, pp. 57-66, 1991 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	██████ ██	10/04/2018	SYN508272 - The Determination of SYN508272 in Rat Blood:Water: (50:50) by LC-MS/MS in Samples Generated in Study 1602600 Report No. 0057/003 Document No. VV-469572 , SYN508272_10923 Test Facility ██████ ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████ ██████ ██████ ██████	13/10/2009	@CSAA798670: Prenatal developmental toxicity study in New Zealand white rabbits - Oral administration (gavage) Report No. 2009/1072507 Document No. VV-393721 , NOA449410_50005   NOA449410_10005 Test Facility ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Shi, Y-L.	31/12/2008	Cytotoxic Response Characteristics and Sensitivity in Mammalian Vero Cells Exposed to 2,4,6-Trichlorophenol Report No. N/A Document No. VV-137984 , NA_13754 Test Facility N/A Asian Journal of Ecotoxicity Vol. 3, 2008, No. 5, 479-487 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	██████ ██████ ██████	24/07/2007	DF-pyrazole acid (CA4312) Salmonella typhimurium and escherichia coli reverse mutation assay Report No. RCC 1077403 Document No. VV-380339 , SYN520453/0096 Test Facility RCC Cytotest Cell Research GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████ ██████	13/03/2014	SYN508272 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay Report No. 1555901 Document No. VV-407008 , SYN508272_10908 Test Facility Harlan Cytotest Cell Research GmbH (Harlan CCR) GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.8.1	██████ ██████ ██████	06/04/2018	SYN548263 - Cell Mutation Assay at the Thymidine Kinase Locus (TKP+/-) in Mouse Lymphoma L5178Y Cells Report No. 1880500 Document No. VV-469505 , SYN548263_10001 Test Facility Envigo CRS GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████ ██████	23/10/2020	CA6519 – Gene Mutation Assay in Chinese Hamster V79 Cells in vitro (V79/HPRT) Report No. 2114300 Document No. VV-877648 Test Facility ICCR-Roßdorf GmbH GLP Unpublished	N	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██████	11/01/2010	CSAA798670 – 28-Day Oral (Dietary) Toxicity Study in the Wistar Rat Report No. ████████ Document No. VV-386115 , NOA449410_10003 Test Facility ████████ ██████████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Stoner, G.	31/12/1986	Comparison of Two Routes of Chemical Administration on the Report No. N/A Document No. VV-138153 , NA_13799 Test Facility N/A Toxicology and Applied Pharmacology 82, 19-31 (1986) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Strobel , K.	31/12/1987	Aliphatic and Aromatic Halocarbons as Potential Mutagens in Drinking Water Report No. N/A Document No. VV-138145 , NA_13775 Test Facility N/A Toxicological and Environmental Chemistry, 1987, Vol. 14, pp. 143-156 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	██████ ██	07/04/2021	CA6519 - 28 Day Oral (Gavage) Toxicity Study in the Rat Report No. ██████ Document No. VV-897862 Test Facility ██████ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	██████ ██	31/12/1992	Cytotoxicity and Mutagenicity of Micropollutants in Drinking Water Report No. N/A Document No. VV-138149 , NA_13779 Test Facility N/A Wal. Sci. Tech. Vol 2S, No. 11. PP- 325-332. 1992. Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Valenc ia, R.	31/12/1985	Chemical Mutagenesis Testing in Drosophila. 111. Results of 48 Coded Compounds Tested for the National Toxicology Program Report No. N/A Document No. VV-138143 , NA_13773 Test Facility N/A Environmental Mutagenesis 7:325-348 (1985) Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Van Delft, J.	31/12/2005	Discrimination of genotoxic from non-genotoxic carcinogens by gene expression profiling Report No. N/A Document No. VV-138065 , NA_13786 Test Facility N/A Carcinogenesis vol.26 no.2 p.511, 2005 and Carcinogenesis vol.25 no.7 pp.1265-1276, 2004 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Van den Berg, K.	31/12/1990	Interaction of chlorinated phenols with thyroxine binding sites of human transthyretin, albumin and thyroid binding globulin Report No. N/A Document No. VV-138061 , NA_13782 Test Facility N/A Chem.-Biol. Interactions, 76 (1990) 63-75 Not GLP Published	N/A	N	N/A	N/A	N

KCA1 5.8.1	■■■■	30/11/2009	CSAA798670 – Cell Mutation Assay at the Thymidine Kinase Locus (TK +/-) in Mouse Lymphoma L5178Y Cells Report No. 1266901 Document No. VV-385865 , NOA449410_10000 Test Facility RCC Cytotest Cell Research GmbH GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	■■■■	18/12/2013	SYN508272 - Cell Mutation Assay at the Thymidine Kinase Locus (TK+/-) in Mouse Lymphoma L5178Y Cells Report No. 1555903 Document No. VV-406536 , SYN508272_10906 Test Facility Harlan Cytotest Cell Research GmbH (Harlan CCR) GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N
KCA1 5.8.1	Yin, D.	31/12/2009	Genotoxic effect of 2,4,6-trichlorophenol on p53 gene in zebrafish liver Report No. N/A Document No. VV-137982 , NA_13752 Test Facility N/A Environmental Toxicology and Chemistry, Vol. 28, No. 3, pp. 603–608, 2009 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.1	Zarn, J.	18/04/2010	The Significance of the Subchronic Toxicity in the Dietary Risk Assessment of Pesticides Report No. N/A Document No. VV-897630 Test Facility _See_Document_Organisation Publication Name: Regulatory Toxicology and Pharmacology ISSN/ISBN: 02732300 Year: 10/2010 Vol (Iss) Pgs: 58 (1) p.72-78 Not GLP Published	N/A	N/A	N/A	N/A	N

KCA1 5.8.1	■■■■	31/12/2014	2,4,6-Trichlorophenol Cytotoxicity Involves Oxidative Stress, Endoplasmic Reticulum Stress, and Apoptosis Report No. N/A Document No. VV-137985 , NA_13755 Test Facility N/A International Journal of Toxicology 2014, Vol. 33(6) 532-541 Not GLP Published	N/A	N	N/A	N/A	N
KCA1 5.8.2	■■■■	07/01/2016	SYN545974 - Position Statement Concerning Immunotoxicity Potential Report No. N/A Document No. VV-134402 , SYN545974_10361 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.8.2	■■■■	11/11/2014	SYN545974 – Effect on Rat Thyroid Peroxidase Activity In Vitro Report No. 5523/1/2/2014 Document No. VV-410417 , SYN545974_10120 Test Facility Leatherhead Food Research (LFR) Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.8.2	■■■■	14/08/2015	SYN545974 - Effect on Hepatic UDPglucuronosyltransferase Activity Towards Thyroxine as Substrate After Dietary Administration for 90 Days to Male Rats Report No. 5522/1/2/2014 Document No. VV-413339 , SYN545974_10264 Test Facility ■■■■■■ ■■■■■■■■■■■■■■■■■■■■ GLP Unpublished	Y	Y	This study is necessary for this regulatory decision and is eligible for data protection	SYN	N

KCA1 5.8.3	[REDACTED]	24/07/2020	Pydiflumetofen - Review for Potential Endocrine Disruption Report No. TK0259197 Document No. VV-639581 , SYN545974_10638 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.8.3	[REDACTED]	24/07/2020	Pydiflumetofen - Endocrine Disruption - Appendix E Update July 2020 Report No. N/A Document No. VV-866644 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished	N	N	N/A	SYN	N
KCA1 5.8.3	[REDACTED]	11/12/2015	SYN545974 – Review for Potential for Endocrine Disruption in Mammalian Species Report No. N/A Document No. VV-414870 , SYN545974_10345 Test Facility Syngenta - Jealott's Hill Not GLP Unpublished This is CONFIDENTIAL INFORMATION	N	N	N/A	SYN	N
KCA1 5.4.2	[REDACTED]	26/05/2017	SYN545974 - Rat Bone Marrow Chromosome Aberration Assay	KCA1 5.4.2	[REDACTED]	26/05/2017	SYN545974 - Rat Bone Marrow Chromosome Aberration Assay	KCA1 5.4.2

\*Syngenta requests data confidentiality for these data. Disclosure of the information might undermine Syngenta's commercial interests by providing access to Syngenta specific know-how used to develop unique positions and approaches to risk assessment.