European Commission

VOLUME 3 – Annex B (AS)

- CHLOROTHALONIL -

B.6b Toxicology and metabolism

Section B.6.8.1 Toxicity studies of metabolites and relevant impurities[1]

Rapporteur Member State: The Netherlands

August 2016

August 2017

October 2017

Renewal Assessment Report and Proposed decision of the Netherlands prepared in the context of the possible approval of chlorothalonil under Regulation (EC) 1107/2009

[1] Due to the large number of metabolites and associated toxicity studies, this separate Volume 3 B.6b has been prepared containing only section B.6.8.1. For the other sections it is referred to Volume 3 B.6a.

Version history page

Date	Version history
May 2016	Draft Renewal Assessment Report
August 2016	Initial Renewal Assessment Report
August 2017	Updated Renewal Assessment Report according to the comments in the Evaluation Table. Revisions are in yellow, except for typo's which are not marked. Due to the large number of metabolites and associated toxicity studies, this separate Volume 3 B.6b has been prepared containing only section B.6.8.1. For the other sections it is referred to Volume 3 B.6a.
October 2017	Revised Renewal Assessment Report according to the comments during the peer review experts' meeting (PPR meeting 162 (session 2), 11-14 September 2017)

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B.6 Toxicology and metabolism data

B.6.1 Absorption, distribution, metabolism and excretion in mammals

Refer to Volume 3, B.6a (AS)

B.6.2 Acute toxicity

Refer to Volume 3, B.6a (AS)

B.6.3 Short-term toxicity

Refer to Volume 3, B.6a (AS)

B.6.4 Genotoxicity

Refer to Volume 3, B.6a (AS)

B.6.5 Long-term toxicity and carcinogenicity

Refer to Volume 3, B.6a (AS)

B.6.6 Reproductive toxicity

Refer to Volume 3, B.6a (AS)

B.6.7 Neurotoxicity

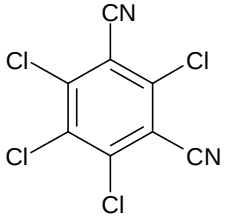
Refer to Volume 3, B.6a (AS)

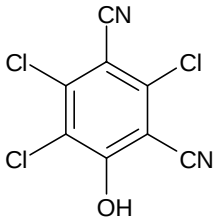
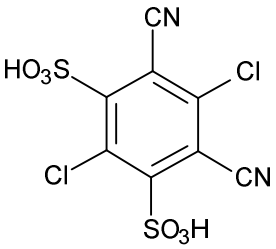
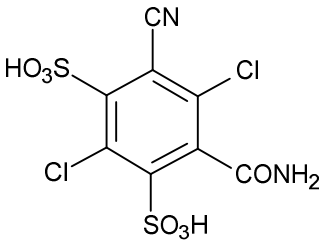
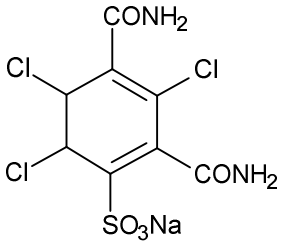
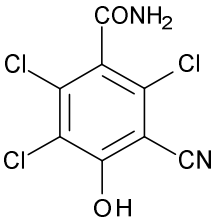
B.6.8 Other toxicological studies

B.6.8.1 Toxicity studies of metabolites and relevant impurities

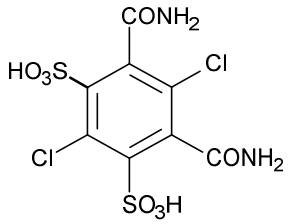
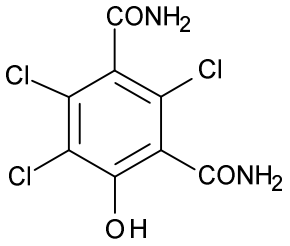
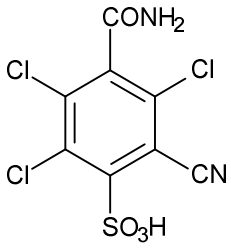
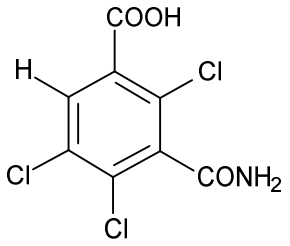
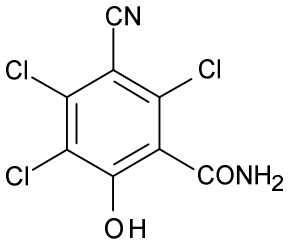
Due to the large number of metabolites and a large number of toxicity studies, the chapter layout of B.6.8.1. has been adjusted using subparagraphs referring to the data requirements..

Table 6.8.1: Substances and metabolites; structures, codes, synonyms

Code Number (Synonyms)	Description	Compound found in:	Structure
Chlorothalonil R044686 SDS 2787 1897-45-6	IUPAC name: 2,4,5,6-tetrachloro-isophtalonitrile		

Code Number (Synonyms)	Description	Compound found in:	Structure
R182281 SDS 3701 R1 Compound 2 C5 28343-61-5 CSCA105253	IUPAC name: 2,5,6-trichloro-4-hydroxyisophthalonitrile	Crop (lettuce, tomato, carrot, wheat, rotated crops) Livestock (hen, goat) Rat	
R418503 M13 R8 Compound 11 CSCA654600 SYN548708 (Na salt)[2]	IUPAC name: 2,5 dichloro-4,6 dicyano-benzene-1,3 disulfonic acid	Soil (aerobic) Groundwater Crop (rotated crops)	
R419492 M8 R15 Compound 12 CSCA655149	IUPAC name: 4-amido-2,5-dichloro-6-cyano benzene-1,3-disulfonic acid	Groundwater Soil (aerobic) Rat	
R471811 M4 R7 Compound 13 CSCA202566 SYN548766	IUPAC name: sodium 2,4-bis-amido-3,5,6-trichlorobenzenesulfonate	Groundwater Soil (aerobic) Crop (rotated crops)	
SYN507900 SDS66882 CSCC210323	IUPAC name: 2,4,5-trichloro-3-cyano-6-hydroxy-benzamide	Groundwater	

[2] used for gentox testing

Code Number (Synonyms)	Description	Compound found in:	Structure
SYN548008 M3 CSCY735822	IUPAC name: 4,6-dicarbamoyl-2,5-dichloro-benzene-1,3-disulfonic acid	Groundwater	
SYN548580 M2 R12 CSDB870985	IUPAC name: 2,4,5-trichloro-6-hydroxy-benzene-1,3-dicarboxamide	Groundwater	
SYN548581 (base) SYN548764 (sodium salt) M11 CSDB870988	~~IUPAC name: 2,3,6-trichloro-5-cyano-4-sulfanyl-benzamide~~ 4-carbamoyl-2,3,5-trichloro-6-cyano-benzenesulfonic acid		
R611965 M5 SDS 46851 R14 Compound 4	IUPAC name: 3-amido-2,4,5-trichlorobenzoic acid	Groundwater Soil (aerobic, anaerobic) Crop (snap beans, rotated crops) Rat	
R611968 M9 SDS 47525 R5	IUPAC name: 2,4,5-trichloro-3-cyano-6-hydroxybenzamide	Groundwater Crop (rotated crops)	

B.6.8.1 – 6.1 Toxicokinetic studies with metabolites

Data requirement 2.23

Applicant to provide results of the toxicokinetic studies with SDS-3701 and SDS-46851 in tabular format.

See also 2(68)
See reporting table 2(67)

NL (August 2017): Additional tables addressing the requested endpoints have been added to the RAR summaries (shown below). The conclusions by the RMS does not change.

B.6.8.1 – 6.1.1 Toxicokinetic study with SDS-3701 - study 1

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	:	Jarrett, 1978	exposure	:	single by gavage
type of study	:	metabolism	doses	:	4.3 and 62.4 mg/kg bw
year of execution	:	1978	vehicle	:	polyethylene glycol 400 (PEG-400)
test substance	:	[^{14}C]-SDS-3701 (radiochem. pur. 97%) and SDS-3701 (pur. 99%)	GLP statement	:	no
route	:	oral	guideline	:	see 'Guidelines and limitations'
species	:	rats (SD)			
group size	:	4 males / group			

Study design

SDS-3701 (R182281) is a soil metabolite of chlorothalonil taken up through the roots by crops. The absorption, tissue distribution and excretion of radiolabel after an oral dose of ^{14}C-SDS-3701 was studied in male rats (SD). Urine and faeces were collected until termination at 96 hours post-dose. Selected tissues were collected at termination. Samples were analysed for radioactivity directly by LSC or by combustion LSC.

Results

The overall recovery of radioactivity was around 100% of the administered dose at both dose levels. Faeces was the major route of elimination and contained 74 and 65% of the administered dose in the low dose and the high dose group. Urine contained 7.5% and 9.7% of the administered dose at the low and high dose, respectively. Total recovery in excreta was 81% and 75% of the administered dose at the low dose and high dose, respectively. The remainder of the administered radiolabel at both dose levels was distributed among body tissues. At 96 h post-dose, highest residue levels (GIT excluded) were determined in blood (3.6 and 41 µg equivalents/g in the low and high dose group), muscle (0.6 and 5.4 µg equivalents/g), and fat (3.1 and 3.6 µg equivalents/g). About 2.1 and 1.2% of

the administered dose was recovered from liver and 0.73 and 0.37% from kidneys of low and high dose animals, respectively. Total recovery from tissues (GIT excluded) accounted for 22 and 16% of the administered radiolabel in the low and high dose group, respectively. Together with the amount of radiolabel excreted via urine, these data indicate an oral absorption of at least 26 to 30%.

Table B.6.8.1 – 6.1.1-1: Total % Administered Dose (± SD) in Tissues Following a Single Oral Dose of [^{14}C]-SDS-3701 to Male Sprague Dawley Rat

	Dose	
Tissue	**4.2 mg/kg**	**62.4 mg/kg**
Adipose	3.05 ± 1.10	3.56 ± 2.71
Blood	6.68 ± 1.41	4.96 ± 0.81
Bone marrow	0.01 ± 0.05	0.01 ± 0.05
Heart	0.18 ± 0.05	0.12 ± 0.03
Kidneys	0.73 ± 0.18	0.37 ± 0.06
Large intestine	3.32 ± 1.25	3.42 ± 0.94
Liver	2.12 ± 0.70	1.22 ± 0.29
Lungs	0.40 ± 0.12	0.32 ± 0.06
Muscle	7.91 ± 1.19	4.71 ± 1.67
Small intestine	1.62 ± 0.33	1.42 ± 0.35
Spleen	0.07 ± 0.05	0.05 ± 0.01
Stomach	0.21 ± 0.05	0.29 ± 0.12
Testes	0.36 ± 0.14	0.25 ± 0.04

For recovery purposes biomass approximations for muscle and adipose were 45% and 5% of the total body weight, respectively, and 64.1 mL/kg for blood.

Table B.6.8.1 – 6.1.1-2: Mean Excretion of Radioactivity (as Percentage of Administered Dose) Following a Single Oral Dose of [14C]-SDS-3701

		4.2 mg/kg	**62.4 mg/kg**
Urine	0-24 h	2.91	4.71
	24-48 h	1.64	2.58
	48-72 h	1.39	1.18
	72-96 h	1.19	0.81
	Subtotal 0-96 h	*7.13*	*9.28*
Faeces	0-24 h	25.27	22.70
	24-48 h	20.84	24.62
	48-72 h	16.24	13.73
	72-96 h	11.19	7.49
	Subtotal 0-96 h	*73.54*	*68.54*

The tissue and excreta data show that SDS-3701 stays predominantly in the central compartment *i.e* blood and with the organs of excretion *i.e*. liver and kidney.

Summary and conclusion of the study

Absorption

Based on the amount of radiolabel recovered from urine and tissues, an oral absorption of at least 26 to 30% was calculated.

Distribution

Approx. 22% and 16% of an administered low and high oral dose of ^{14}C-SDS-3701 to rats, respectively, was retained in non-GIT tissues 96 h post-dose. Highest levels were found in blood,

muscle, fat, and kidney. From the kidney, 0.73% and 0.37% of the dose was recovered in the low and high dose animals.

Excretion

Radiolabel of ^{14}C-SDS-3701 orally dosed rats was mainly excreted via faeces (74% and 65% for low and high dose, respectively within 96 h). Within 96 h, 7.5% and 9.7% of the doses was excreted via the urine.

Guidelines and limitations

No bile-duct cannulation was performed, indicating that actual absorption of SDS-3701 may be higher than the 26 to 30% of the administered dose that was excreted in urine.

B.6.8.1 – 6.1.2 Toxicokinetic study with SDS-46851- study 2

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	:	Savides et al., 1990c	exposure	:	single by gavage
type of study	:	absorption, elimination	doses	:	10, 1000 mg/kg bw
year of execution	:	1988	vehicle	:	0.75% methylcellulose/water (w/v)
test substance	:	[^{14}C]-SDS-46851 (radiochem. pur. 95.2%) and SDS-46851 (pur. 99.7%)	GLP statement	:	yes
route	:	oral	guideline	:	see 'Guidelines and limitations'
species	:	rats (SD)			
group size	:	5/sex/dose group/time of sacrifice			

Study design

SDS-46851 is a soil metabolite of SDS-2787 (chlorothalonil), taken up through the roots by crops. In order to determine the absorption and elimination of radiolabel following an oral administration of SDS-46851 to male and female rats, ^{14}C-SDS-46851 was administered by single oral gavage at 10 and 1000 mg/kg bw doses. Blood was sampled at various time points and urine and faeces were collected until sacrifice. Animals were sacrificed at 36, 42 or 48 h post-dose and liver, kidneys and GIT-tissues and contents were collected. Samples were analysed for radioactivity by LSC or combustion LSC.

Results

A summary of the excretion and recovery data is presented in Table 6.8.1 – 6.1.2.

Table 6.8.1 – 6.1.2: Excretion and recovery of radioactivity following oral administration of ^{14}C-SDS-46851 [1]

Dose (mg/kg)	Sex [2]	Urine [3]	Faeces	Recovered approx. [4]
10	m (16)	10-17	79-87	77-100
10	f (12)	9.5-20	61-81	77-100
1000	m (14)	3.6-6.7	54-64	61-95
1000	f (14)	3.2-6.2	44-80	61-95

[1] In percentage of the administered dose. Presented are ranges of amounts detected at the three different times of sacrifice.
[2] Numbers in parentheses indicate number of animals. Because for some animals, the total amount of radiolabel recovered was greater than 112% of the administered radiolabel, these were omitted from the results. Therefore some subgroups are <15 animals.
[3] Values include cage washes which contained and average of ≤2.3% of the administered dose.
[4] Percentage recovered includes gastrointestinal tracts, livers, and kidneys as well as excreta and are approximate values. Since the carcasses and the majority of tissues were not analysed, and there were losses of urine and faeces during sampling of the blood, an accurate material balance could not be calculated.

The results indicate that faeces was the major route for excretion of radiolabel for both sexes at both dose levels. Up to 61-87% and 44-80% of the administered radiolabel was excreted via the faeces within 48 h in the low and high dose groups, respectively. Up to 9.5-19.5% and 3.2-6.7% of the administered radiolabel was recovered from the urine within 48 h in the low and high dose groups, respectively. The high dose resulted in a prolonged excretion of radiolabel via the urine and via the faeces, which may have been due to an effect on the motility of the GIT resulting in a prolonged time period in which absorption occurred. Analyses of the GIT contents also indicated that more than 48 hours were needed to complete the excretion of the radiolabel. At termination (36, 42 or 48 hours), only trace amounts of radiolabel were detected in liver (≤ 0.30% of the administered dose) and kidneys (≤ 0.03%) at both dose levels. Since the carcasses and the majority of tissues were not analysed, and there were losses of urine and faeces during sampling of the blood, an accurate material balance could not be calculated.

The concentration of the radiolabel in the blood was used to calculate the half-life, the time at which the peak radiolabel concentration occurred, and the area under the blood concentration versus time curve (AUC). There were no statistical differences in these parameters between sexes within a given dose group. The ratio of the AUC values at low and high doses (277000 / 4050 = 68) was rather comparable to the actual dose ratio (1000 / 10 = 100) at the 5% level of significance for both sexes. These data suggest that bioavailability was proportional to dose level. Peak blood concentrations were estimated to occur within 1.5 and 2 h at the low and high dose level, respectively. The half-life for elimination of radiolabel from blood was calculated to be 2.5 h and was estimated to be 6.2 h, at 10 and 1000 mg/kg dose, respectively.

B.6.8.1 – 6.1.3 Toxicokinetic study with SDS-46851 - study 3

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. This study is the same study as study 4 (Ho M D, Marciniszyn J P and Killeen J C (1990), which was evaluated in the addendum 14 of the DAR (april 2004)

Characteristics

reference	: Ho, 1990	exposure	: single by gavage
type of study	: distribution and excretion	doses	: 10, 1000 mg/kg bw
year of execution	: 1990	vehicle	: 0.75% methylcellulose/water (w/v)
test substance	: [^{14}C]-SDS-46851 (radiochem. pur. 95.2%) and SDS-46851 (pur. 99.3%)	GLP statement	: yes
route	: oral	guideline	: see 'Guidelines and limitations'
species	: rats (SD)		
group size	: 5/dose/sex		

Study design

In order to determine the amount of radiolabel excreted in expired air, urine and faeces, and to determine the distribution of radiolabel in tissues following a single oral administration, ^{14}C-SDS-46851 was administered by single oral gavage at 10 and 1000 mg/kg bw doses. Expired air was collected for 6, 12 and 24 hours after dosing. Urine and faeces were collected until sacrifice. Animals were sacrificed 168 h post-dose. Blood, various tissues, and the carcass were sampled at necropsy. Samples were analysed by LSC or combustion LSC. At termination, cages were rinsed to collect any residual radioactivity that might have adhered to the cage walls.

Results

The average total recovery of radiolabel ranged from 91% to 99% of the administered dose. Essentially no radiolabel (<0.02%) was found in expired air during 24 h post-dose sampling and <0.3% was found in tissues and carcasses. In low dose animals, 90% of the faecal excretion occurred within 48 h (68% of the administered dose by males to 74% by females). The same occurred with urinary excretion (22% by males to 24% by females). At the high dose level, 90% of the total faecal excretion (69% of the administered dose by males, 76% by females) occurred within 72 h post-dose, and 90% of the total urinary excretion (26% by males and 17% by females) occurred within 96 h post-dose.

Table B.6.8.1 – 6.1.3-1: Mean Excretion of Radioactivity Expressed as Percentage of Administered Dose, Following a Single Oral Dose of [14C]-SDS-46851 to Rats

	Dose			
	10 mg/kg		1000 mg/kg	
	Male	Female	Male	Female
Urine	21.77	24.00	26.37	16.68
Faeces	68.48	73.70	69.38	76.40
Exp. Air	0.00	0.00	0.02	0.00
Tissues	0.10	0.20	0.13	0.05
Cage Wash	0.34	0.79	0.70	0.89
Total Recovery	90.68	98.69	96.60	94.02

(results expressed as % administered dose)

The mean % of dose excreted in urine was between 17 and 26%. Therefore, the fraction of dose that is systemically available is >17%.

Summary and conclusion on SDS-46851 (Savides, 1990c, and Ho, 1990).

Absorption

Upon a single oral administration of 10 or 1000 mg/kg bw ^{14}C-SDS-46851 to male and female rats (SD), absorption was at least 17-26%, based on the recovered radiolabel in urine.

Distribution

Peak blood levels upon a single oral administration of 10 or 1000 mg/kg bw ^{14}C-SDS-46851 to rats (SD) of both sexes occurred at <1.5 and 2 h at the low and high dose level, respectively. The half-life for elimination of radiolabel from blood was 2.5 h at 10 mg/kg bw. Only trace amounts of radiolabel remained in liver (≤ 0.30%), kidneys (≤ 0.03%) and carcass (≤0.61%) at both dose levels.

Excretion

In rats (SD), upon a single oral administration of 10 or 1000 mg/kg bw, about ~~15~~ 17-26% and 68-80% of the administered dose were excreted via urine and faeces, respectively, within 48 h and 168 h for both dose groups. Only trace amounts were expired by air (<0.02% of the administered dose).

Guidelines and limitations

The combined experiments as described by Savides (1990c), and Ho (1990) were performed mainly in compliance OECD 417. No bile-duct cannulation was performed to establish the absorption more reliable.

B.6.8.1 – 6.1.4 Balance study with SDS-46851 - study 4

Previous evaluation	In addendum 14 DAR (april 2004)
Evaluation RMS	No remarks on original assessment. This study is the same study as study 3 (Ho, 1990)

Characteristics

Reference	:	Ho M D, Marciniszyn J P and Killeen J C (1990).	exposure	:	single dose by gavage
type of study	:	Absorption, distribution and excretion	Doses	:	10 or 1000 mg a.i./kg bw
year of execution	:	1990	Vehicle	:	aqueous 0.75% methylcellulose (w/v) (10 mL/kg bw)
test substances	:	Unlabelled R611965 (SDS-46851) Lot No. 0207; Purity 99.3% w/w. [U-14C]-ring labelled R611965; Specific activity 3.51 mCi/mM; Radiochemical purity: 98.6%.	GLP statement	:	Yes
Route	:	Oral	Guideline	:	EPA 85-1
Species	:	rat, CD SD	acceptability	:	acceptable
group size	:	5 M & 5 F			

Study design

The study investigated the absorption, distribution and excretion of ^{14}C-R611965 (SDS-46851) after a single oral dose of 10 or 1000 mg a.i./kg bw. Each dose was administered to 5 rats of each sex suspended in dose vehicle. Two male and two female rats were administered a single oral dose of the dose vehicle to provide control samples. All animals received water and food *ad libitum* throughout the study. Exposure and sampling of urine, cage wash, faeces, expired air and tissues was as

described in Table 6.8.1 – 6.1.4-1. At termination, blood and selected tissues and organs (see footnote (A) of table 6.8.1 – 6.1.4-1) were collected from all animals. No bile was sampled.

Radioactivity in all sample was quantified by LSC, either directly or following combustion.

Table 6.8.1 – 6.1.4-1: Experimental groups for each dose level

Group	No. of animals/ sex	Treatment	Sampling times (h after dosing)	Sacrifice time (h after last dose)
A	5	Single oral dose at 10 mg/kg [U-^{14}C]-R611965	0-6, 6-12, 12-24, thereafter every 24 hour interval up to 168 hours: urine; 0-12, 12-24, thereafter every 24 hour interval up to 168 hours: faeces; 0-168: cage wash: 0-6, 6-12, 12-24: expired air(B).	168(A)
B	5	Single oral dose at 1000 mg/kg [U-^{14}C]-R611965	As above	As above

(A) At sacrifice. the following tissues and organs were taken from all animals: blood, heart, lungs, kidney, spleen, adrenals, liver, mesenteric fat, testes/ovaries, leg muscle, brain, bone and residual carcass.
(B) 10% KOH trap for CO_2 followed by Chromosorb polymer trap for organic volatiles.

Results

The report did not provide information on the occurrence of any signs of toxicity among the study animals. The excretion and tissue distribution results are presented in Tables 6.1.2 and 6.1.3. The average total recovery of the administered radiolabelled dose ranged from 90.7% to 98.7%. At the most 0.02% of the dose was expired as [^{14}C]-carbon dioxide and ≤0.2% of the dose was present in tissues and residual carcasses at 7 days.

There were no significant differences in the excretion of radioactivity between the sexes. At both doses the predominant route of elimination was in faeces, accounting for 68-76% of the dose, with urine representing 17-26% of dose. Following a 1000 mg/kg bw dose, the elimination rate was slower: 83-87% and 67-73% AR was excreted within 48 hours in urine and faeces of rats receiving a 10 and 1000 mg/kg bw dose, respectively. For rats receiving a 1000 mg/kg bw dose, it took 72 hours to excrete over 80% (86-88%) of the dose.

Tissue concentrations after 7 days were low with the highest concentration in the liver (67-95 and 4687-5917 ng eq./kg at 10 and 1000 mg/kg bw respectively), although this residue represented only 0.02-0.05% of the dose. Lower concentrations were present in kidneys of both sexes (39-51 and 722-1823 ng eq./kg at 10 and 1000 mg/kg bw respectively) and in adrenal glands (77 and 489-1116 ng eq./kg at 10 and 1000 mg/kg bw respectively), with the exception of female adrenals following a 10 mg/kg bw dose, which contained negligible residues (1 ng eq./kg). Residue levels in other tissues and organs were ≤7 and ≤689 ng eq./kg at 10 and 1000 mg/kg bw respectively.

Table 6.8.1 – 6.1.4-2: Mean percentage recoveries of administered radioactivity over 7 days after a single oral dose of 10 or 1000 mg [^{14}C]-R611965/kg bw

	10 mg/kg bw	1000 mg/kg bw

Sample	Males	Females	Males	Females
Urine	21.8	24.0	26.4	16.7
Faeces	68.5	73.7	69.4	76.4
Cage wash	0.3	0.8	0.7	0.9
Expired air	0.00	0.00	0.02	0.00
Tissues & carcass	0.09	0.20	0.13	0.04
Total	90.7	98.7	96.6	94.0

Table 6.8.1 – 6.1.4-3: Mean tissue concentrations of radioactivity 7 days after a single oral dose of 10 or 1000 mg [14C]-R611965/kg bw

	10 mg/kg bw				1000 mg/kg bw			
	Males		Females		Males		Females	
Tissue	% dose	ng eq/g	% dose	ng eq/g	% dose	ng eq/g	% dose	ng eq/g
Whole blood	0.00	2	0.00	1	0.00	80	0.00	109
Heart	0.00	7	0.00	5	0.00	310	0.00	187
Lungs	0.00	2	0.00	1	0.00	33	0.00	113
Spleen	0.00	0	0.00	2	0.00	375	0.00	0
Adrenal glands	0.00	77	0.00	1	0.00	1116	0.00	489
Kidneys	0.00	39	0.00	51	0.00	1823	0.00	722
Liver	0.05	95	0.03	67	0.03	5917	0.02	4687
Mesenteric fat	0.01	7	0.01	7	0.01	689	0.00	266
Gonads	0.00	5	0.00	5	0.00	80	0.00	146
Muscle (leg)	0.01	2	0.04	7	0.00	0	0.00	23
Brain	0.00	2	0.00	0	0.00	121	0.00	42
Bone	0.00	6	0.00	0	0.00	243	0.00	479
Carcass	0.05	4	0.17	16	0.10	1004	0.03	256

Conclusions

Following administration of a single oral dose of 10 or 1000 mg ^{14}C-R611965/kg/bw to male and female rats, at the most 0.02% of the dose was expired as [^{14}C]-carbon dioxide and ≤0.2% of the dose was present in tissues and residual carcasses at 7 days. There were no significant differences in the excretion of radioactivity between the sexes. At both doses the predominant route of elimination was in faeces, accounting for 68-76% of the dose, with urine representing 17-26% of dose. Following a 1000 mg/kg bw dose, the elimination rate was slower. At 10 and 1000 mg/kg bw respectively, after 7 days the highest tissue concentration was found in the liver (67-95 and 4687-5917 ng eq./kg), followed by kidneys (39-51 and 722-1823 ng eq./kg) and adrenal glands (77 and 489-1116 ng eq./kg), with the exception of female adrenals following a 10 mg/kg bw dose, which contained negligible residues (1 ng eq./kg). Residue levels in other tissues and organs were ≤7 and ≤689 ng eq./kg at 10 and 1000 mg/kg bw respectively.

Acceptability

The study was conducted in agreement with OECD 417 and is considered acceptable for the purpose for which it was conducted (determination of absorption, distribution and excretion).

B.6.8.1 – 6.1.5 Reactivity study towards GHS with SDS-417888 - study 5

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Seville, 1999a	test system	: enzyme assay
type of study	: reactivity towards GSH	GLP statement	: no
year of execution	: 1999	guideline	: see 'Guidelines and limitations'
test substance	: chlorothalonil (R044686) and R417888		

Study design

The electrophilic reactivity towards glutathion (GSH) of R417888 and chlorothalonil were compared using an in vitro assay, in the absence or presence of glutathion S-transferase. The reaction constants were normalised to 25°C and pH 7 using experimentally derived algorithms, published in the scientific literature. Experimental conditions are not described in detail in the report.

Results

Comparing the second order reaction rate constants of chlorothalonil (0.335 M^{-1} s^{-1}) and R417888 (1.1 x 10^{-5} M^{-1} s^{-1}), a 29000 fold lower reactivity is determined for the metabolite. This enables the prediction that in vivo R417888 will conjugate at a much lower rate to glutathion in gut and liver, thus not reaching the kidney to the same extent as chlorothalonil, whose nephrotoxicity is primarily caused by metabolites formed in the kidney.

Guidelines and limitations

The experiments were conducted in accordance with Clarke ED, Greenhow DT and Adams D, Metabolism-related assays and their application to agrochemical research: reactivity of pesticides with glutathion and glutathion transferases, Pesticide Science 1998, 54, 385-393.

B.6.8.1 – 6.1.6 Reactivity study towards GHS with SDS-46851 (R611965) - study 6

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Seville, 1999b	test system	: enzyme assay
type of study	: reactivity towards GSH	GLP statement	: no
year of execution	: 1999	guideline	: see 'Guidelines and limitations'
test substance	: chlorothalonil (R044686) and R611965 (SDS-46851)		

Study design

The electrophilic reactivity towards glutathion (GSH) of R611965 (SDS-46851) and chlorothalonil were compared using an in vitro assay, in the absence or presence of glutathion S-transferase. The reaction constants were normalised to 25°C and pH 7 using experimentally derived algorithms, published in the scientific literature. Experimental conditions are not described in detail in the report.

Results

Comparing the second order reaction rate constants of chlorothalonil (0.335 M^{-1} s^{-1}) and R611965 (2.2 x 10^{-8} M^{-1} s^{-1}), a 15 million fold lower reactivity is determined for the metabolite. This enables the prediction that in vivo R611965 will conjugate at a much lower rate to glutathion in gut and liver, thus not reaching the kidney to the same extent as chlorothalonil, whose nephrotoxicity is primarily caused by metabolites formed in the kidney.

Guidelines and limitations

The experiments were conducted in accordance with Clarke ED, Greenhow DT and Adams D, Metabolism-related assays and their application to agrochemical research: reactivity of pesticides with glutathion and glutathion transferases, Pesticide Science 1998, 54, 385-393.

B.6.8.1 – 6.1.7 Degradation of chlorothalonil by bovine rumen fluid - study 7

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Duane, 1971	test system	: in vitro assay
type of study	: degradation of chlorothalonil by bovine rumen fluid	GLP statement	: no
year of execution	: 1971	guideline	: see ‘Guidelines and limitations’
test substance	: [^{14}C]-chlorothalonil		

Study design

The conversion of chlorothalonil when incubated with bovine rumen fluid was investigated using an in vitro assay with Holstein cows.

Results

After 20 hours, 90 percent of the chlorothalonil was converted, 77 percent of which to water soluble metabolites, the remainder 33 percent associated with solid matter. One of the metabolites was identified as 4-hydroxy-2,5,6-trichloroisophthalonitrile (SDS-3701). After 20 hours of incubation, the substance was generated at approximately 5 percent of the chlorothalonil dose (20 ppm).

Conclusions

The presence of 4-hydroxy-2,5,6-trichloroisophthalonitrile in the milk of cows fed chlorothalonil (0.2% of the dose) can be explained by its generation in bovine rumen fluid in vitro.

Guidelines and limitations

No guidelines exist for the experiments.

B.6.8.1 – 6.2 Acute toxicity studies with metabolites

B.6.8.1 – 6.2.1 Acute oral toxicity study in rat with SDS-3701 - study 1

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. Classification was adjusted to CLP.

Characteristics

reference	: Wazeter, 1971a	exposure	: once by gavage
type of study	: acute oral toxicity study	doses	: 100, 147, 215, 316, 464, and 681 mg/kg bw
year of execution	: 1971		
test substance	: SDS-3701, white powder,	vehicle	: 0.5% aqueous methocel
route	: oral	GLP statement	: not compulsory at the time of execution
species	: rat, Carworth; males	guideline	: not in accordance with OECD 401
group size	: 5/dose	**LD_{50}**	: 332 mg/kg bw

Study design

The study was performed before OECD guideline 401 became effective and therefore, several deviations are noted (e.g. too limited data on environmental conditions and clinical sings were provided, and no pathology was performed).

Results

Mortality: animals died within 4 hours and up to four days after dosing at dose levels 215 mg/kg bw and above (Table 6.8.1).

Signs of toxicity: no data on clinical signs were presented.

Pathology: no pathology was performed.

Conclusions

The acute oral LD50 of SDS-3701, administered by gavage in 0.5% aqueous methocel, was calculated to be 332 mg/kg bw for male rats.

Table 6.8.5

Dose (mg/kg bw)	Mortality
100	0/5
147	0/5
215	1/5
316	2/5
464	4/5
681	5/5

Guidelines and limitations

The study has been performed before GLP regulations and OECD guideline 401 became effective. Although very few data have been submitted on study design and environmental conditions and no clinical and pathologic observations have been made, the results of this study are considered suitable for the overall toxicological evaluation.

B.6.8.1 – 6.2.2 Acute oral toxicity study in dog with SDS-3701 - study 2

Previous evaluation	In DAR (2000). Applicant re-submitted the study for the purpose of renewal.
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Wazeter, 1971b	exposure	: once, packed in gelatine capsules
type of study	: acute oral toxicity study	doses	: ~~0~~, 46.4, 68.1, 100, 147, 215, and 316 mg/kg bw
year of execution	: 1971	vehicle	
test substance	: SDS-3701, white powder,	GLP statement	: not applicable
route	: oral	guideline	: not compulsory at the time of execution
species	: dog, Beagle; male and female	**LD_{50}**	: not in accordance with OECD 401
group size	: 1/sex/dose		: -

Study design

The study was performed before OECD guideline 401 became effective and therefore, deviations may occur. However, too limited data on environmental conditions and clinical sings were provided, no pathology was performed. Furthermore, the number of animals and selection of species are not considered adequate for evaluation.

Results

Mortality: two animals died within 4 days after dosing at dose levels of 100 and 215 mg/kg bw.

Signs of toxicity: no data on clinical signs were presented.

Pathology: no pathology was performed.

Conclusions

An acute oral LD_{50} of SDS-3701 for dogs, administered by gelatine capsule, could not be determined, as nine out of twelve dogs vomited within one to four hours and could not used for the calculation of the oral LD_{50}.

Guidelines and limitations

The study has been performed before GLP regulations and OECD guideline 401 became effective. As very few data have been submitted on study design and environmental conditions and no clinical and pathologic observations have been made, the results of this study are excluded from the overall evaluation. However, it should be stressed that only one of the three animals which did not vomit (two dosed at 100 mg/kg bw and one at 215 mg/kg bw), survived.

B.6.8.1 – 6.2.3 Acute oral toxicity study in rat with SDS-3701 - study 3

Previous evaluation	In DAR (2000). Applicant re-submitted the study for the purpose of renewal.
Evaluation RMS	No remarks on original assessment. Classification was adjusted to CLP.

Characteristics

reference	: Hastings, 1973	exposure	: once by gavage
type of study	: acute oral toxicity study	doses	: 100, 150, 225, 337.5 and 506 mg/kg bw
year of execution	: 1973	vehicle	: 10% hydroxypropylcellulose (klucel) in water
test substance	: SDS-3701, white powder		
route	: oral	GLP statement	: not compulsory at the time of execution
species	: rats, Sprague Dawley; male and female	guideline	: not in accordance with OECD 401
group size	: 4-6/sex/dose	**LD_{50}**	: 422 mg/kg bw for males and 242 mg/kg bw for females

Study design

The study was performed before OECD guideline 401 became effective. Therefore several deviations can be noted, e.g. the study design (environmental conditions, individual clinical and pathological data) was described very limited.

Results

Mortality: animals died within 4 days after dosing at dose levels of 150 mg/kg bw and above (Table 6.8.2).

Signs of toxicity: tremors, extreme jerks on stimulation, salivation, followed by ataxia and terminal convulsions with a number of cases of nasal haemorrhage and cyanosis (based on summary of the notifier).

Pathology: a slight darkening of the kidney of those that had died.

Conclusions

The acute oral LD50 of SDS-3701 in rats, administered by gavage, was found to be 422 and 242 mg/kg bw for male and females, respectively.

Table 6.8.1 – 6.2.3

Dose (mg/kg bw)	Mortality (males)	Mortality (females)
100	-	0/6
150	0/4	1/6
225	0/4	3/6
337.5	1/4	5/6
506	3/4	5/6

Guidelines and limitations

The study has been performed before GLP and OECD guideline 401 became effective. Although very limited data have been submitted on study design and environmental conditions and the individual clinical and pathological observations have not been submitted, the results of this study are considered relevant for the overall toxicological evaluation.

B.6.8.1 – 6.2.4 Acute oral toxicity study in rat with SDS-3701 - study 4

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant with regard to the oral LD50 of SDS-3701. A different conclusion is drawn with regard to classification. Based on CLP the substance needs classification with H301 “Toxic if swallowed”, instead of R22 “Harmfull if swallowed”.

Report:	K-CA 5.8.1/01. Beerens-Heijnen, C.G.M. (2005), Assessment of acute oral toxicity with SDS-3701 in the rat (acute toxic class method), testing facility: Notox B.V, 5231 DD’s-Hertogenbosch, The Netherlands. Report No. 402479, Unpublished. (Syngenta File Number R182281_10019)

GUIDELINES: OECD Guideline 423

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

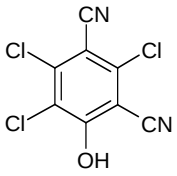
Acceptability: There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

The test substance, SDS-3701, was administered in a stepwise procedure by oral gavage to groups of three fasted female rats at subsequent dose levels of 2000, 300, 50 and 50 mg/kg bw.
Animals were observed for gross toxicity, behavioural changes and/or mortality at periodic intervals on the day of dosing (day 1) and twice daily, thereafter, until day 15. Body weights were determined on day 1 (pre-administration), day 1, day 8 and day 15. All animals were subjected to macroscopic gross examination consisting of opening the abdominal and thoracic cavities.

Test Material: SDS-3701

	4-hydroxy-2,5,6-trichloroisophtalonitrile; 4-OH-2,5,6-trichloro-1,3-dicyanobenzene
Description:	White Powder
Lot/Batch#:	Batch 51955-15-21
Purity:	99%



Vehicle and/or positive control: Propylene glycol

Results

Table 6.8.1 – 6.2.4 Doses, Mortality / Animals Treated, Clinical Signs

Dose (mg/kg bw)	Mortalities	Clinical signs
2000	3/3	Hunched posture, tremors, salivation and/or urine flow
300	2/3	Restless behaviour, clonic spasms, general tremor, lateral recumbency, hunched posture, flat gait, laboured respiration, salivation, piloerection and/or ptosis
50	0/3	Hunched posture, uncoordinated movements, piloerection, lethargy and/or hyperthermia
50	0/3	

The decedents and moribund animals were found within 4 h post treatment. Details are provided in Table 6.8.1 – 6.2.4. The surviving animals had recovered from the symptoms between days 3 and 15.

The mean body weight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain.
No abnormalities were found upon macroscopic post mortem examination.

Conclusions

The oral LD_{50} value of SDS-3701 in Wistar rats was established to be within the range 50-300 mg/kg bw.

B.6.8.1 – 6.2.5 Skin irritation study with SDS-3701 - study 5

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/02. Beerens-Heijnen, C.G.M. (2005a), Primary skin irritation/corrosion study with SDS-3701 in the rabbit, testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands. Report No. 411108. Unpublished. (Syngenta File Number R182281_10021)

GUIDELINES: OECD 404 (2002)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

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

Study design

In a primary dermal irritation study, 3 adult male New Zealand rabbits were exposed *via* the dermal route to 0.5 g of SDS-3701 each. Initially, one animal was exposed for 3 minutes, 1 hour and 4 hours. In absence of sever skin reactions, two further animals were exposed for 4 hours. The test material was applied to the clipped skin of one flank, using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours after exposure.

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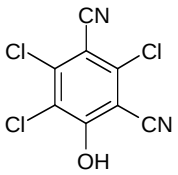
Test Material: **SDS-3701**
4-hydroxy-2,5,6-trichloroisophtalonitrile; 4-OH-2,5,6-trichloro-1,3-dicyanobenzene
Description: White Powder
Lot/Batch#: Batch 51955-15-21
Purity: 99%
Vehicle and/or positive control: No vehicle



Results

No symptoms of systemic toxicity were observed in the animals during the test period and no mortality occurred. No dermal response was observed at the test site of any animal during the 72-hour study period.

Conclusion

SDS-3701 was not irritating to rabbit skin.

B.6.8.1 – 6.2.6 Acute oral toxicity study in rat with SDS-46851- study 6

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Long et al, 1985	exposure	: once by gavage
type of study	: acute oral toxicity study	doses	: 200, 1 000, and 5 000 mg/kg bw
year of execution	: 1984	vehicle	: 0.5% methylcellulose
test substance	: SDS-46851, white powder, 95% pure	GLP statement	: yes
route	: oral	guideline	: in accordance with OECD 401
species	: rats, Sprague Dawley; male and female	**LD_{50}**	: > 5 000 mg/kg bw
group size	: 2/sex/dose		

Study design

The study was performed in accordance with OECD guideline 401.

Results

Mortality: no animals died during the study.

Signs of toxicity: soft stool and mucus and tan coloured particles in the stool, anogenital staining, red nasal discharge and/or chromodacryorrhea was observed.

Pathology: mottled kidney and dilated renal pelvis and concretion in the lumen of the urinary bladder.

Conclusions

The acute oral LD_{50} of SDS-46851 in rats, administered by gavage, was found to be more than 5 000 mg/kg bw.

Guidelines and limitations

This study was performed in accordance with OECD guideline 401 and is of relevance for the overall toxicological evaluation.

B.6.8.1 – 6.2.7 Acute oral toxicity study in rat with SDS-46851 - study 7

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/41 Beerens-Heijnen, C.G.M. (2005b), Assessment of acute oral toxicity with SDS 46851 In the rat (acute toxic class method), testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands, published: no, report No. 402558. (Syngenta File Number R611965_10014)

GUIDELINES: OECD 423

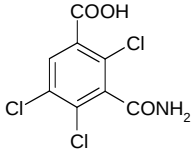
GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

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

Study design

Following an overnight fast, 3 female rats were given a single dose of 2000 mg SDS-46851per kg bw by oral gavage suspended in aqueous carboxymethyl cellulose (1% w/w). Animals were observed for gross toxicity, behavioural changes and/or mortality at periodic intervals on the day of dosing (day 1) and twice daily, thereafter, until day 15. Body weights were determined on day 1 (pre-administration), day 1, day 8 and day 15. All animals were subjected to macroscopic gross examination consisting of opening the abdominal and thoracic cavities.

Test Material:	SDS-46851
	2, 4,5-trichloro-isophthalamic acid, 3-carbamyl-2,4,5-trichlorobenzoic, 3-carboxy-2,5,6-trichlorobenzamide
Description:	White Powder
Lot/Batch#:	Batch 52031-13-23
Purity:	99%



Vehicle and/or positive control: 1% (w/w) aqueous carboxymethy cellulose

Results

No mortalities occurred. Clinical signs observed included hunched posture, on day 1 and/or 2 observed in all animals.
The mean body weight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain.
No abnormalities were found upon macroscopic post mortem examination.

Conclusion
The oral LD50 value of SDS 46851 in Wistar rats was established to be exceeding 2000 mg/kg bw.

B.6.8.1 – 6.2.8 Skin irritation study in rabbit with SDS-46851 - study 8

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/42 Beerens-Heijnen, C.G.M. (2005c), Primary skin irritation/corrosion study with SDS-46851 in the rabbit, testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands, published: no, report No. 411119 (Syngenta File Number R611965_10015)

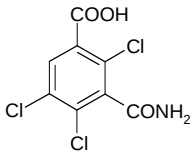
GUIDELINES: OECD 404 (2002)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

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

Study design
In a primary dermal irritation study, 3 adult male New Zealand rabbits were exposed *via* the dermal route to 0.5 g of SDS-46851 each. Intially, one animal was exposed for 3 minutes, 1 hour and 4 hours. In absence of sever skin reactions, two further animals were exposed for 4 hours. The test material was applied to the clipped skin of one flank, using a semi-occlusive dressing. Observations were made 1, 24, 48 and 72 hours after exposure.

Test Material:	SDS-46851
	2, 4,5-trichloro-isophthalamic acid, 3-carbamyl-2,4,5-trichlorobenzoic, 3-carboxy-2,5,6-trichlorobenzamide
Description:	White Powder
Lot/Batch#:	Batch 52031-13-23
Purity:	99%



Vehicle and/or positive control: No vehicle

Results

No symptoms of systemic toxicity were observed in the animals during the test period and no mortality occurred.

No dermal response was observed at the test site of any animal during the 72-hour study period.

Conlcusion

SDS-46851 was not irritating to rabbit skin.

B.6.8.1 – 6.2.9 Acute oral toxicity study in rat with R417888- study 9

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Johnson, 1999	exposure	: once by gavage
type of study	: acute oral toxicity study	doses	: 2 000 mg/kg bw
year of execution	: 1999	vehicle	: deionised water
test substance	: R417888, white solid, 97% pure	GLP statement	: yes
route	: oral	guideline	: in accordance with OECD 401
species	: rats, Alpk:AP$_f$SD; male and female	**LD_{50}**	: > 2 000 mg/kg bw
group size	: 5/sex/dose		

Study design

The study was performed in accordance with OECD guideline 401 (1987).

Results

Mortality: no animals died during the study.

Signs of toxicity: no signs of systemic toxicity.

Pathology: pelvic dilatation of the kidney in one female.

Conclusions

The acute oral LD_{50} of R417888, 2-amido-3,5,6-trichloro-4-cyanobenzene sulfonic acid in rats, administered by gavage, was found to be more than 2 000 mg/kg bw.

Guidelines and limitations

This study was performed in accordance with OECD guideline 401 and is of relevance for the overall toxicological evaluation.

B.6.8.1 – 6.2.10 Acute oral toxicity study in rat with R417888 (VIS 01)- study 10

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/14 van Huygevoort, A.H.B.M. (2005), Assessment of acute oral toxicity with VIS 01 in the rat (acute toxic class method), testing facility: Notox B.V, 5231 DD's-Hertogenbosch, The Netherlands. Report No. 402547. Unpublished. (Syngenta File Number R417888_10023)

GUIDELINES: OECD 423

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

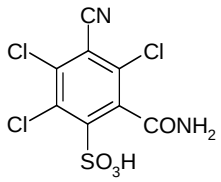
Acceptability: There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

The test substance, VIS 01, was administered by oral gavage to two subsequent groups of three female rats at a dose levels of 2000 mg/kg bw. Animals were observed for gross toxicity, behavioural changes and/or mortality at periodic intervals on the day of dosing (day 1) and twice daily, thereafter, until day 15. Body weights were determined on day 1 (pre-administration), day 1, day 8 and day 15. All animals were subjected to macroscopic gross examination consisting of opening the abdominal and thoracic cavities.

Materials

Test Material: VIS 01, 1-cyano-1, 5, 6-trichloro-3-amido-benzene-sulphonate = R417888



Description: White powder
Lot/Batch#: Batch 52168-11-16
Purity: 99.5%

Vehicle and/or positive control: 1% (w/w) aqueous carboxymethyl cellulose

Results

No mortalities occurred. Clinical signs observed included hunched posture, on day 1 and/or 2 observed in all animals

The mean body weight gain shown by the animals over the study period was considered to be similar to that expected of normal untreated animals of the same age and strain.

No abnormalities were found upon macroscopic post mortem examination.

Conclusions

The oral LD_{50} value of VIS 01 in Wistar rats was established to be exceeding 2000 mg/kg bw.

B.6.8.1 - 6.3 Short-term toxicity studies

B.6.8.1 – 6.3.1 Oral 60-day toxicity study in rat with SDS-3701 - study 1

Previous evaluation	In DAR (2000). Applicant re-submitted the study for the purpose of renewal.
Evaluation RMS	Acceptable. Based on a re-evaluation of the data, the RMS does not agree with the conclusions drawn by the applicant. The RMS concludes that based on the increased incidence and increase in severity in inactivity and piloerection at 10 mg/kg and above, a NOAEL could not be established. I In the DAR of 2000, the following was concluded: *At 10 mg/kg bw, slight but treatment-related changes, namely piloerection (both sexes), decreased MCHC (females) and increased relative liver weight (females), were seen. At higher dose levels these changes were more severe and/or accompanied by clear signs of anaemia and hepatotoxicity. Based on these findings, 10 mg/kg bw is considered a MOAEL.*

Characteristics

reference	: Ford et al., 1982a	exposure	: 60 days
type of study	: oral 60 day study in the rat	doses	: 0, 10, 20, 40, 75, 125, 250, 500 and 750 mg/kg bw
year of execution	: 1978		
test substance	: DS-3701 (9813-30-3), fine white powder, purity 99.6%	vehicle	: food
		GLP statement	: yes
route	: oral	guideline	: in compliance with OECD 407
species	: rat (COBS^{R}CD^{R}(SD))	**NOAEL**	: <10 mg/kg bw
group size	: 10/sex/dose		

Study design

The study is in accordance with OECD 407 (adopted 12 May 1981). The duration of the study was relatively long (60 days).

Results

The results are summarized in Table 6.8.1 – 6.3.1-1.

Table 6.8.1 – 6.3.1-1 Summary of results, Study 1

Dose (mg/kg bw)[1]	10		20		40		75		125		250		500/750		dr
	m	f	m	f	m	f	m	f	m	f	m	f	m	f	
Mortality								3	5	10	9	10	9/10	10	m,f
Clinical signs															
Inactivity	+	+	+	+	+	+	+	+	+	++	++	++	++	++	m,f
Piloerection	+	+	+	+	++	+	++	++	++	++	++	++	++	++	m,f
Pallor			+				++	++	++	++	++	++	++	++	m,f
Hyperirritability			+		+		+	+	+		+	+	++	++	
Body weight					dc	dc	dc	dc	dc	dc	dc	dc	dc	dc	m,f
Food consumption						dc	dc	dc		dc	d	dc	dc	dc	
Haematology											)[2]	)[2]	)[2]	)[2]	
MCHC		dc	dc	dc	dc	dc		dc							
MCH/MCV/haematocrit/ haemoglobin				dc		dc	dc	dc	dc						m,f
RBC						dc	dc	dc	dc						m,f
nucleated RBC					ic	ic	i	ic	ic						m,f
WBC/lymphocyte						ic									
prothrombin time								ic							
Clinical chemistry											)[2]	)[2]	)[2]	)[2]	
ASAT			dc		dc			ic	ic						
ALAT				dc	dc			ic	ic						
glucose				dc	dc	d	dc	dc	d						
total protein				dc	dc	dc	dc	dc	dc						m,f
albumin						dc		dc	dc						
globulin					dc		dc	dc	dc						m
A/G ratio			ic		ic		ic		ic						m
BUN						ic		ic	ic						f
ALP				dc				ic							

Dose (mg/kg bw)[1]	10		20		40		75		125		250		500/750		dr
	m	f	m	f	m	f	m	f	m	f	m	f	m	f	
Organ weights[3]											)[2]	)[2]	)[2]	)[2]	
heart					dc^a	dc^a, ic^r	dc^a, ic^r	$ic^{a,r}$	dc^a, ic^r						m,f
kidneys			dc^a	dc^a	dc^a	dc^a	dc^a, ic^r	dc^a	dc^a, ic^r						$m^{a,r}$
adrenals					ic^r	dc^a	dc^a, ic^r	dc^a, ic^r	dc^a						m^r
liver		ic^r		ic^r	dc^a	ic^r	dc^a, ic^r	dc^a, ic^r	dc^a, ic^r						m,f
spleen					ic^r	ic^r	dc^a, ic^r	ic^r	dc^a, ic^r						m^r
testes					ic^r		dc^a, ic^r		dc^a, ic^r						m
ovaries						$dc^{a,r}$		$dc^{a,r}$							f
brain					dc^a,ic^r	dc^a, ic^r	dc^a,ic^r	dc^a, ic^r	dc^a, ic^r						m,f
(para)thyroids				ic^r		ic^r	ic^r	ic^r	ic^r						m,f
pituitary				ic^r	ic^r		dc^a, ic^r	ic^r	dc^a, ic^r						m^r
Pathology															
macroscopy[3]						+	++	++	++	++	++	++	++	++	
microscopy															
bone marrow:															
- erythroid hyperplasia					+	+	+	+							
- erythroid hypoplasia									+	+	++	++	++	++	
- decreased granulopoiesis					+	+	+	+	++	++	++	++	++	++	
spleen:															
- erythroid hyperplasia					+	+	+	+							
- erythroid hypoplasia							+	+	++	++	++	++	++	++	
- decreased granules/															
lymphopoiesis					+	+	+	+	++	++	++	++	++	++	
- hemosiderosis	+			++			+		++	++	+	++	++	++	
liver:															
- centrolob. tox. hepatitis							+	++	++	++	++	++	++	++	
- hemosiderosis								+	++	++	+	++		+	
- parenchymal atrophy													+	+	
heart:															
- myocardial degeneration						+	+	++	+	++	+	++	++	++	
- thrombosis in auricles									+	++	+				
adrenals:															
- cortical necrosis and															
vacuolative degeneration								+	+	++	+	++	+	++	
testes:															
- bilateral tub.					+		++		++		++		++		m
degeneration															
ovaries:															
- degen. and necrosis of						++		++		++		++		++	f
follicular epithelium															
kidneys:															
- cortical tubular nephrosis								+	+	+	+	+	++	+	
- cortical atrophy						+	++	++	++	++	++	+	++	++	
- mineralisation in															
pelvis and/or medulla								+	+	+	+	+		+	
stomach:															
- erosive ulcer. gastritis										+	+	+	+	+	

dr dose related
dc/ic statistically significantly decreased/increased
d/i decreased/increased, but not statistically significantly
\+ present in one/a few animals
++ present in most/all animals
a absolute organ weight
r relative organ weight
1 all groups were compared to a vehicle control group.
2 not performed because of the mortality prior to the scheduled examination.
3 No individual macroscopic data were presented. According to the summary, the principal alterations in decedents of the 75 through 750 mg/kg bw dose groups were paleness of the bone marrow, heart, kidneys, brain, pituitary, liver and pancreas (indicative of anaemia); gastric erosions and/or ulcerations and discolorations of urine (pink/red), liver and kidney; foam in the trachea, pulmonary congestion and oedema. The survivors of these groups showed flabby hearts, thin pale blood and pale yellow-brown livers and/or kidneys. In the 40 mg/kg group, 3 females had pale brown kidneys, one female had a pale brown liver and one had a flabby heart (not accompanied by microscopic alterations).

Mortality: Mortalities attributed to the administration of SDS-3701 were observed in groups of rats at dose levels of 75 to 750 mg/kg bw/day. No mortalities were observed at dosage levels of 10, 20 and 40 mg/kg.

Table 6.8.1 – 6.3.1-2: Mortalities

Test group	Dose level (mg/kg bw/day)	Deaths male	Deaths female
I (control)	0	0/10	0/10
II	10	0/10	0/10
III	20	0/10	0/10
IV	40	0/10	0/10
V	75	0/10	3/10
VI	125	5/10	10/10
VII	250	9/10	10/10
VIII	500	9/10	10/10
IX	750	10/10	10/10

Clinical observations: Hyperirritability, inactivity, piloerection, and pallor were observed in rats receiving diets containing SDS-3701. The incidence of hyperirritability for rats receiving SDS-3701 was variable, and no relationship to the administration of SDS-3701 was apparent. Inactivity was observed in all groups of rats fed diets containing SDS-3701 and in 3 control females at days 57-end and in 1 control male male at days 43-49 and 2 control males at days 57-end. An increased in incidence and severity was seen with increasing doses. Incidence of inactivity was associated with those groups of animals in which increased moribundity and spontaneous deaths occurred, and thus was probably a result of the generally poor condition of these animals. Piloerection was observed in all groups of rats fed diets containing SDS-3701 and in one control group female. The occurrence of piloerection appeared to be related to the dosage level and to the duration of administration. Paleness of the skin and/or eyes (pallor) was observed in groups of female rats at dosage levels > 75 mg/kg and in groups of male rats at dosage levels > 20 mg/kg. In male rats, the incidence of pallor was low and variable at the 20 and 40 mg/kg dosage levels and was evident only after seven weeks of SDS-3701 administration. Data on clinical observations are given in Table 6.8.1 – 6.3.1-3.

Table 6.8.1 – 6.3.1-3: Intergroup incidence of selected clinical observations

Observation/days		Dose level (mg/kg bw/day)								
Male		**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Inactivity	**1-7**	0	0	0	0	0	1	5	9	10
	22-28	0	0	3	10	10	10	10	7	-
	43-49	0	9	7	10	10	10	10	2	-
	57-end	3	10	10	10	9	10	2	1	-
Piloerection	**1-7**	0	0	0	0	0	0	0	6	8
	22-28	0	0	2	5	9	10	10	7	-
	43-49	0	7	7	10	10	10	10	1	-
	57-end	0	7	10	10	10	10	2	1	-
Pallor	**15-21**	0	0	0	0	0	1	8	9	2
	22-28	0	0	0	0	0	9	9	7	-
	43-49	0	0	0	0	8	10	10	1	-
	57-end	0	0	3	1	8	10	2	1	-
Female		**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Inactivity	**1-7**	0	0	0	0	1	1	3	9	10
	22-28	0	1	3	7	7	10	10	9	-
	43-49	1	6	7	10	10	10	9	3	
	57-end	2	5	8	8	9	7	-	-	-
Piloerection	**1-7**	0	0	0	0	0	0	0	6	7
	15-21	0	1	2	0	5	9	10	9	2
	22-28	0	1	1	5	7	10	10	9	-
	43-49	0	2	4	8	10	10	9	3	-
	57-end	1	1	4	7	8	5	-	-	-
Pallor	**15-21**	0	0	0	0	0	0	10	9	2
	22-28	0	0	0	0	0	6	10	9	-
	43-49	0	0	0	0	10	10	9	3	-
	57-end	0	0	0	0	9	6	-	-	-

- = all animals dead

Body weight and weight gain: Significantly decreased body weights were observed in groups of male and female rats fed diets containing SD5-3701 at dose levels ≥ 40 mg/kg, when compared with

concurrent control values. The decreased body weights occurred in a dosage-related manner. The mean body weights of the high dose (750 mg/kg) animals were decreased significantly after only one week of dietary administration of SDS-3701. The mean body weights of the 40 mg/kg dose level animals were significantly decreased (17% for males and 19% for females) when compared with control values after seven weeks of SDS-3701 administration. The mean body weights of rats at the 10 and 20 mg/kg dose levels were comparable with the control values throughout the study. Data on body weight (gain) are given in Table 6.8.1 – 6.3.1-4.

Table 6.8.1 – 6.3.1-4: Intergroup comparison of body weights (g) - selected timepoints

Week	Dose level (mg/kg bw/day)								
Males	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
1	189	194	196	191	191	191	185	191	189
2	247	249	245	228	205*	195**	168**	144**	130** (8)
3	298	298	295	267	219**	197**	175**	161**	146b (2)
4	339	336	331	293	226**	198**	186**	145** (7)	-
6	398	388	388	338	233**	214**	185**	132b (3)	-
9d	450 (9)	444 (9)	437	372	237** (8)	199** (8)	157b (2)	132b (1)	-
Females	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
1	132	144	141	139	144	138	139	137	140
2	159	165	160	152	148	136	130*	118**	109** (5)
3	180	186	180	167	157	135**	137**	132** (9)	117b (2)
4	200	200	194	174	158**	135**	138**	121** (9)	-
6	221	220	213	190	162**	158**	145**	121** (7)	-
9d	238 (9)	234 (9)	225 (9)	191** (9)	157** (7)	141b (3)	-	-	-

** Statistically significant difference from control group mean p≤ 0.01 (Dunnett's t-test)
* Statistically significant difference from control group mean p≤ 0.05 (Dunnett's t-test)
n = 10 unless shown otherwise by a number in parentheses
b = not analysed statistically when n ≤ 3
- = all animals dead
d = animals fasted for urinalysis excluded from means

Food consumption and compound intake: In groups of male and female rats fed diets containing SDS-3701 at dose levels of 250, 500 and 750 mg/kg, the mean food consumption was variable and at some intervals significantly decreased when compared with control values. The mean food consumption of male and female rats at dose levels of 75 and 125 mg/kg were generally decreased and at times statistically significantly decreased when compared with controls. In females a decreased food consumption was seen at 40 mg/kg dose level in week 6 and 8. Food consumption in groups of rats fed diets containing SDS-3701 at dose levels of 10 and 20 mg/kg were comparable with control values throughout the study. Data on food consumption are given in Table 6.8.1 – 6.3.1-5.

Table 6.8.1 – 6.3.1-5: Intergroup comparison of food consumption (g/kg/day) - selected timepoints

Week	Dose level (mg/kg bw/day)								
Males	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
1	96	98	98	93 (9)	75	64 (8)	60 (8)	33** (9)	45** (8)
2	89	90	89	82	69	69 (9)	70 (8)	81 (9)	156b (1)
3	79	81	81	80	64 (7)	70 (6)	73 (7)	50* (6)	-
6	66	67 (9)	68	66	55 (8)	59 (8)	57 (6)	72b (1)	-
8	59 (9)	60 (9)	60	58	51 (8)	53 (8)	48b (2)	52b (1)	-
Females	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
1	112	112	106	103	82 (9)	65*	62** (8)	67** (9)	76 (5)
2	106	104	94	86	81	75	74 (9)	108 (7)	79b (1)
3	95	96	90	86 (9)	68	81	66 (9)	83 (8)	-
6	85 (9)	88	81	71*	63** (7)	60**	62**(9)	77b(3)	-
8	76 (9)	76 (8)	69 (9)	60** (9)	53** (7)	61b (3)	-	-	-

** Statistically significant difference from control group mean p≤ 0.01 (Dunnett's t-test)
* Statistically significant difference from control group mean p≤ 0.05 (Dunnett's t-test)
n = 10 unless shown otherwise by a number in parentheses (less than 10 due to mortality or food spillage)

b = not analysed statistically when n ≤ 3; - = all animals dead

The calculated mean compound (SDS-3701) consumption for groups of rats at dose levels of 10, 20, 40 and 75 mg/kg were generally within ± 20% of the intended dosage levels. At dosage levels of 125, 250, 500 and 750 mg/kg, the calculated compound consumptions were more variable, probably due to the reduced food consumption and poor general condition of the animals in these groups. The mean compound intake values for each group are given in Table 6.8.1 – 6.3.1-6.

Table 6.8.1 – 6.3.1-6: Mean compound intake values (mg/kg/day) - selected timepoints

Week	Dose level (mg/kg bw/day)								
Males	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
1	0	12	22	43 (9)	66	93 (8)	166 (8)	203 (9)	387 (8)
2	0	11	25	44	68	117 (9)	250 (8)	480 (9)	1377 (1)
3	0	10	21	42	69 (7)	143 (6)	264 (7)	601 (6)	-
6	0	10 (9)	20	40	71 (8)	119 (8)	224 (6)	437 (1)	-
8	0	10 (9)	19	38	66 (8)	102 (8)	202 (2)	390 (1)	-
Females	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
1	0	12	21	44	66 (9)	94	163 (8)	375 (9)	614 (5)
2	0	11	23	35	65	96	189 (9)	515 (7)	636 (1)
3	0	10	19	37 (9)	65	151	253 (9)	529 (8)	-
6	0	10	20	40	102 (7)	100	208 (9)	527 (3)	-
8	0	9 (8)	17 (9)	34 (9)	66 (7)	109 (3)	-	-	-

n = 10 unless shown otherwise by a number in parentheses (less than 10 due to mortality or food spillage)

Clinical pathology: Because of the high mortality and/or clotted blood samples, the terminal clinical laboratory studies could be conducted on only a limited number of animals at dose levels ≥ 125 mg/kg for females and ≥ 250 mg/kg for males. Statistical comparisons with control values were not conducted for these groups.

Haematology: When compared with control groups, significantly decreased mean values for the red blood cell count, haematocrit, haemoglobin, mean corpuscular volume and mean corpuscular haemoglobin were observed in groups of male rats at dose levels of 75 and 125 mg/ kg and for female rats at dose levels of 40 and 75 mg/ kg. A decrease in MCHC and MCH was seen in females at 10, 29, 40 and 75 mg/kg however, the decrease at 10, 20 and 40 mg/kg was only slight and not considered adverse. The number of nucleated red blood cells observed in peripheral blood smears was increased in males and females at dose levels of 40 and 75 mg/kg and in males at a dose level of 125 mg/kg. The number of reticulocytes observed in peripheral blood smears was increased in males at a dose level of 75 mg/kg and in females at a dose level of 40 mg/kg. The increased numbers of immature red blood cells observed in the blood smears is an indication of a regenerative response in the bone marrow and correlates with the increased erythroid activity noted in the bone marrow smears. Statistically significant decreased mean haematocrit, haemoglobin, mean corpuscular haemoglobin concentration, mean corpuscular volume and mean corpuscular haemoglobin values were observed in female rats at the 20 mg/kg dose level, when compared with controls. Although these values were statistically decreased when compared with the concurrent control values, they were within the normal limits of variation. In addition, the mean red blood cell count, reticulocyte count and number of circulating nucleated red cells were comparable with control values. Thus, the

statistical differences observed in the haematology parameters of females at the 20 mg/kg dose level were not considered related to the administration of SDS-3701. For male rats at dose levels of 10, 20 and 40 mg/kg, and for female rats at dose levels of 10 and 20 mg/kg, the haematology parameters were comparable with control values, and no relationship to the administration of DS-3701 was evident. Results for relevant heamatology parameters are given in Table 6.8.1 – 6.3.1-7.

Table 6.8.1 – 6.3.1-7: Intergroup comparison of haematology selected parameters and groups

Parameter						
Males	**0**	**10**	**20**	**40**	**75**	**125**
MCHC %	33	33	32**	32**	35	32
MCH µµg	20	22	20	19	17*	17**
RBC x10^6/mm^3	7.47	6.99	7.42	7.28	4.44** (6)	1.77b (3)
Haematocrit %	45	45	46	44	21** (7)	12** (4)
Haemoglobin g/dL	14.8	14.8	14.7	14.0	7.5** (7)	3.8** (4)
MCV μ^3	60	68	62	61	49** (6)	55** (3)
MCHb µµg	20	22	20	19	17* (6)	17** (3)
Nucleated RBC %	0.1	0	0.1	2.2*	6.2 (6)	10.1 * (4)
Females	**0**	**10**	**20**	**40**	**75**	**125**
MCHC %	33	32*	32*	32*	28**	-
MCH µµg	21	21*	20**	18**	15**	-
RBC x10^6/mm^3	7.04	6.95	6.86	5.83**	2.82** (3)	-
Haematocrit %	45	46	42**	33**	15** (3)	-
Haemoglobin g/dL	14.9	14.8	13.6**	10.4**	4.2** (3)	-
MCV μ^3	64	66	62**	56**	52** (3)	-
MCHb µµg	21	21	20**	18**	15** (3)	-
Nucleated RBC %	0.4	0.2	1.1	9.9*	20.7 (3)	-

n = 10 unless shown otherwise by a number in parentheses (less than 10 due to mortality or clotted sample)
- = all animals dead
** Statistically significant difference from control group mean p≤ 0.01 (Student's t-test)
* Statistically significant difference from control group mean p≤ 0.05 (Student's t-test)

Blood clinical chemistry: At termination, significantly increased mean values for alkaline phosphatase, blood urea nitrogen, glutamic pyruvic transaminase and glutamic oxaloacetic transaminase were observed in male rats at the 125 mg/kg bw/day dose level and for females at the 75 mg/kg dose level. When compared with control values, the mean total protein, albumin and globulin values for males and females at the 75 mg/kg dose level were significantly decreased while the A/G ratio was increased at 20 mg/kg and above in the male groups only. The observed changes in globulin and A/G ration in females at 10 mg/kg were not considered treatment related in absence of a dose-response. No changes were observed in the serum chemistry values for males or females at the 10 mg/kg dosage levels, when compared with concurrent controls. Results for relevant clinical biochemistry parameters are given in Table 6.8.1 – 6.3.1-8.

Table 6.8.1 – 6.3.1-8: Intergroup comparison of blood biochemistry selected parameters and groups

Parameter						
Males	**0**	**10**	**20**	**40**	**75**	**125**
Glucose (mg/dL)	150 (9)	135	140	125	123	125 (5)
Total protein (g/dL)	5.8	5.8	5.6	5.5	5.2**	4.7 ** (5)
Albumin (g/dL)	3.8	3.9	3.8	3.9	3.7	3.5** (5)
Globulin (g/dL)	2.0	1.9	1.8	1.6	1.5**	1.2** (5)
A/G ratio	1.9	2.1	2.2*	2.4**	2.7**	2.9** (5)
ALP (IU/L)	66	75	62	56	78	100 (5)
Urea nitrogen (mg/dL)	17	17	17	18	19	29** (5)
SGOT activity (IU/L)	34	32	27	26	45	66** (5)
SGPT activity (IU/L)	23	18	15	13	37	61** (5)
Females	**0**	**10**	**20**	**40**	**75**	**125**
Glucose (mg/dL)	131	122	119*	119	120* (7)	-
Total protein (g/dL)	5.8	5.8	5.6*	5.3**	5.1** (7)	-
Albumin (g/dL)	4.0	3.9	4.0	3.7**	3.7* (7)	-
Globulin (g/dL)	1.7	1.9*	1.6	1.6	1.4** (7)	-
A/G ratio	2.4	2.1*	2.5	2.4	2.4 (7)	
ALP (IU/L)	50	44	39	58	73* (7)	-
Urea nitrogen (mg/dL)	16	16	18	19**	30** (7)	-
SGOT activity (IU/L)	30	27	25	29	57** (7)	-
SGPT activity (IU/L)	14	12	10**	11	29** (7)	-

n = 10 unless shown otherwise by a number in parentheses (less than 10 due to mortality or clotted sample)
- = all animals dead
** Statistically significant difference from control group mean p≤ 0.01 (Student's t-test)
* Statistically significant difference from control group mean p≤ 0.05 (Student's t-test)

Urinalysis: The urinalysis parameters for male and female rats fed diets containing DS-3701 at dose levels of 10, 20, 40 and 75 mg/kg were comparable with concurrent controls at the termination of the study

Macroscopic findings: No individual macroscopic data were presented. According to the summary, the principal alterations in decedents of the 75 through 750 mg/kg bw dose groups were paleness of the bone marrow, heart, kidneys, brain, pituitary, liver and pancreas (indicative of anaemia); gastric erosions and/or ulcerations and discolorations of urine (pink/red), liver and kidney; foam in the trachea, pulmonary congestion and oedema. The survivors of these groups showed flabby hearts, thin pale blood and pale yellow-brown livers and/or kidneys. In the 40 mg/kg group, 3 females had pale brown kidneys, one female had a pale brown liver and one had a flabby heart (not accompanied by microscopic alterations).

Organ weights: Due to poor survival for males at dose levels ≥ 250 mg/kg and for females at dose levels ≥ 125 mg/kg meaningful comparisons of organ weights with control values were not possible. Decreased absolute organ weights were observed in both male (at dose levels of 40, 75 and 125 mg/kg) and female rats (at dose levels of 40 and 75 mg/kg) when compared with concurrent control values. These decreased absolute organ weights were generally observed in conjunction with significantly decreased body weights. Although statistically significantly decreased absolute brain weights were observed for males at the 40, 75, and 125 mg/kg dose levels and for females at the 40

and 75 mg/kg dose levels, the decreases were small (5 to 12%) when compared with the decreased terminal body weights (18 to 56%). Thus, the organ weight to brain weight ratios were considered appropriate for evaluating changes in organ weights. Heart and kidney to brain weight ratios for male rats were decreased at dose levels of 40, 75 and 125 mg/kg. For males at dose levels of 75 and 125 mg/kg, the liver, spleen and testes to brain weight ratios were significantly decreased when compared with control values. The observed statistically significant decrease in testes wiehgt at 20 mg/kg was not considered treatement related in absence of a dose response. Kidney and ovary to brain weight ratios for female rats at dose levels of 40 and 75 mg/kg were significantly decreased when compared with control values. For females at a dose level of 75 mg/kg, the mean liver weight to brain weight ratio was significantly decreased when compared with controls. Other statistically significant changes were observed in the absolute and relative organ weights of males and females at dose levels > 40 mg/kg, but they occurred sporadically, and no relationship to the administration of SDS-3701 was apparent. The terminal body weights and absolute and relative organ weights of groups of rats fed diets containing SDS-3701 at dose levels of 10 and 20 mg/kg were comparable with control values. Selected absolute organ weight and organ to brain weight ratios are given in Table 6.8.1 – 6.3.1-9.

Table 6.8.1 – 6.3.1-9: Intergroup comparison of organ weights (g) and organ:brain weight ratios selected organs and groups

Organ	Dose level (mg/kg bw/day)					
Males	**0**	**10**	**20**	**40**	**75**	**125**
Heart	1.32	1.27	1.29	1.06**	0.88**	1.02** (5)
Heart:Brain x 10^{-1}	6.36	6.19	6.29	5.35*	4.69**	5.57** (5)
Kidney	3.24	3.10	3.01*	2.60**	1.80**	1.66** (5)
Kidney:Brain x 10	1.56	1.51	1.46	1.31**	0.94**	0.90** (5)
Liver	12.21	12.27	12.19	10.43*	6.89**	6.01** (5)
Liver:Brain x 10	5.90	5.98	5.93	5.26	3.59**	3.26** (5)
Spleen	0.67	0.65	0.63	0.64	0.49**	0.48** (5)
Spleen:Brain x 10^{-1}	3.25	3.16	3.07	3.21	2.50**	2.59* (5)
Testes	3.44	3.22**	3.37	3.31	2.50**	1.70** (5)
Testes:Brain x 10	1.67	1.57	1.63	1.67	1.31*	0.91** (5)
Females	**0**	**10**	**20**	**40**	**75**	**125**
Kidney	1.72	1.61	1.55*	1.35**	1.40** (7)	-
Kidney:Brain x 10	0.91	0.86	0.84	0.76**	0.79** (7)	-
Liver	6.07	6.18	5.96	5.49	4.80** (7)	-
Liver:Brain x 10	3.22	3.32	3.23	3.07	2.71* (7)	-
Ovaries	0.122	0.116	0.106	0.074**	0.052** (7)	
Ovary:Brain x 10^{-2}	6.48	6.28	5.77	4.16**	3.02** (7)	-

n = 10 unless shown otherwise by a number in parentheses (less than 10 due to mortality or clotted sample)
- = all animals dead
** Statistically significant difference from control group mean p≤ 0.01 (Student's t-test)
* Statistically significant difference from control group mean p≤ 0.05 (Student's t-test)

Microscopic findings

Histopathological examination of tissues from animals receiving diets containing DS-3701 revealed compound-related changes in the bone marrow (sternum), spleen, liver, heart, adrenal glands, kidneys, stomach and gonads. Depressed bone marrow and splenic (granulocytic and erythroid) activity were observed in rats at dose levels of 125, 250, 500 and 750 mg/kg. Slight to moderate

erythroid hyperplasia and slightly depressed granulopoiesis were observed in the bone marrow and spleen at dose levels of 40 and 75 mg/ kg. A low incidence of ulcerative gastritis was noted in groups of male and female rats at dosage levels ≥ 125 mg/ kg. Mild hepatic haemosiderosis, toxic centrilobular hepatitis, myocardial degeneration and occasional renal cortical atrophy were observed in groups of male rats at dose levels ≥ 75 mg/kg. Focal adrenal cortical necrosis and vacuolative degeneration, renal cortical tubular nephrosis and testicular degeneration were observed in groups of male rats at dose levels ≥ 125 mg/kg. In female rats, myocardial degeneration, renal cortical atrophy and ovarian follicular degeneration and necrosis were observed at dose levels ≥ 40 mg/kg. At dosage levels ≥ 75 mg/kg toxic centrilobular hepatitis, focal adrenal cortical necrosis and vacuolative degeneration and renal cortical tubular nephrosis were noted in female rats. Evaluation of tissues from male and female rats at dose levels of 10 and 20 mg/kg revealed no histopathological changes which could be attributed to the administration of DS-3701.Histopathological findings are summarized in Tables 6.8.1 – 6.3.1-10, -11 and -12.

Table 6.8.1 – 6.3.1-10: Total incidence of treatment-related findings in the spleen

Organ/finding	Dose level (mg/kg bw/day)								
Males	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Number examined	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Erythropoiesis									
Normal	6	8	9	1	3	2	0	0	0
Very slight increase	2	1	1	5	1	0	0	0	0
Slight increase	1	1	0	2	1	0	0	0	0
Moderate increase	1	0	0	2	1	0	0	0	0
Marked increase	0	0	0	0	0	0	0	0	0
Very slight decrease	0	0	0	0	4	1	0	1	1
Slight decrease	0	0	0	0	0	2	2	4	2
Moderate decrease	0	0	0	0	0	5	6	2	3
Marked decrease	0	0	0	0	0	0	2	3	4
Granulopoiesis									
Normal	10	10	10	8	0	1	0	0	0
Very slight decrease	0	0	0	2	1	0	0	0	0
Slight decrease	0	0	0	0	8	4	4	3	1
Moderate decrease	0	0	0	0	1	4	4	3	5
Marked decrease	0	0	0	0	0	1	2	4	4
Lymphopoiesis									
Normal	10	10	10	10	10	1	1	0	0
Very slight decrease	0	0	0	0	0	0	1	0	1
Slight decrease	0	0	0	0	0	4	6	3	3
Moderate decrease	0	0	0	0	0	4	2	4	5
Marked decrease	0	0	0	0	0	1	0	3	1

Organ/finding	Dose level (mg/kg bw/day)								
Females	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Number examined	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Erythropoiesis									
Normal	9	8	7	0	4	0	0	1	0
Very slight increase	1	1	3	1	1	0	0	0	0
Slight increase	0	1	0	4	0	0	0	0	0
Moderate increase	0	0	0	4	1	0	0	0	0
Marked increase	0	0	0	1	0	0	0	0	0
Very slight decrease	0	0	0	0	1	3	0	1	1
Slight decrease	0	0	0	0	2	1	0	1	4
Moderate	0	0	0	0	0	4	6	6	5
Marked decrease	0	0	0	0	1	2	4	1	0
Granulopoiesis									
Normal	10	10	9	6	1	0	0	0	0
Very slight increase	0	0	0	0	1	0	0	0	0
Very slight decrease	0	0	1	4	0	0	0	0	0
Slight decrease	0	0	0	0	6	2	0	2	4
Moderate	0	0	0	0	2	4	5	7	5
Marked decrease	0	0	0	0	1	4	5	1	1
Lymphopoiesis									
Normal	10	10	10	10	5	0	0	0	0
Very slight decrease	0	0	0	0	2	0	0	0	1
Slight decrease	0	0	0	0	1	5	4	4	3
Moderate decrease	0	0	0	0	2	5	5	5	5
Marked decrease	0	0	0	0	0	0	1	1	1

Table 6.8.1 – 6.3.1-11: Total incidence of treatment-related findings in the bone marrow (sternum)

Organ/finding	Dose level (mg/kg bw/day)								
Males	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Number examined	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Bone marrow erythropoiesis									
Normal	6	4	1	0	1	0	1	0	0
Very slight increase	3	2	3	1	2	1	1	0	0
Slight increase	1	3	6	3	3	3	0	0	0
Moderate increase	0	1	0	5	2	0	0	0	0
Marked increase	0	0	0	1	0	0	0	0	0
Very slight decrease	0	0	0	0	1	2	2	0	1
Slight decrease	0	0	0	0	1	4	3	1	1
Moderate	0	0	0	0	0	0	2	6	3
Marked decrease	0	0	0	0	0	0	1	3	4
Bone marrow erythropoiesis	0	0	0	0	0	0	0	0	0
Bone marrow granulopoiesis									
Normal	7	7	8	1	2	1	0	0	0
Very slight increase	0	0	0	0	1	0	0	0	0
Very slight decrease	3	2	2	4	3	3	1	0	0
Slight decrease	0	1	0	5	3	1	1	0	2
Moderate	0	0	0	0	1	3	7	4	6
Marked decrease	0	0	0	0	0	2	1	6	2
Organ/finding	**Dose level (mg/kg bw/day)**								
Females	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**

Number examined	9	10	10	10	10	10	10	10	10
Bone marrow erythropoiesis									
Normal	2	4	0	0	0	0	0	0	0
Very slight increase	3	2	1	3	2	5	1	1	1
Slight increase	3	3	7	4	3	0	0	0	0
Moderate increase	0	1	2	2	4	0	0	0	0
Marked increase	0	0	0	1	0	0	0	0	0
Very slight decrease	1	0	0	0	1	3	2	2	0
Slight decrease	0	0	0	0	0	2	4	2	1
Moderate	0	0	0	0	0	0	3	2	4
Marked decrease	0	0	0	0	0	0	0	2	4
Bone marrow granulopoiesis									
Normal	4	6	4	6	0	0	0	0	0
Very slight increase	0	0	0	0	1	0	0	0	0
Very slight decrease	4	1	3	0	2	0	0	0	0
Slight decrease	1	3	3	4	5	1	2	1	1
Moderate	0	0	0	0	2	6	2	5	8
Marked decrease	0	0	0	0	0	3	6	4	1

Table 6.8.1 – 6.3.1-12: Total incidence of treatment-related microscopic pathology findings

Organ/finding	Dose level (mg/kg bw/day)								
Males	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Number examined	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Liver									
Centrilobular hepatocyte hepatitis	0	0	0	0	4	9	10	7	4
Haemosiderosis	0	0	0	0	0	6	4	0	0
Atrophy	0	0	0	0	0	0	0	1	3
Heart									
Myocardial degeneration	0	0	0	0	2	4	5	7	8
Thrombus in auricle	0	0	0	0	0	3	1	0	0
Adrenals									
Cortical necrosis +/- degeneration	0	0	0	0	0	5	4	1	1
Testis									
Bilateral tubular degeneration	1	0	0	2	6	10	10	10	5
Kidney									
Mineralisation pelvis and/or medulla	0	1	0	1	1	4	3	1	0
Cortical atrophy	0	0	0	0	6	9	9	10	4
Cortical tubular necrosis	0	0	0	0	0	4	3	6	8
Stomach									
Erosive ulcerative gastritis	0	0	0	0	0	0	1	2	5
Organ/finding	**Dose level (mg/kg bw/day)**								
Females	**0**	**10**	**20**	**40**	**75**	**125**	**250**	**500**	**750**
Number examined	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**
Liver									
Centrilobular hepatocyte hepatitis	0	0	0	0	8	10	9	10	3
Haemosiderosis	0	0	0	0	4	7	7	2	0
Atrophy	0	0	0	0	0	0	0	1	2
Heart									
Myocardial degeneration	0	0	0	1	6	9	6	7	5
Thrombus in auricle	0	0	0	0	0	6	0	0	0
Adrenals									
Cortical necrosis +/- degeneration	0	0	0	0	3	8	6	4	6
Ovary									
Degeneration/necrosis of follicular epithelium	3	2	2	7	10	8/8+	9/9+	8/9+	10
Kidney									
Mineralisation pelvis and/or medulla	0	1	0	1	3	3	3	4	0
Cortical atrophy	0	0	0	5	7	7	4	7	1
Cortical necrosis +/- degeneration	0	0	0	0	2	5	7	5	3
Stomach									
Erosive ulcerative gastritis	0	0	0	0	0	3	2	0	4

+ indicates where less than 10 animals examined

Conclusions

According to the study author and applicant, groups of rats fed diets containing SDS-3701 at dose levels of 10 and 20 mg/ kg were comparable with the control group and no relationship to the administration of SDS-3701 was evident. The no observed effect level (NOEL) was, therefore, 20 mg/kg bw/day. However, the RMS concludes that based on the increased incidence and increase in severity in inactivity and piloerection at 10 mg/kg and above, a relationship to treatment with the test substance could not be excluded and, therefore, a NOAEL could not be established. In the DAR of 2000, a MOAEL was set at 10 mg/kg bw/day.

Guidelines and limitations

The study was in accordance with OECD 407 (adopted 12 May 1981).

B.6.8.1 – 6.3.2 Oral 5-week toxicity study in mouse with SDS-3701 - study 2

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristic

reference	: Ford et al., 1980	exposure	: 35 days
type of study	: oral 5-week toxicity study in the mouse	doses	: 0, 50, 125, 250, 500, 750, 1500, 3000 and 6000 mg/kg food (equivalent to 0, 7.5, 18.8, 37.5, 75, 112.5, 225, 450 and 900 mg/kg bw)
year of execution	: 1978		
test substance	: SDS-3701 (batch 8307-26A-1), fine textured white powder, purity 99.6%		
route	: oral	vehicle	: food
species	: mouse (CD-1)	GLP statement	: no
group size	: 10/sex/group	guideline	: not in compliance with OECD 407
		NOAEL	: see guidelines and limitations

Results and conclusions

- Systemic effects (decreased body weights and centrilobular and pericentral hypertrophy of the liver) were observed in both sexes at concentrations ≥500 mg/kg food (≥75 mg/kg bw).
- A NOAEL was not established (see guidelines and limitations).

Guidelines and limitations

The study has not been carried out in compliance with OECD guideline 407 (adopted 12 May 1981), because no haematology, clinical chemistry and organ weighing were performed. Furthermore, only liver, kidney and gross lesions were taken from the animals for gross- and histopathology, animals were older than 6 weeks and the study design (5 weeks) is deviant. Based on these shortcomings, this study is not considered suitable to establish a NOAEL and the study is therefore considered inappropriate for the overall evaluation.

B.6.8.1 – 6.3.3 Oral 30-day toxicity study in rat with SDS-46851- study 3

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Wilson et al., 1986d	exposure	: 30 days
type of study	: 30 day oral toxicity study	doses	: 0, 500, 2000 mg/kg bw
year of execution	: 1985	vehicle	: food
test substance	: SDS-46851 (batch: SDS-46851-0202, tan microcrystals, purity > 94%	GLP statement	: yes
		guideline	: not in compliance with OECD 407
route	: oral	**NOAEL**	: 500 mg/kg bw/day
species	: rat (Sprague-Dawley)		
group size	: 5/sex/dose		

Study design

The study fulfils OECD 407 with the exception that two treatment groups were included instead of three. Dose levels were obtained from a 14 day study (Wilson et al., 1985d), in which up to 2000 mg/kg bw/day no treatment-related findings were observed.

Results

The results are summarized in Table 6.8.1 – 6.3.3

Table 6.8.1 – 6.3.3 Summary of results, Study 3

Dose (mg/kg bw)	0		500		2000		dr
	m	f	m	f	m	f	
Mortality	none						
Clinical signs							
ears							
- redness, swelling, scabbing			++	++	++	++	
Body weight	no treatment-related findings						
Food consumption	no treatment-related findings						
Haematology	no treatment-related findings						
Clinical chemistry							
total protein			ic	ic			
globulin				ic			
Urinalysis	no treatment-related findings						
Organ weights							
liver				ic^{r}	$ic^{a,r}$	ic^{r}	f
Pathology							
macroscopy							
kidneys							
- dilated renal pelvis						+	
microscopy							
liver							
- centrilobular hepatocellular hypertrophy			$+^{1}$		++	++	
kidneys							
- pelvic dilatation						+	

dr	dose related
ic	statistically significantly increased
+	present in one/a few animals
++	present in most/all animals
a	absolute organ weight
r	relative organ weight
1	observed in one animal

Conclusions

- According to the authors, the effects observed on the ears might have been due to an excessively tight attachment of the tags to the ears. However, this does not explain why these effects were not observed in the control animals. According to the RMS, the effects observed were probably the result of test substance settling in-between ears and tag, causing local effects due to the irritating properties of the test substance.
- The changes in clinical chemistry were observed in the 500 mg/kg bw dose group only and were not supported by gross or histopathological findings. Therefore, these findings were not considered to be of toxicological relevance.
- The statistically significant increases in relative liver weights (respectively 3.088 and 3.294 g/100g), observed in female rats at 500 and 2000 mg/kg bw, could be explained by the relatively low liver weights in the controls (2.816 g/100g, compared to the presented historical control group-data: 3.004-3.364 g/100g).
- In the 2000 mg/kg bw/day dose group compound related effects were judged to have occurred, based on the increases in weights and the histopathological changes in the liver in both sexes.
- The NOAEL was considered to be 500 mg/kg bw/day, although one male rat out of five had very slight centrilobular hepatocellular hypertrophy.
- It is noted that local effects were observed at the lowest dose level (500 mg/kg bw/day).

Guidelines and limitations

The study is in accordance with OECD 407 (adopted 12 May 1981), with the exception of the two included treated groups instead of three. Furthermore, the body weights of the animals at the start of the study were not reported. Despite these shortcomings, the study is considered suitable for evaluation.

B.6.8.1 – 6.3.4 Oral 28-day toxicity study in mouse with SDS-46851- study 4

Previous evaluation	In DAR (2000)
Evaluation RMS	In the DAR, the MOAEL of 46 mg/kg bw/d, was indicated as equivocal, since the effect is minimal and not dose related. After re-evaluation, the NOAEL is set at 2028 mg/kg bw/d, the highest dose tested, since no consistent, dose-related effects were observed.

Characteristics

reference	: Mizens et al., 1990	exposure	: 28 days
type of study	: oral 28-day toxicity study	doses	: 0, 250, 500, 1000, 5000, 10000 mg/kg food (equal to 0, 46, 96, 188, 963 and 2028 mg/kg bw for males and 0, 54, 108, 217, 1023 and 2112 mg/kg bw for females)
year of execution	: 1990		
test substance	: SDS 46851 (SDS 46851-0207), off- white powder, purity 99.3%		
route	: oral		
species	: mouse (Cr1: CD1-1(1 CR) VAF plus)	vehicle	: food
		GLP statement	: yes
group size	: 5/sex/dose	guideline	: not in compliance with OECD 407
		NOAEL	: 2028 mg/kg bw/day (see conclusions)

Study design

The study was in accordance with OECD 407 (adopted 12 May 1981) with the exception that no clinical chemistry was performed, the adrenals were not weighed and the adrenals, spleen and heart were not examined microscopically.

Results

The results are summarized in Table 6.8.1 – 6.3.4-1 and -2

Table 6.8.1 – 6.3.4-1 Summary of results, Study 4

Dose (mg/kg food)	0		250/500		1000/5000		10000		dr
	m	f	m	f	m	f	m	f	
Mortality	none								
Clinical signs	no treatment-related findings								
Body weight	no treatment-related findings								
Food consumption							ic[1]		
Haematology	no treatment-related findings								
Clinical chemistry	not conducted								
Organ weights	no treatment-related findings								
Pathology									
macroscopy	no treatment-related findings								
microscopy									
kidneys									
- hyperplasia renal tubule epithelium			+[2]		+[3]		+		

dr dose related
ic statistically significantly increased
\+ present in one/a few animals
1 food consumption relative to body weight
2 in the 250 mg/kg bw group only
3 in the 1000 mg/kg bw group only

Table 6.8.1 – 6.3.4-2 Pathology results – study 4

Dose (mg/kg food)	0		250		500		1000		5000		10000	
	M	F	M	F	M	F	M	F	M	F	M	F
Pathology												
microscopy												
kidneys												

- hyperplasia renal tubule epithelium (slight/mild)	0	0	1	0	0	0	1	0	0	0	2	0

Conclusions

On the basis of the hyperplasia of the renal tubule in males of the 250, 1000, and 10000 mg/kg food dose group, a MOAEL of 250 mg/kg food (equal to 46 mg/kg bw) was established in thr DAR (2000). The relationship of this alteration to treatment is considered equivocal due to the lack of a dose response or clearly increased incidence in high dose animals. Therefore, in this renewal, the NOAEL is set at 2028 mg/kg bw/d, the highest dose tested, since no consistent, dose-related effects were observed.

coRMS-BE: agrees with RMS-NL that the renal hyperplasia data do not suggest a dose-response relationship. It also appears that a similar pathology was apparently not observed in the rat study (see Study 3 hereabove). This event is likely to be an incidental finding. The setting of NOAEL to 2028 mg/kg bw/day is supported.

Guidelines and limitations

The study was not in accordance with OECD 407 (adopted 12 May 1981) because no clinical chemistry was performed, the adrenals were not weighed and the adrenals, spleen and heart were not examined microscopically. Despite these shortcomings, the study is considered suitable for evaluation.

B.6.8.1 – 6.3.5 Oral 37/38-day toxicity study in dog with SDS-46851- study 5

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. A table with individual body weights is added, for the discussion on the ARfD.

Characteristics

reference	: Serrone et al., 1989	exposure	: 37/38 days
type of study	: 37/38-day oral toxicity study	doses	: 0, 100, 500 and 1000 mg/kg bw
year of execution	: 1987	vehicle	: gelatine capsules
test substance	: SDS-46851 (SDS-46851-0202), beige powder, purity 98%	GLP statement	: yes
		guideline	: no OECD available
route	: oral (capsule)	**NOAEL**	: see guidelines and limitations
species	: dog (Beagle)		
group size	: 2/sex/dose		

Study design and conclusions

Based on the fact that the number of animals used in this study was too small (2/sex/dose), this study is not considered suitable for establishing a NOAEL. In this study, dose-related effects on body weight, food consumption, pH in the urine and organ weights (testes/epididymides, liver and heart) were observed at levels ≥500 mg/kg bw. Microscopical findings included multifocal centrilobular to a diffuse hepatocytomegaly, diffuse hypoplasia of the prostate and micro-follicles in the thyroid (≥500 mg/kg bw). Effects on liver, prostate and thyroid were observed at the lowest dose level tested (100 mg/kg bw).

INDIVIDUAL BODY WEIGHT (KILOGRAMS)
WEEK

Animal Number	Sex	Dose	0	1	2	3	4	5
1001	M	Control	8.1	8.4	8.5	8.9	9.1	9.2
1002	M	Control	7.8	8.6	9.0	9.3	9.5	9.6
2001	M	100 mg/kg	8.0	8.5	8.4	8.6	8.6	9.1
2002	M	100 mg/kg	8.6	8.8	9.4	9.3	9.5	9.5
3001	M	500 mg/kg	8.2	8.5	8.6	8.8	8.8	9.0
3002	M	500 mg/kg	7.2	7.5	7.9	7.8	7.4	6.2
4001	M	1000 mg/kg	7.7	8.2	8.2	8.3	8.4	8.3
4002	M	1000 mg/kg	8.2	8.8	8.8	8.5	7.2	5.9
1501	F	Control	4.7	4.9	5.1	5.4	5.7	5.7
1502	F	Control	7.1	7.7	8.0	8.4	8.4	8.8
2501	F	100 mg/kg	5.2	5.4	5.7	5.8	6.2	6.1
2502	F	100 mg/kg	6.6	7.1	7.4	7.6	8.0	8.2
3501	F	500 mg/kg	5.5	5.7	5.8	5.9	6.2	6.2
3502	F	500 mg/kg	5.7	6.1	6.2	6.2	6.3	6.1
4501	F	1000 mg/kg	5.8	6.2	6.3	6.3	6.5	6.7
4502	F	1000 mg/kg	5.3	5.6	5.5	5.1	4.5	4.8

B.6.8.1 – 6.3.6 Oral 90-day toxicity study in rats with SDS-3701- study 6

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant. After the commenting round, the notifier provided an argumentation regarding the NOAEL setting (refer to data requirement 2.41 below). The RMS agrees with the notifier. The NOAEL remains unchanged. In the PPR Expert meeting (162 – session 2, September 2017), the NOAEL was set at 15 ppm (1 mg/kg bw/day) based on a decrease in body weight gain in females at 4 mg/kg bw/day.

Report:	K-CA 5.8.1/07 Van-Otterdijk F (2007) SDS3701: 90 Day oral toxicity study with SDS3701 by dietary administration in the rat. NOTOX B.V., Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands. Laboratory Report No. 481646, 18 December 2007. Unpublished. (Syngenta File Number R182281_10023)

GUIDELINES: Repeated dose oral toxicity (rat): OECD 408 (1998): OPPTS 870.3100 (1998): EC Directive 67/548/EEC B.26 (2001): Japanese Chemical Substances Control Law 1987, Notification of Nov. 21 2003 by MHLW (No. 1121002), METI (No. 2) and ME (No. 031121002).

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

Acceptability: The study was performed in accordance with OECD guideline 408 of 1998. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

In a subchronic toxicity study SDS3701 (purity 99.9%) was administered for at least 90 days by dietary administration to SPF-bred Wistar rats. One control group and three treated groups (15, 50 and 250 ppm) were tested, each group consisting of 10 males and 10 females. Chemical analyses of diet preparations were conducted during the study to assess accuracy, homogeneity and stability over 2 weeks at room temperature under normal laboratory light conditions. The following parameters were evaluated: clinical signs daily; functional observation tests in week 12-13; body weight weekly; food consumption twice weekly (weekly from week 6 onwards); ophthalmoscopy pretest and in week 12; clinical pathology (haematology, clinical biochemistry and urinalysis); macroscopy at termination; organ weights (adrenal glands, brain, epididymis, kidnyes, heart, liver, ovaries, spleen, testes, thymus and uterus) and histopathology on all tissues collected at the scheduled sacrifice from all treated animals and all gross lesions.

Materials:

Test Material:	SDS3701
Description:	White powder
Lot/Batch number:	51955-15-21
Purity:	99.9%
CAS#:	Not reported
Stability of test compound:	Expiry date 06 November 2008 (stored refrigerated in the dark)

Results

Dietary analyses confirmed that diets were prepared accurately and homogenously, and were stable over at least 2 weeks at room temperature under normal laboratory light conditions. The mean test article intake over the study period was as follows: 1, 3, 16 mg/kg bw/day for males and 1, 4, 17 mg/kg bw/day for females for dietary inclusion levels of 15, 50 and 250 ppm, respectively.

No mortality occurred during the observation period and no clinical signs of toxicity were noted. Hearing ability, pupillary reflex, static righting reflex and grip strength were normal in all animals. The variation in motor activity did not indicate a relation with treatment. There were no toxicologically relevant ophthalmology findings at pre-dose and in week 12.

Body weight and weight gain: Body weights and body weight gain were reduced in females at 250 ppm, achieving a level of statistical significance throughout the treatment period. Total weight gain difference over the treatment period compared to control weight gain was approximately 35%. Among other dose groups, no changes in body weights and body weight gain were noted that were clearly associated with treatment and toxicologically significant. The statistically significant lower body weight gain of males at 50 and 250 ppm in the first week of treatment and of females at 15 ppm in week 12 was of a temporary and slight nature. Body weights and body weight gain data are presented in Tables 6.8.1 – 6.3.6-1 and -2.

Table 6.8.1 – 6.3.6-1: Intergroup comparison of body weight (g) - selected timepoints

	Dietary Concentration (ppm) Males				Females			
Day	**0**	**15**	**50**	**250**	**0**	**15**	**50**	**250**
1	304	307	309	307	208	213	204	203
8	357	358	354	349	224	228	218	209*
15	382	390	386	381	237	235	224	217**
29	430	439	431	428	247	253	234	226**
64	507	516	511	496	272	277	261	240**
90	544	559	545	530	282	283	269	251**

* Statistically significant difference from control group mean, p<0.05
** Statistically significant difference from control group mean, p<0.01

Table 6.8.1 – 6.3.6-2: Intergroup comparison of body weight gains (%) - selected timepoints

	Dietary Concentration (ppm) Males				Females			
Day	**0**	**15**	**50**	**250**	**0**	**15**	**50**	**250**
8	18	17	15*	13**	8	7	7	3**
15	26	27	25	24	14	10	10	7**
29	41	43	40	39	19	18	15*	11**
64	67	68	65	61	31	30	28	18**
90	79	82	77	73	36	33	32	23**

* Statistically significant difference from control group mean, p<0.05
** Statistically significant difference from control group mean, p<0.01

Food consumption and compound intake: Food consumption was slightly reduced in females at 250 ppm throughout the treatment period. Relative food consumption of these females (i.e. after correction for body weight) showed a slight reduction in the first week of treatment only. Food consumption and relative food consumption of other groups was similar to control levels. Food consumption data are given in Table 6.8.1 – 6.3.6-3.

Table 6.8.1 – 6.3.6-3: Intergroup comparison of food consumption (g/animal/day) - selected timepoints

	Dietary Concentration (ppm) Males				Females			
Days	**0**	**15**	**50**	**250**	**0**	**15**	**50**	**250**
1-4	26	24	24	24	17	18	17	14
4-8	27	26	27	25	16	17	16	13
18-22	28	28	28	27	16	17	16	14
36-43	24	28	29	28	19	19	18	16
64-71	27	28	29	28	18	19	18	16
85-90	29	29	31	29	19	19	19	17
Overall (a)	27	27	28	27	18	18	18	15

No statistically significant differences from control group mean
(a) mean of means over treatment period

Mean test article intake over the study period was as follows:

Table 6.8.1 – 6.3.6-4: Mean Dose Received (mg/kg bw/day)

Dietary concentration (ppm)	15	50	250
Males	1	3	16
Females	1	4	17

Haematology: In females at 250 ppm, reduced red blood cell counts, haemoglobin and haematocrit levels, and increased red cell distribution width were noted. Red cell distribution width was also increased in males at 250 ppm, without concurrent changes in haematological parameters. These changes achieved a level of statistical significance.
Statistically significant lower haemoglobin and mean corpuscular haemoglobin concentration levels in females at 15 ppm occurred in the absence of a dose-related distribution. One female at 15 ppm showed a notably increased red cell distribution width (RDW), mean corpuscular volume (MCV) and reticulocyte count, along with a lower haemoglobin and red blood cell count. Similar changes were

absent among the other animals of this dose group, and of the next higher dose group. Therefore, no toxicological significance was ascribed to these alterations. Haematology data are given in Table 6.8.1 – 6.3.6-5.

Table 6.8.1 – 6.3.6-5: Intergroup comparison of selected haematology parameters

Parameter	Dietary Concentration (ppm) Males				Females			
	0	15	50	250	0	15	50	250
Red blood cells (10E12/L)	8.90	9.04	8.63	8.73	8.19	7.82	8.00	7.63*
Haemoglobin (mmol/L)	9.7	9.9	9.6	9.4	9.4	9.0*	9.2	8.3**
Haematocrit (L/L)	0.456	0.470	0.448	0.442	0.437	0.429	0.430	0.389**
RDW (%)	13.3	12.8	13.1	14.4**	12.3	13.5	12.4	14.6**

* Statistically significant difference from control group mean, p<0.05
** Statistically significant difference from control group mean, p<0.01

Blood clinical chemistry: Reduced total bilirubin levels in males at 50 and 250 ppm, and in females at 15 ppm and higher, increased creatinine level in males at 50 and 250 ppm, and reduced sodium level in males and females at 250 ppm were considered treatment-related changes. Other statistically significant changes were considered to be of no toxicological significance as they occurred in the absence of a treatment-related distribution. Clinical biochemistry data are given in Table 6.8.1 – 6.3.6-6.

Table 6.8.1 – 6.3.6-6: Intergroup comparison of selected clinical chemistry parameters

Parameter	Dietary Concentration (ppm) Males				Females			
	0	15	50	250	0	15	50	250
Total bilirubin (umol/L)	2.5	2.2	1.9**	1.4**	3.3	2.2**	1.8**	1.5**
Creatinine (umol/L)	39.1	41.7	44.9**	47.4**	47.2	49.7	51.1*	49.8
Sodium (mmol/L)	143.9	143.3	144.3	142.6*	142.6	141.8	142.4	140.8**

* Statistically significant difference from control group mean, p<0.05
** Statistically significant difference from control group mean, p<0.01

Urinalysis: There were no differences in urine clinical chemistry parameters which were considered to be related to treatment.

Macroscopic findings: A small number of lesions were observed, none of which was related to treatment.

Organ weights: Males at 250 ppm showed an increased liver to body weight ratio (12% when compared to controls), achieving a level of statistical significance. Statistically significant alterations in organ weights and organ to body weight ratios of females at 250 ppm (lower heart, thymus and kidney weight, and higher brain to body weight ratio) were considered to be related to the reduced terminal body weight. Organ weight data are given in Table 6.8.1 – 6.3.6-7.

Table 6.8.1 – 6.3.6-7: Intergroup comparison of selected organ weights and organ weight ratios

Parameter	Dietary Concentration (ppm) Males				Females			
	0	15	50	250	0	15	50	250
Absolute heart weight (gram)	1.503	1.488	1.550	1.503	0.935	0.954	0.868	0.802*
Absolute thymus weight (gram)	0.302	0.342	0.308	0.289	0.264	0.240	0.232	0.194**
Absolute kidney weight (gram)	3.38	3.43	3.30	3.31	1.80	1.90	1.69	0.19**
Relative brain weight (%)	0.43	0.42	0.44	0.43	0.76	0.80	0.81	0.85**
Relative liver weight (%)	2.49	2.52	2.53	2.50**	2.67	2.80	2.66	2.89

* Statistically significant difference from control group mean, p<0.05
** Statistically significant difference from control group mean, p<0.01

Microscopic findings: A small number of spontaneous lesions were observed, none of which was related to treatment. In males the incidence of vacuolation of the zona fasciculata of the adrenal cortex appeared slightly increased. In group 4 (250 ppm) vacuolation was recorded in 9/10 animals (six animals: minimal and three animals: slight). In males of group 1 (control) this was seen in 6/10 animals (four animals: minimal and two animals: slight). In group 2 (15 ppm) vacuolation of the adrenal cortex was recorded in 7/10 animals (six animals: minimal, one animals slight) and in group 3 (50 ppm) in 5/10 animals (three animals: minimal, two animals: slight). As there was no meaningful increase in either severity or incidence with dose, the finding was considered not treatment related.

Conclusion:

The effects seen at 50 ppm on clinical biochemistry were not accompanied by any further histopathological changes or an effect on organ weight. Therefore, the observed changes at 50 ppm in clinical biochemistry were not considered toxicological relevant. Based on the number of changes in haematological and clinical biochemistry parameters primarily at 250 ppm, and in particular the reduced body weights in females at 250 ppm, a No Observed Adverse Effect Level (NOAEL) for SDS3701 of 50 ppm was established, corresponding to 3 and 4 mg/kg bw/day for males and females respectively.

Data requirement 2.41 / Experts' consultation 2.15

- Applicant to provide a technical position on the 90-day study in rats with SDS-3701.
- MSs experts to discuss the NOAEL of the 90-day rat study performed with SDS-3701 in an experts' meeting.

See reporting table 2(90)

NL (August 2017): The argumentation from the notifier and the response by the RMS regarding the NOAEL setting in the 90-day study in rats with SDS-3701 is provided below.

Response by Task Force :

"Task Force agrees with the position of the RMS that effects seen at 50 ppm in clinical biochemistry were not accompanied by any further histopathological changes or an effect on organ weight and are therefore not considered toxicological relevant. It is noteworthy that in a 2yr study with rats (Ford, 1983), no statistically significant differences in bilirubin were seen following 6 months exposure in males up to 15mg/kg bw/day and in females only small changes were evident at 6 months at 3mg/kg bw/day however, this did not show a clear dose response relationship."

Response by RMS:

In line with the above technical position provided by the notifier, the RMS remains of opinion that the NOAEL should remain at 50 ppm as the observed effect in bilirubin at this dose level was not accompanied by any further histopathological changes or effects on organ weight. In addition, the RMS agrees with the notifier that in the 2-year study in rats (Ford, 1983; refer to B.6.8.1 – 6.5.1), at a similar dose level (3 mg/kg bw/day, approx. 50 ppm) no clear treatment-related effects were seen on bilirubin levels .

The NOAEL for this 90-day rat study with SDS-3701 will be discussed during the experts' meeting (Expert's consultation 2.15).

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the PPR Expert meeting , the NOAEL for this 90-rat study was discussed. The NOAEL proposed by the RMS was 50 ppm based on BW gain and haematological changes. The bilirubin effect (decrease) was not accompanied by any histopathological effect and then not considered adverse. However, there was an increase in creatinine in males and a decrease in BW gain in females at 50 ppm. The effect on BW gain (around 10%) in females was considered adverse and therefore the NOAEL was set at 15ppm (1 mg/kg bw per day).

B.6.8.1 – 6.3.7 Oral 120-day toxicity study in rat with SDS-3701 - study 7

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: Hastings et al., 1975a	exposure	: 120 days
type of study	: 120-day dietary toxicity study	doses	: 0, 10, 50, 100 and 200 mg/kg food[2]
year of execution	: 1975	vehicle	: diet
test substance	: SDS-3701[1]	GLP statement	: no
route	: oral	Guideline	: not in accordance with OECD 408
species	: rat (Sprague-Dawley)	**NOAEL**	: see guidelines and limitations
group size	: 10 males/dose, 20 females/dose		

[1] 4-hydroxy 2,5,6-trichloroisophthalonitrile, batch no.: 8180-70, white crystalline powder, > 99%
[2] 1 equivalent to 0, 0.5, 2.5, 5 and 10 mg/kg bw/day

Study design

The study lasted for 120 days. After 70 days the rats were mated and pregnant females went through gestation and lactation. Blood samples were taken at 120 days from 10 male and 20 female rats from the 0, 10, 50 and 100 mg/kg food groups and from 5 males and 10 females of the 200 mg/kg food group for haematological and clinical chemistry investigations. Urine samples were taken at 120 days from 4 males and 8 females from each of the 0, 10, 50 and 100 mg/kg food groups.

Guidelines and limitations

- This study has been performed prior to GLP regulations and OECD guideline 408 became effective. As a consequence there are several deviations from this guideline. Only limited clinical chemistry parameters were investigated, no data on the clinical investigations were presented and no ophthalmoscopy was performed.

- Although is was stated that the rats went through a reproduction phase, no data on reproductive performance were presented. Pregnancy or non-pregnancy may have compromised the study results.
- The results are poorly presented. No statistics were presented.
- On the basis of the above considerations, the study is unsuitable for evaluation.

B.6.8.1 – 6.3.8 Oral 90-day toxicity study in dog with SDS-3701- study 8

Previous evaluation	In DAR (2000). Re-submitted for the purpose of renewal.
Evaluation RMS	In the DAR (2000), a NOAEL of < 50 mg/kg food was established (equivalent to < 1.25 mg/kg bw/day), based on statistically significant increase in blood glucose and decrease in ASAT and ALAT in the 50 and 100 mg/kg bw/day dose groups. However, these changes in glucose, ALAT and ASAT are in general not consistent over time or sex and, according to the study author, were within the normal range (historical control data not provided). The NOAEL is set at 1.25 mg/kg bw/d, based on dose-related clinical signs at 100 ppm (2.5 mg/kg bw/d).

Characteristics

reference	: Bundy et al., 1975	exposure	: 90 days
type of study	: 90-day feeding study	doses	: 0, 50, 100 and 200 mg/kg food[2]
year of execution	: 1975	vehicle	: diet
test substance	: SDS-3701[1]	GLP statement	: no
route	: oral	Guideline	: not in accordance with OECD 409
species	: dog, beagle	**NOAEL**	: 1.25 mg/kg bw/day
group size	: 4/sex/dose		

[1] 4-hydroxy 2,5,6-trichloroisophthalonitrile, batch no.: 8180-70, white crystalline powder, > 99%
[2] equivalent to 0, 1.25, 2.5 and 5 mg/kg bw/day

Study design

The animals were observed daily for overt signs of toxicity throughout the study. Body weights and food consumption were recorded weekly. Blood samples were taken at pre-study and at 4 and 13 weeks for haematological and clinical chemistry investigations. Urine samples were taken at pre-study and at 4 and 13 weeks. All animals were subjected to a gross *post-mortem* examination. Weights of organs were recorded and histopathological investigations were carried out on all animals.

Results

The results are presented in Table 6.8.1 – 6.3.8-1.

Table 6.8.1 – 6.3.8-1 Summary of results, Study 8

Dose (mg/kg food)	0		50		100		200		dr[6]
	m	f	m	f	m	f	m	f	
Mortality	0/4	0/4	0/4	0/4	0/4	0/4	4/4	4/4	
Clinical signs[1]									
tarry stools					+	+	++	++	
anorexia							++	++	
Body weight							dc	dc	
Food consumption							dc	dc	
Ophthalmoscopy	not performed								
Haematology									
eosinophils			dc[4]	ic[5]	dc[5]	ic[3]	dc[3]		
WBC			ic[4]		ic[4]				
HCT					dc[4]			ic[3]	
RBC					dc[4]				
haemoglobin					dc[4]				
Clinical chemistry									
glucose			ic[4]		ic[4]			dc[3]	
bilirubin								ic[3]	
ASAT			dc[4]		dc[4]			ic[3]	
ALAT			dc[4]		dc[4]				
calcium					ic[4]				
Urinalysis	no treatment-related findings								
Organ weights									
spleen							dc[ar]	dc[ar]	
kidneys							dc[ar]	ic[r]	
heart							dc[a], ic[r]	dc[a], ic[r]	
liver					i[r]	i[r]	dc[a]	dc[a]	
testes							dc[ar]		
ovaries								ic[r]	
thyroid							dc[a]	dc[a]	
adrenals							ic[r]	ic[r]	
Pathology									
macroscopy									
emaciation							++	++	
microscopy[1]									
liver:									
bilistasis							++	++	
parenchymatous degeneration							++	+	
acute diffuse necrosis								+	
focal necrosis									
kidneys:							++	++	
tubular degeneration									
stomach:								++	
diffuse oedema in (sub)mucosa									

dr	dose related
dc/ic	statistically significantly decreased/increased
d/i	decreased/increased, but not statistically significantly
a/r	absolute/relative organ weight
+	present in one/a few animals
++	present in most/all animals
1	based on description in results section, no summary table or individual data presented
2	statistically significant according to the authors, however no statistics were presented
3	observed at 4 weeks only
4	observed at 13 weeks only
5	observed at 4 and 13 weeks
6	only for the 50 and 100 dose groups, since all animals died in the high dose group

`

Blood clinical chemistry: Females at 200 ppm had increased bilirubin values, decreased glucose and increased SGOT (ASAT) at the 4 week assessment. Decreased food consumption and catabolism of body tissues probably account for the glucose values and, in part, the SGOT values. The increased bilirubin values are considered to be due to the compound-induced liver toxicity confirmed by histopathology. Changes in blood biochemistry values are presented in Table 6.8.1 – 6.3.8-2. Statistically significant decrease in SGOT (ASAT) and SGPT (ALAT) and an increased in blood glucose were noted in males at 50 and 100 mg/kg food, although in general these findings were not consistent over time or sex and, according to the study author, were within the normal range (historical control data not provided). Moreover, these effects were, except for ALAT, not reproduced in a more recent 1-year dog study (study 9, B.6.8.1 – 6.3.9), but in that study no toxicological significance is adhered to a decrease in tha decrease in ALAT.

Table 6.8.1 – 6.3.8-2: Intergroup comparison of selected clinical chemistry parameters

	Dietary Concentration (ppm)							
	Males				Females			
Parameter	**0**	**50**	**100**	**200**	**0**	**50**	**100**	**200**
Bilirubin								
Week 4	0.51	0.27	0.34	0.69	0.33	0.32	0.20	1.11*
Week 13	0.34	0.31	0.31	-	0.28	0.40	0.26	-
Glucose								
Week 4	85.7	85.4	86.5	69.0	82.6	86.4	87.3	55.1*
Week 13	89.0	101.8*	106.1	-	106.9	99.1	98.5	-
SGOT (ASAT)								
Week 4	21.0	21.3	16.8	34.0	22.8	18.8	20.5	57.5*
Week 13	35.7	22.3*	24.8*	-	31.5	24.8	40.5	-
SGPT (ALAT)								
Week 4	16.7	15.0	17.8	16.3	24.0	15.0	14.0	20.1
Week 13	24.7	13.0*	10.8*	-	18.3	14.5	11.0	-

* 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)

Table 6.8.1 – 6.3.8-3: Intergroup comparison of selected mean organ weights and organ/final body weight ratios

		Dietary Concentration (ppm)							
		Males				Females			
Organ		0	50	100	200	0	50	100	200
Spleen	abs (g)	62.77	54.85	44.18	7.9*	54.75	51.28	45.15	7.1*
	bw ratio (%)	0.60	0.60	0.56	0.22*	0.71	0.66	0.59	0.23*
Kidney	abs (g)	58.87	55.4	51.28	35.5*	42.03	40.2	39.7	40.4
	bw ratio (%)	0.57	0.61	0.64	0.98*	0.54	0.52	0.53	1.34*
Heart	abs (g)	81.7	81.7	76.1	43.0*	61.1	65.1	59.7	32.6*
	bw ratio (%)	0.78	5.69	0.94	1.17*	0.78	0.84	0.80	1.09*
Liver	abs (g)	359.5	302.7	346.0	145.2*	287.0	290.1	309.8	132.6*
Thyroid	abs (g)	1.016	0.812	1.412	0.580*	0.908	1.066	0.855	0.418*
Adrenal	bw ratio (%)	0.010	0.011	0.016	0.028*	0.014	0.014	0.012	0.038*

bw ratio = organ to final body weight ratio, abs = absolute weight

* 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)

Microscopic findings: At a dietary concentration of 200 ppm, treatment-related microscopic findings were present in the liver and kidney. The four male dogs died spontaneously and two of them showed extensive *post-mortem* changes, especially in the kidneys and liver. In both livers, however, evidence of *in vivo* bile stasis was present. Scattered throughout the livers was accumulation of bile pigment. Because of the marked *post-mortem* changes it was extra cellularly. The other two males showed extensive parenchymatous degeneration of the liver with bile retention. Also, degeneration of the epithelium of the renal tubules was present.

Diffuse acute liver necrosis was present in one female and the kidneys showed degeneration of the convoluted tubules. There was submucosal fibrosis in the gastric wall. One female exhibited focal necrosis of the liver with deposition of bile pigments and degeneration of the renal tubules was present. This animal was the only dog in this group which was not found dead. It was, however, sacrificed early *in extremis*. The other two females showed *post-mortem* changes in their viscera. There was accumulation of bile pigment in the liver and diffuse oedema in the mucosa and submucosa of the stomach.

Conclusions

- At the highest dose level all animals died. Before death, effects on body weight, food consumption, bilirubin and histopathological changes of the liver, kidney and stomach and effects on organ weights (spleen, kidneys, heart, liver, testes, ovaries, thyroid and adrenals), were observed.
- According to the authors, the NOAEL should be placed at 100 mg/kg food based on mortality, effect on organ weights, bilirubin, ASAT and histopathological changes of the kidneys and liver, at a level of 200 mg/kg food.
- However, the changes in glucose, ALAT and ASAT are in general not consistent over time or sex and, according to the study author, were within the normal range (historical control data not provided).

- The NOAEL is set at 50 ppm (1.25 mg/kg bw/d), based on dose-related clinical signs at 100 ppm (2.5 mg/kg bw/d).

Co-RMS

The LOAEL in this study is set by the RMS-NL to 2.5 mg/kg bw/day based on “tarry stools”, the sole clinical sign mentioned at this dose. Should we regard this effect as an adverse event susceptible to support a LOAEL? Or should we support the author’s view (NOAEL 2.5 mg/kg bw/d).

Reply RMS

Tarry stools were noted in a dose dependent way. The RMS agrees that the finding is not severe, but should still be considered test substance related and adverse.

Guidelines and limitations

This study has been performed prior to GLP regulations and OECD guideline 409 became effective. As a consequence there are several minor deviations from this guideline, which do not affect the relevance of the results in the context of Annex II. The report does not contain information on the stability of the test substance mixed with the food, no data on the clinical investigations were presented and no ophthalmoscopy was performed. Nevertheless, the study is considered appropriate for the overall evaluation.

B.6.8.1 – 6.3.9 Oral one-year toxicity study in dogs with SDS-3701- study 9

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Data requirement 2.24 / Experts' consultation 2.13

- Applicant to provide a technical position on dogs studies with SDS-3701.
- MSs experts to discuss the short term NOAEL of SDS-3701 in dogs in an experts' meeting.

See reporting table 2(70)

NL (August 2017):

EFSA provided the following comment regarding the relevant NOAEL based on 90-day/1-year dog studies with metabolite SDS-3701 for ADI setting : "*In our view, the relevant NOAEL for short term dog studies should be set at 0.83 mg/kg bw per day from the 1-year study since it represent a longer treatment period and the LOAEL of the 1-year study (1.8 mg/kg bw per day) is too close to the NOAEL of the 90-day study (1.25 mg/kg bw per day)."*

The current NOAEL setting in the 1-year dog study for SDS-3701 is, however, based on minimal effects in male dogs at the 30 ppm dose level (0.83 mg/kg bw/day). The notifier is of the opinion that the NOAEL from this study should be 60 ppm (1.8 mg/kg/day) for male dogs instead of 30 ppm (0.83 mg/kg bw/day) and remains 30 ppm (0.95 mg/kg/day) for female dogs. The current NOAEL is based on minimal effects in male dogs, however, the observed increase in blood glucose was not considered an adverse effect of the test material by the study authors as no other blood chemistry alterations were seen. In addition, also the slight alterations in RBC and MHC concentration in male dogs were not considered adverse as there were no other effects on the haemotological parameters. Then for male dogs the NOAEL is set at 60 ppm (1.8 mg/kg bw/day) and the relevant NOAEL is 0.95 mg/kg/day based on the effects observed in females. The RMS agrees with the notifier, however this point will be discussed during the expert meeting. Additional results tables have been added to the summary below (haematology and blood clinical chemistry).

In this case, the NOAEL in this specific 1-year dog study is thus increased to 0.95 mg/kg/day based on the observations in female dogs. Based on the EFSA comment, this results in an unchanged ADI of 0.01 mg/kg bw/day using a safety factor of 100 (Volume 1, point 2.6.11). However, the relevant NOAEL for ADI setting will also be discussed at the Expert meeting.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

During the expert meeting it was concluded that the NOAEL should be set at 0.83 mg/kg bw/day, based on reduced red blood cells in both sexes and reduced BW gain in females only at 1.86 mg/kg bw per day.

Characteristics

reference	: Schetter et al., 2000	exposure	: 52 weeks
type of study	: oral one-year toxicity study with dogs	doses	: 0, 30, 60 and 120 ppm[1]
year of execution	: 1997-1998	vehicle	: diet
test substance	: SDS-3701, 4-hydroxy-2,5,6-trichloro-isophthalonitrile, light grey powder, purity 96.6%	GLP statement	: yes
		guideline	: in compliance with OECD 409
		NOAEL	: 30 ppm (0.8395 0.83 mg/kg/day)
route	: dietary		
species	: dog, Beagle		
group size	: 6/sex/dose		

[1] average daily intake males: 0, 0.83, 1.80 and 3.26 mg/kg bw, females: 0, 0.95, 1.86 and 3.35 mg/kg bw

Study design

Study in accordance with OPP 83-1 and OPPTS 870.4100 (in accordance with OECD guideline 409).

Results

The results are summarised in Table 6.8.1 – 6.3.9-1

Table 6.8.1 – 6.3.9-1 Summary of results, Study 9

Dose (ppm)	0		30		60		120		dr
	M	F	M	F	M	F	M	F	
Mortality	none								
Clinical signs									
emaciation							++(1-12)		
Body weight						dc(6-12)	dc(3-12)	d(3-12)	
body weight gain						dc(1-12)	dc(3-12)	dc(3-12)	F
Food consumption							dc(3-12)		
Ophthalmoscopy									
retinal thinning and/or depigmentation							+	+	
Haematology									
red blood cell count					dc(6-12)	dct(6)	dc(6-12)	dc(6-12)	M,F
hemoglobin							dc(6-12)	dc(6,12)	
hematocrit							dc(6-12)	dc(6,12)	
reticulocyte count							ict(6)		
MCV						ict(6)	ic(6-12)	ic(3-12)	F
MCH					ic(6,12)	ic(6,12)	ic(6-12)	ic(6-12)	M,F
MCHC						ict(6)	ic(6,12)		
platelets							dct(3-6)	dc(3-12)	
Clinical chemistry									
ALAT			dc(3)	dc(9)	dc(3-12)	dct(3-9)	dc(3-12)	dc(3-12)	M,F
ASAT							ic(3-12)	ict(6)	
Alkaline phosphatase							ic(3-12)	ic(3-12)	M,F
Total bilirubin							ict(3-6)	ic(12)	
Glucose					ic(3-12)	ic(3-12)	ic(3-12)	ic(3-12)	M,F
Gamma-GT							ict(6)	ic(6,12)	
Urinalysis	no treatment-related findings								
Organ weights									
liver							dc[arb]		
testis							dc[a]		
thyroid/parathyroid			dc[arb]				dc[arb]	dc[arb]	
spleen						dc[arb]	dc[a]		
kidney							ic[r]	ic[r]	
brain							ic[r]		
Pathology									
macroscopy									
testis, small							+		
microscopy									
liver, hepatocyte necrosis and hypertrophy, hemorrhage, nodular hyperplasia, periportal fibrosis, pigment deposition, macrophage, lymphocyte and neutrophil infiltrates	+		+	+	+	+	++	++	M,F
kidney, glomerular fibrosis and cortical tubular regeneration				+			++	++	
testis, seminiferous tubule degeneration	+		+		+		++		M

dr dose related
dc/ic statistically significantly decreased/increased
d/i decreased/increased, but not statistically significantly
t transient
(…) observation time in months
+ present in one/a few animals
++ present in most/all animals
a absolute organ weight
r organ weight relative to body weight
rb organ weight relative to brain weight

Table 6.8.1 – 6.3.9-2 Intergroup comparison of selected haematology parameters

Parameter		Dietary Concentration (ppm)							
		Males				Females			
	wk	0	30	60	120	0	30	60	120
RBC	11	6.653	6.210	6.078	5.507**	6.920	6.700	6.723	6.322
x10˜6	24	7.022	6.390	6.172*	4.918**	7.118	6.793	6.505*	5.697**
/mm3	39	6.898	6.490	5.973**	4.732**	6.400	6.720	6.572	5.642**
	52	7.152	6.648	6.322*	4.690**	6.898	6.893	6.637	5.598**
HGB	11	14.55	14.03	13.80	12.95*	15.52	14.97	15.75	14.92
g/dL	24	15.27	14.27	14.23	12.30**	16.08	15.40	15.63	14.30**
	39	15.62	15.02	14.37	12.37**	15.17	15.37	15.98	14.60
	52	15.93	15.23	14.85	12.13**	16.27	15.95	16.28	14.52**
HCT	11	42.88	40.82	40.33	37.28**	45.82	44.17	45.85	43.58
%	24	45.62	42.20	41.90	35.52**	47.53	45.33	45.38	41.57**
	39	46.03	43.72	41.55	35.50**	43.93	44.87	45.72	41.40
	52	47.18	44.42	43.28	34.60**	47.83	47.37	47.33	42.07**
MCV	11	64.53	65.78	66.35	67.73*	66.22	65.85	68.18	68.93*
µm3	24	64.97	66.18	67.83	72.25**	66.80	66.70	69.75**	72.93**
	39	66.80	67.42	69.57	74.95**	68.67	66.82	69.57	73.37**
	52	65.97	66.85	64.48	73.68**	69.40	68.68	71.33	75.15**
MCH	11	21.90	22.60	22.70	23.52*	22.45	22.33	23.43	23.58
pg	24	21.77	22.37	23.03*	25.02**	22.60	22.63	24.03**	25.12**
	39	22.68	23.17	24.05	26.08**	23.72	22.87	24.32	25.87**
	52	22.30	22.92	23.50*	25.83**	23.60	23.13	24.53*	25.93**
RETIC	24	0.4	0.4	0.4	1.5**	0.4	0.3	0.8	1.4
%	39	0.2	0.1	0.1	0.7	0.5	0.6	1.1	2.2
	52	0.2	0.2	0.2	0.5	0.6	0.7	1.1	1.5
PLT	11	342.5	329.7	312.2	240.3*	366.2	372.8	387.2	231.0**
x10˜3	24	344.3	315.2	305.8	238.0**	329.8	372.7	379.5	222.0**
/mm3	39	329.0	293.2	295.3	267.7	333.3	333.7	370.5	244.2*
	52	303.8	288.5	290.8	252.7	353.0	364.0	373.0	248.0**
PT	11	6.68	7.45	6.85	7.00	7.28	7.67	6.97	6.87
	24	6.83	6.82	7.30	7.62*	7.72	7.38	6.93	6.43
	39	7.02	7.62	7.22	7.32	7.63	7.63	7.20	7.08
	52	7.03	7.25	7.17	7.20	7.48	7.63	7.20	7.03

* Statistically significant difference from control group mean at the 0.05 level

** Statistically significant difference from control group mean at the 0.01 level

Table 6.8.1 – 6.3.9-3 Intergroup comparison of selected blood clinical chemistry parameters

Parameter		Dietary Concentration (ppm)							
		Males				Females			
	wk	0	30	60	120	0	30	60	120
ALT U/L	11	42.5	30.8*	25.7**	16.3**	43.7	32.2	24.8**	15.7**
	24	40.2	31.3	25.5**	12.5**	40.2	29.3	24.8**	14.7**
	39	44.0	32.2	27.7**	13.7**	38.2	26.5*	24.8**	13.0**
	52	39.5	29.3	26.2*	11.3**	35.3	26.8	23.3	12.8**
AST U/L	11	34.0	29.7	32.0	44.3*	33.0	33.0	35.3	41.2
	24	30.7	30.3	32.0	41.8**	30.8	32.2	33.7	43.0*
	39	28.0	29.0	30.3	41.7**	28.0	29.5	30.0	33.8
	52	28.8	30.8	33.5	45.8**	27.8	31.5	37.2	38.8
GLU mg/dL	11	107.2	110.5	123.0**	135.5**	106.3	111.5	125.8**	128.2**
	24	104.0	103.2	119.7**	128.5**	106.7	113.0	126.8**	132.8**
	39	101.0	104.7	117.3**	123.8**	103.5	105.3	121.0	125.8**
	52	104.2	105.7	117.0*	122.7**	106.2	107.3	119.3**	124.2**
TB mg/dL	11	0.08	0.10	0.10	0.30**	0.15	0.15	0.13	0.22
	24	0.10	0.10	0.10	0.28**	0.18	0.17	0.13	0.23
	39	0.13	0.10	0.10	0.23	0.13	0.17	0.12	0.18
	52	0.15	0.10	0.10	0.23	0.12	0.12	0.10	0.22*
ALP U/L	11	74.8	78.7	67.0	298.5	78.8	89.0	96.2	183.3
	24	53.2	51.5	53.3	209.0*	54.3	64.7	84.3	161.7**
	39	46.2	46.7	50.0	148.2*	48.7	66.2	81.2	128.8*
	52	43.5	42.8	52.0	170.2*	45.3	62.3	83.7	143.5*
GGT U/L	11	1.45	1.02	0.85	4.10	0.73	1.00	1.08	2.45
	24	2.93	2.73	2.72	5.48*	2.72	3.07	3.88	5.02*
	39	2.57	2.25	2.37	5.33	2.40	3.10	3.08	4.32
	52	2.70	2.22	2.15	7.27	2.08	2.65	3.23	5.95*

* Statistically significant difference from control group mean at the 0.05 level

** Statistically significant difference from control group mean at the 0.01 level

Conclusions

- The retinal thinning and/or depigmentation in one male and one female of the top dose group were without histological correlation.
- The average terminal body weight of male animals in the 120 ppm dose group was 24 percent below the control. In females of the 60 ppm dose groups, the difference with the control group was 21 percent. Females of the top dose group showed a 14 percent lower terminal body weight compared with the control (not statistically significant).
- Blood cell morphology of top dose animals revealed increased incidences of abnormal erythrocytes (e.g. poikilocytosis, Howell-Jolly bodies). The hematological findings are indicative of macrocytic anemia and possibly hemolytic anemia in view of the increased serum bilirubin.

- Although a dose related, statistically significant change in serum ALAT activity was observed in males and females, no toxicological significance is adhered to a decrease in this enzyme. Moreover, at the start of the study, ALAT activities in all treatment groups were below the control value.
- The decreased thyroid/parathyroid and spleen weights are not considered toxicologically relevant in the absence of correlating histopathological findings in these organs.
- The increased weights of kidney and brain to the body weight in the top dose group is considered to be due to the decreased body weight since the absolute weights of these organs and the kidney weight relative to the brain weight were not different from the control.
- ~~Based on the effects on the hematopoietic system, liver, kidney and testis at 120 ppm in both sexes, on the decreased body weight gain in females at 60 ppm and the dose-related increase of serum glucose at 60 ppm and above in both sexes, the NOAEL is placed at 30 ppm (0.83 mg/kg bw/day for males and 0.95 mg/kg bw/day for females).~~
- Based on the effects on the hematopoietic system, liver and kidney in both sexes at 120 ppm, effects on testis in males at 120 ppm, and on the decreased body weight gain in females at 60 ppm, the NOAEL is placed at 30 ppm (0.95 mg/kg bw/day) for females and at 60 ppm (1.80 mg/kg bw/day) for males.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the expert meeting it was concluded that the NOAEL should be set at 0.83 mg/kg bw/day, based on reduced red blood cells in both sexes and reduced BW gain in females only at 1.86 mg/kg bw per day.

Guidelines and limitations

This study was performed in accordance with OECD guideline 409 and is of relevance for the toxicological evaluation.

B.6.8.1 – 6.3.10 Oral 90-day toxicity study in rat with SDS-46851- study 10

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/46 van Otterdijk F, 2007b. SDS46851: 90 Day oral toxicity study with SDS46851 by dietary administration in the rat. NOTOX B.V., Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands. Laboratory Report No. 481624, 19 December 2007. (Syngenta File Number R611965_10019)

GUIDELINES: Repeated dose oral toxicity (rat): OECD 408 (1998): OPPTS 870.3100 (1998): EC Directive 67/548/EEC B.26 (2001): Japanese Chemical Substances Control Law 1987, Notification of Nov. 21 2003 by MHLW (No. 1121002), METI (No. 2) and ME (No. 031121002).

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

Acceptability: The study was performed in accordance with OECD guideline 408 of 1998. There were no deviations from the guideline of 1998 considered to compromise the scientific validity of the study.

Study design

In a subchronic toxicity study SDS46851 (purity 99.1%) was administered for at least 90 days by dietary administration to SPF-bred Wistar rats. One control group and three treated groups (150, 600 and 2400 ppm) were tested, each consisting of 10 males and 10 females. Chemical analyses of diet preparations were conducted during the study to assess accuracy, homogeneity and stability over 2 weeks at room temperature under normal laboratory light conditions. The following parameters were evaluated: clinical signs daily; functional observation tests in week 12-13; body weight weekly; food consumption weekly (twice weekly in weeks 3-9); ophthalmoscopy pretest and in week 13; clinical pathology (haematology, clinical biochemistry and urinalysis) and macroscopy at termination; organ weights and histopathology on a selection of tissues.

Test Material:	SDS46851
Description:	White powder
Lot/Batch number:	52717-14-9
Purity:	99.1%
CAS#:	Not reported
Stability of test compound:	Expiry date 24 January 2008 (stored refrigerated in the dark)

Results

Dietary analyses confirmed that diets were prepared accurately and homogenously, and were stable over at least 2 weeks at room temperature under normal laboratory light conditions. The mean test article intake over the study period was as follows: 14, 57 and 197 mg/kg bw/day for males and 14, 60 and 223 mg/kg bw/day for females for dietary inclusion levels of 150, 600 and 2400 ppm, respectively.

No toxicologically significant changes were noted in any of the parameters investigated in this study (i.e. clinical appearance, functional observations, ophthalmologic examinations, body weight, food consumption, clinical laboratory investigations, macroscopic examination, organ weights, and microscopic examination).

Conclusion

In a 90-day oral toxicity study in rats, a No Observed Adverse Effect Level (NOAEL) for SDS46851 of ≥2400 ppm was established, corresponding to ≥197 and ≥223 mg SDS46851/kg bw/day for males and females respectively.

B.6.8.1 – 6.3.11 Oral 90-day toxicity study in dog with SDS-46851- study 11

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. Some details are added to the result table.

Characteristics

reference	: Auletta et al., 1990	exposure	: 90 days, gavage
type of study	: 90-day feeding study	doses	: 0, 5, 15, 50 and 500 mg/kg bw/day
year of execution	: 1988	vehicle	: gelatine capsule
test substance	: SDS-46851[1]	GLP statement	: yes
route	: oral	Guideline	: in accordance with OECD 409
species	: dog, beagle	**NOAEL**	: 50 mg/kg bw/day
group size	: 4/sex/dose		

[1] 3-carbamyl-2,4,5-trichlorobenzoic acid, tan/off white powder, > 99%

Study design

The study was performed in accordance with OECD guideline 409.

Results

The results are presented in Table 6.8.1 – 6.3.11.

Table 6.8.1 – 6.3.11 Summary of results, Study 11

Dose (mg/kg bw/day)	0		5		15		50		500		dr
	m	f	m	f	m	f	m	f	m	f	
Mortality	none										
Clinical signs watery stool									+[1]	+[1]	
Body weight gain Week 0 Week 1 Week 14	9.7 10.1 12.9	8.6 8.9 10.5	9.4 9.7 12.4	8.5 8.6 10.5	9.4 9.5 12.7	8.5 8.4 10.4	10.1 9.8 13.2	8.6 8.9 11.7	9.6 9.6 11.2 (-13%)	8.6 8.5 10.3	
Food consumption	no treatment-related findings										
Ophthalmoscopy	no treatment-related findings										
Haematology	no treatment-related findings										
Clinical chemistry glucose					ic[2]		i[2]	ic[2]	ic[2]	ic[2]	↓
Urinalysis pH										d	
Organ weights liver, absolute live, relative brain			+1% +2%	-1% -2%	+6% +5%	+1% +1%	+6% +4%	+14% +2%	+10% +28%* i[r]	+23% +23%	
Pathology macroscopy	no treatment-related findings										

Dose (mg/kg bw/day)	0		5		15		50		500		dr
	m	f	m	f	m	f	m	f	m	f	
microscopy	no treatment-related findings										

dr dose related
dc/ic statistically significantly decreased/increased
d/i decreased/increased, but not statistically significantly
a/r absolute/relative organ weight
1 watery stool was observed in all dose group, however the incidence per animal was increased in the highest dose group
2 only observed at 8 weeks
* significantly different from control

Conclusions

- In the highest dose level an increased incidence of watery stool was observed in dogs of both sexes. A decreased body weight gain and urine pH was observed in high dose male and female dogs, respectively. At 8 weeks a statistically increase in glucose was observed in males of the mid and high dose groups and in females of the two highest dose groups, however this increase was not observed at 13 weeks and is therefore considered of no toxicological relevance. Absolute liver weight was increased, but not statistically significant, in females of the two-highest dose group and relative liver weight was increased in females and males of the highest dose group.
- The reviewer agrees with the authors that the NOAEL is placed at 50 mg/kg bw/day, based on the observed statistically significantly increased in relative liver weight, increased incidence of watery stools and decreased body weight at 500 mg/kg bw/day.

Guidelines and limitations

The study meets the requirements of OECD guideline 409 and is considered suitable for evaluation.

B.6.8.1 – 6.3.12 Oral 90-day toxicity study in rat with R417888 - study 12

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	:	Noakes, 2001	exposure	:	90 days, in diet
type of study	:	90-day oral toxicity study	doses	:	0, 65, 390, 780 mg/kg food[1]
year of execution	:	2000	vehicle	:	none
test substance	:	**R417888**, batch no. P8 (WRC#16882-46-01), purity 97%, white solid	GLP statement	:	yes
route	:	oral	guideline	:	in accordance with OECD 408
species	:	rat, Alpk:AP$_f$SD (Wistar-derived)	acceptability	:	acceptable
group size	:	12/sex/dose	**NOAEL**	:	780 mg/kg food (59 mg/kg bw/d)

[1] Equivalent to 0, 5, 29.5, and 59.1 mg/kg bw/d for males and 0, 5.8, 35.0, and 71.5 mg/kg bw/d for females.

Study design

The study was performed in accordance with OECD 408. A minor deviation was that no blood urea nitrogen concentrations were measured. In addition, all animals were tested using the Functional Observational Battery (FOB) and tested on motor activity.

Dose levels were based on the results of a 28-day range-finding study (CTL Study No. KR1389).

Results

Table 6.8.1 – 6.3.12

Dose (mg/kg food)	0		65		390		780		dr
	m	f	m	f	m	f	m	f	
Mortality (n=12)	0	0	0	1 (wk 2)	0	0	0	0	
Clinical signs	no treatment-related findings								
Body weight gain	no treatment-related findings								
Food consumption	no treatment-related findings								
Ophthalmoscopy	no treatment-related findings								
Haematology	no treatment-related findings								
Clinical chemistry	no treatment-related findings								
Organ weights	no treatment-related findings								
Pathology macroscopy	no treatment-related findings								
microscopy	no treatment-related findings								
Functional observational battery	no treatment-related findings								
Motor activity measurements	no treatment-related findings								

dr dose related

Acceptability

The study is considered acceptable.

Conclusions

Since no significant treatment-related effects were found when administering **R417888** up to the highest dose group (780 mg/kg food), the NOAEL is set at 780 mg/kg food which is equivalent to 59 mg/kg bw/d.

B.6.8.1 – 6.3.13 Oral 90-day toxicity study in rat with R417888 - study 13

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/19. van Otterdijk F, 2007a. VIS01: 90 Day oral toxicity study with VIS01 by dietary administration in the rat. NOTOX B.V., Hambakenwetering 7, 5231 DD 's-Hertogenbosch, The Netherlands. Laboratory Report No. 481602, 18 December 2007. (Syngenta File Number R417888_10027)

GUIDELINES: Repeated dose oral toxicity (rat): OECD 408 (1998): OPPTS 870.3100 (1998): EC Directive 67/548/EEC B.26 (2001): Japanese Chemical Substances Control Law 1987, Notification of Nov. 21 2003 by MHLW (No. 1121002), METI (No. 2) and ME (No. 031121002).

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

Acceptability: The study was performed in accordance with OECD guideline 408 of 1998. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

In a subchronic toxicity study VIS01 (purity 100%) was administered for at least 90 days by dietary administration to SPF-bred Wistar rats. One control group and three treated groups (150, 600 and 2400 ppm) were tested, each consisting of 10 males and 10 females. Chemical analyses of diet preparations were conducted during the study to assess accuracy, homogeneity and stability over 2 weeks at room temperature under normal laboratory light conditions. The following parameters were evaluated: clinical signs daily; functional observation tests in week 12; body weight weekly; food consumption weekly (twice weekly in weeks 4-9); ophthalmoscopy pretest and in week 13; clinical pathology (haematology, clinical biochemistry and urinalysis) and macroscopy at termination; organ weights (adrenal glands, brain, epididymis, kidnyes, heart, liver, ovaries, spleen, testes, thymus and uterus) and histopathology on all tissues collected at the scheduled sacrifice from all treated animals and all gross lesions.

Test Material:	VIS01
Description:	White powder
Lot/Batch number:	52717-15-13
Purity:	100%
CAS#:	Not reported
Stability of test compound:	Expiry date 24 January 2008 (stored refrigerated in the dark)

Vehicle: The test substance was administered via standard powder rodent diet (SM RIM-Z from SSNIFF® Spezialdiaten GmbH, Soest, Germany).

Results

No mortality occurred during the observation period and no clinical signs of toxicity were noted. Hearing ability, pupillary reflex, static righting reflex and grip strength were normal in all animals. The variation in motor activity did not indicate a relation with treatment. There were no toxicologically relevant ophthalmology findings at pre-dose and in week 12. There were no treatment-related effects on body weight, body weight gain, food consumption or compound intake.

Mean test article intake over the study period was as follows:

Table 6.8.1 – 6.3.13: Mean Dose Received (mg/kg bw/day)

Dietary concentration (ppm)	**150**	**600**	**2400**
Males	13	54	192
Females	15	56	218

There were no treatment-related effects on haematology parameters, . blood clinical chemistry parameters and urinalysis parameters.
Macroscopic findings: A small number of lesions were observed, none of which was related to treatment.
Organ weights: There were no treatment-related effects on organ weights or organ to body weight ratios.
Microscopic findings: A small number of spontaneous lesions were observed, none of which was related to treatment.

Conclusion

No toxicologically significant changes were noted in any of the parameters investigated in this study (i.e. clinical appearance, functional observations, ophthalmologic examinations, body weight, food consumption, clinical laboratory investigations, macroscopic examination, organ weights, and microscopic examination).Therefore, a No Observed Adverse Effect Level (NOAEL) for VIS01 of 2400 ppm was established, corresponding to 192 and 218 mg VIS01/kg/day for males and females respectively.

B.6.8.1 - 6.4 Genotoxicity

Genotoxicity SDS-3701 – in vitro

B.6.8.1 – 6.4.1 Reverse mutation assay in vitro with SDS-3701 - study 1

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: reverse mutation assay, with bacteria, with and without metabolic activation; plate incorporation assay

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
B: *S. typh.* TA98 TA100 TA1535 TA1538 TA1537	 point mut. point mut. point mut. point mut. point mut.	 * * * * *	 * * * * *	rat liver	Aroclor 1254	1-100 µg/plate vehicle: dimethylsulphoxide (DMSO)	Kouri, Parmar and Banzer, 1977
Test substance: SDS-3701, 4-hydroxy-2,5,6-trichloroisophthalonitrile, 99%, white amorphous powder GLP - statement: no According to OECD 471: no *: inconclusive							

Conclusions

The test substance was not found to be mutagenic when tested in the *Salmonella*/microsome test in the presence and the absence of a liver homogenate from Aroclor-treated rats. However, the limitations listed hereunder make the study unacceptable for evaluation. Therefore, the outcome of the study may not be regarded as evidence for the absence of a mutagenic effect of the test substance in the *Salmonella*/microsome test.

Guidelines and limitations

The study has not been carried out according to OECD 471, since no information was provided on the inoculum size and on the growth of the back-ground lawn, the preliminary toxicity test was inadequate (see below) and the test was only performed once.

The result cannot be accepted as evidence for the lack of mutagenic activity of the test substance in the *Salmonella*/microsome test, mainly because there is no information on the toxicity of the test substance under the conditions of the test. The preliminary toxicity test was carried out in a liquid culture, which has a limited predictive value as regards the toxicity under “plate-incorporation” conditions, while the influence of S9 fraction was not accounted for in this test. Furthermore, the growth of the back-ground lawn was not inspected. Thus, it cannot be ruled out that toxicity would not have prevented testing of higher doses. Such higher doses might have given rise to a mutagenic response.

B.6.8.1 – 6.4.2 Reverse mutation assay in vitro with SDS-3701 - study 2

Previous evaluation:	Submitted for the purpose of renewal, new data

RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/03 Verspeek-Rip, C.M. (2004), Evaluation of the mutagenic activity of SDS-3701 in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat), testing facility: Notox B.V, 5231 DD's-Hertogenbosch, The Netherlands. Report No. 402468. Unpublished. (Syngenta File Number R182281_10022)

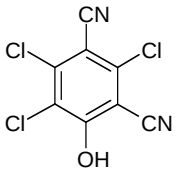
GUIDELINES: OECD 471 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

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

Study design
In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA1535 and TA1537 of *Salmonella typhimurium* and WP_2uvrA of *E.coli* were exposed to SDS-3701, using dimethyl sulfoxide as a vehicle at concentrations up to 5000 µg /plate, with and without S-9 activation.
The test article was tested at six dose levels along with appropriate vehicle and positive controls on the tester strains employed in the presence and absence of S9-mix. All dose levels, vehicle and positive controls were plated in triplicate.

Test Material: SDS-3701
4-hydroxy-2,5,6-trichloroisophtalonitrile; 4-OH-2,5,6-trichloro-1,3-dicyanobenzene



Description: White Powder
Lot/Batch#: Batch 51955-15-21
Purity: 99%

Control Material
Negative: Vehicle (Dimethyl sulfoxide)
Solvent: DMSO at 0.1 mL/plate

Results
No cytotoxicity was observed in a dose range finding assay.
On basis of the preliminary cytotoxicity test a mutation assay was performed in the presence or absence of rat liver S9 at dose levels of 3 - 5000 µg SDS-3701/plate.

Triplicate plates per strain, dose and condition were used. No positive responses were observed with any of the strains used, in the presence as well as in the absence of microsomal enzymes. The results are summarized in Table **6.8.2 – 6.4.2-1**.

Table **6.8.1 – 6.4.2-1**: Mutagenic Response of SDS-3701, in Different *Salmonella* and *E.Coli* Strains

SDS-3701 µg/plate	Average revertants per plate									
Strain:	TA98		TA100		TA1535		TA1537		WP_2uvrA	
S9-mix:	-	+	-	+	-	+	-	+	-	+
Vehicle*	19	29	127	119	10	13	6	6	12	12
3	nt	nt	93	123	nt	nt	nt	nt	13	11
10	nt	nt	100	122	nt	nt	nt	nt	13	18
33	nt	nt	109	126	nt	nt	nt	nt	11	13
100	18	27	114	128	12	11	6	6	14	18
333	22	25	117	123	10	13	4	7	10	16
1000	20	20	114	117	11	8	4	5	11	17
3330	10	14	98	122	7	7	2	4[2]	10	9
5000	10	16	71	97	7[2]	7	3	3[2]	9	8
Pos. control**	1109	584	1013	605	439	197	315	285	580	422

* DMSO

** 2-nitrofluorene	10 g/plate -	TA98 (-)
2-aminoanthracene	1-10 µg/plate	TA 98(+), TA100 (+), TA1535 (+), TA1537 (+), TA1538 (+)
Sodium azide	5 µg/plate -	TA 1535 (-)
9-Aminoacridine	60 µg/plate	TA1537 (-)
Methylmethansulfonate	10 µg/plate	TA100 (-)
4-Nitroquinoline-N-oxide	10 µg/plate	WP_2uvrA (-)

nt not tested

In a second independent experiment these results were confirmed.

Conclusions
SDS-3701 was not mutagenic when tested on S. *typhimurium* strains TA98, TA100, TA1535 or TA1537 and E.coli strain WP_2uvrA, with or without S9-mix activation.

B.6.8.1 – 6.4.3a In vitro gene mutation assay with SDS-3701 - study 3a

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/04 Verspeek-Rip. C.M. (2005), Evaluation of the mutagenic activity of SDS-3701 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat, testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands. Report No. 402457. Unpublished. (Syngenta File Number R182281_10020)

GUIDELINES: OECD 476 (1997)

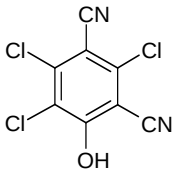
GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design
The test substance SDS-3701 was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and presence of an S9-activation system. Test substance doses ranged from 1-80 µg/mL in the absence of S9 and 10-350 µg/mL in the presence of S9.

Test Material: SDS-3701

	4-hydroxy-2,5,6-trichloroisophtalonitrile; 4-OH-2,5,6-trichloro-1,3-dicyanobenzene
Description:	White Powder
Lot/Batch#:	Batch 51955-15-21
Purity:	99%



Results

Cytotoxicity Assay:

The cytotoxicity was determined by counting the cells after exposure and by measuring the relative suspension growth and cloning efficiency of the L5178Y cells 24 and 48 hours after treatment – compared to negative control and the relative total growth (RTG: product of RSG and the relative colony-forming ability ["cloning efficiency"] 48 hours after treatment). Cell toxicity became obvious at concentrations from 100 µg/mL and 333 µg/mL onwards in non-activated and in S9-activated systems, respectively. The results are summarized in Table 6.8.1 – 6.4.3-1.

Table 6.8.1 – 6.4.3-1: Cytotoxicity of SDS-3701 in Mouse Lymphoma L5178Y Cells

Concentration SDS-3701 (µg/mL)	% relative survival (relative suspension growth)	
	Absence of S9	Presence of S9
0	100	100
33	93	115
100	3	89
333	[2)]	8
1000	[2)]	[2)]
2475[1)]	[2)]	[2)]

[1)] precipitation of the test substance ,
[2)] cell death

Mutation Assays:

The results of the mutagenicity assay are summarized in Table 6.8.1 – 6.4.3-2. In the absence or presence of S9 mix, SDS-3701 did induce a dose related increase in mutant frequency at 50-80 µg/mL and 250-350 µg/mL in the absence and presence of S9, respectively. However, pronounced cytotoxicity was observed at these concentrations which may have caused this observation.
Both positive control compounds fulfilled the requirements for a valid test.

Table 6.8.1 – 6.4.3-2: Effects of SDS-3701 on Gene Mutations at the TK-Locus of Mouse Lymphoma L5178Y Cells in the Absence or Presence of S9-Mix

Concentration SDS-3701 (µg/mL)	Absence of S9-mix		Presence of S9-mix	
	Mutation frequency x10^6	Survival (%)	Mutation frequency x10^6	Survival (%)
0	80	100	111	100
1	83	95		
3	77	118		
10	74	101	81	98
30	80	68		
40	86	62		
50	116	50	70	98
65	147	36		
80	232	14		
100			77	93
150			93	80
200			81	83
250			269	51
300			341	9
350			365	6
MMS (15 µg/mL)	**692**	**97**		
CP (10 µg/mL)			**1087**	**77**

Conclusions

Based on the overall results of this study it is concluded, that the test substance SDS3701 is mutagenic at the TK-locus of mouse lymphoma L5178Y cells. However mutagenicity was observed only at highly cytotoxic concentrations.

B.6.8.1 – 6.4.3b In vitro gene mutation assay with SDS-3701 - study 3b

Data requirement 2.44

Applicant to submit available genotoxicity studies performed on SDS-3701.

See reporting table 2(100)

NL (August 2017): The notifier submitted an in vitro gene mutation assay in Chinese Hamster cells (V79/HPRT). The RMS agrees with the conclusion drawn by the applicant.

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/09 Chang S (2017) R182281 – Gene Mutation Assay in Chinese Hamster V79 Cells In Vitro (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1800300 issue date: 22 March 2017. Unpublished. Syngenta File No. R182281_10071).

STUDY TYPE: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT) OECD 476 (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.

TEST MATERIAL (PURITY): R182281 (98.7% w/w)

SYNONYMS: SDS-3701

SPONSORS: Syngenta Ltd, Jealott's Hill International Research Centre, Bracknell, Berkshire RG42 6EY, United Kingdom

EXECUTIVE SUMMARY

The test item R182281 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. The assay was performed in two experiments, using two

parallel cultures throughout. The experiments were performed with and without liver microsomal activation and a treatment period of 4 hours.

Cytotoxic effects indicated by an adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred in the first experiment at 11.7 µg/mL and above without metabolic activation and at 125.0 µg/mL and above with metabolic activation.
In Experiment I, the mean mutant colony numbers exceeded the 95% control limit at the concentration of 15.6 without metabolic activation, and at the lowest concentration of 7.8 µg/mL up to the highest evaluated concentration of 125.0 µg/mL with metabolic activation.
In the absence of metabolic activation a t-test evaluating the mean data of both parallel cultures showed no statistically significant increase in MF, while in the presence of metabolic activation a t-test evaluating the mean data of both parallel cultures showed significant increases versus the corresponding solvent controls at 31.3 µg/ml.
A linear regression analyses showed no concentration related trend in the mean mutant frequency for neither the conditions with and without S9.
To further examine the result of Experiment I, a second experiment was performed with and without metabolic activation.

In Experiment II, the mean mutant colony numbers exceeded the 95% control limit at 7.8, 15.6, and 23.4 µg/mL in the absence of metabolic activation. In the presence of metabolic activation the control limit was exceeded at 15.6 µg/mL and above. The t-test indicated statistically significant higher mutation frequencies at these concentrations. Within both experiments there was notable inter-culture variability in MF. In the majority of cases an increase in MF above the 95% control limit (MF 29.4) in one culture was not seen in the parallel culture under the same conditions.

In the absence of S9, neither Experiment I nor II clearly met the conditions for a positive response in this assay, and despite concentrations being used in Experiment II at up to twice that of Experiment I (31.3 vs 15.6 µg/mL) no similar increase in MF was observed. Hence, the increases in MF observed without a consistent concentration related trend are considered to be of questionable biological significance.
In the presence of S9, neither Experiment I nor II met the conditions for a positive response in this assay, in the two experiments no concentration related trend was noted. Hence, the apparent increases in MF are considered to be of questionable biological significance.
In the experiments conducted in the presence of S9 an examination of the individual culture data indicates the responses in MF seen are not concentration related within cultures, or consistently reproducible between cultures. This may be taken to demonstrate a lack of a true mutagenic response in the presence of S9.

EMS and DMBA were used as positive controls and showed a distinct increase in induced mutant colonies.

In conclusion, the test substance R182281 was examined in two independent experiments in the absence and presence of metabolic activation. Although increases in mean MF were observed the data in the absence of S9 do not fully meet the requirements for a clear positive response.

In the presence of S9 the collected data may indicate a negative response in this assay.

MATERIALS AND METHODS

Materials:

Test Material:	R182281
Description:	White solid
Lot/Batch number:	P2
Molecular weight:	247.5 g/mol
Purity	98.7% w/w, concentration calculation not adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: Ethylmethane sulfonate (EMS), 0.30 mg/mL Presence of S9 mix: 7,12-dimethylbenz(a)anthracene (DMBA), 2.3 µg/mL = 8.6 µM

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

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

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ 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:

Experiment I	Absence of S9 mix	2.0; 3.9; 7.8; 11.7; 15.6; and 23.4µg/mL
	Presence of S9 mix	7.8; 15.6; 31.3; 62.5; and 125.0 µg/mL
Experiment II	Absence of S9 mix	7.8; 11.7; 15.6; 23.4; and 31.3 µg/mL
	Presence of S9 mix	7.8; 15.6; 31.3; 62.5; and 125.0 µg/mL

Study Design and Methods:

In-life dates: Start: 25 October 2016, End: 14 February 2017

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.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment. Colonies with more than 50 cells were counted. If in doubt the colony size was checked with a preparation microscope.

Statistical Methods:

A linear regression analysis (least squares, using a validated Excel sheet) was performed to assess a possible dose dependent increase of mutant frequencies. A trend is judged as significant whenever the p-value (probability value) is below 0.05. However, both, biological and statistical significance were considered together.

Evaluation Criteria:

The colonies are counted manually.

A test chemical is considered to be clearly positive if, in all of the experimental conditions examined all of the following criteria are met:

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent solvent control in both parallel cultures,

b) the increase is dose-related when evaluated with an appropriate trend test,

c) any of the results are outside the distribution of the historical solvent control data.

When all of these criteria are met, the test chemical is then considered able to induce gene mutations in cultured mammalian cells in this test system.

RESULTS AND DISCUSSION

Preliminary toxicity assay:

The pre-experiment was performed in the presence and absence of metabolic activation. Test item concentrations between 15.6 µg/mL and 2000 µg/mL were used. The highest concentration was chosen with respect to the current OECD Guideline 476.

Relevant toxic effects were observed without metabolic activation at concentrations of 125 µg/mL and above. In the absence of metabolic activation the cell growth was completely inhibited at all the concentrations tested. No precipitation or phase separation occurred up to the maximum concentration with and without metabolic activation.
The concentrations used in experiment I were selected based on the data of the pre-experiment. The maximum concentration was limited by cytotoxicity of the test item. In experiment II the concentrations were based on the cytotoxicity data of experiment I.
The cultures at the three lowest concentrations in experiment I and II without metabolic activation were not plated for colony counting as a minimum of only four analysable concentrations is required by the test guidelines. The cultures at the three highest concentrations in experiment I and II with metabolic activation were not continued due to exceedingly severe cytotoxic effects.

Mutation assay:
The test item R182281 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. Two parallel cultures were used throughout the assay. The treatment period was 4 hours with and without metabolic activation.

Table B.6.8.1 – 6.4.3b-1 Results of Experiment I and II

	conc. µg/mL	S9 mix	relative cloning efficiency I %	relative cell density %	rel adjusted cloning efficiency I %	mutant colonies/ 10^6 cells
Column	1	2	3	4	5	6
Experiment I / 4 h treatment			**mean values of culture I and II**			
Saolvent control with DMSO		-	100.0	100.0	100.0	27.2
Positive control (EMS)	300.0	-	95.6	87.1	82.6	375.2
Test item	0.24	-	culture was not continued#			
Test item	0.49	-	culture was not continued#			
Test item	0.98	-	culture was not continued#			
Test item	2.0	-	102.4	78.7	79.3	#
Test item	3.9	-	100.1	77.0	76.3	25.0
Test item	7.8	-	86.6	74.4	62.4	28.0
Test item	11.7	-	41.9	84.6	34.0	26.1
Test item	15.6	-	22.8	68.6	15.4	35.9
Saolvent control with DMSO		+	100.0	100.0	100.0	27.9
Positive control (DMBA)	2.3	+	95.1	91.3	86.8	134.0
Test item	7.8	+	99.4	89.4	90.2	39.1
Test item	15.6	+	97.4	82.9	81.1	33.2
Test item	31.3	+	93.0	86.9	80.9	39.7
Test item	62.5	+	59.3	92.5	55.0	34.3
Test item	125.0	+	12.2	82.1	9.7	32.3
Test item	187.5	+	3.3	40.2	1.3	##
Test item	250.0	+	##	5.2	##	##
Test item	375.0	+	culture was not continued##			
Experiment II / 4 h treatment			**mean values of culture I and II**			
Saolvent control with DMSO		-	100.0	100.0	100.0	22.3
Positive control (EMS)	300.0	-	100.7	129.6	129.2	190.5
Test item	0.98	-	99.4	104.7	104.1	#
Test item	2.0	-	106.7	106.8	113.9	#
Test item	3.9	-	108.4	91.3	89.5	#
Test item	7.8	-	101.9	102.4	104.3	31.9
Test item	11.7	-	99.9	129.0	128.1	29.7
Test item	15.6	-	97.1	141.6	136.8	47.9
Test item	23.4	-	100.2	105.3	105.8	32.9
Test item	31.3	-	97.8	95.2	93.1	27.3
Saolvent control with DMSO		+	100.0	100.0	100.0	18.7
Positive control (DMBA)	2.3	+	99.3	88.2	89.1	105.2
Test item	7.8	+	104.1	98.7	102.5	24.5
Test item	15.6	+	100.2	101.6	99.8	32.1
Test item	31.3	+	95.2	100.1	94.1	37.7
Test item	62.5	+	70.4	103.5	72.9	34.2
Test item	125.0	+	16.8	87.2	14.7	31.2
Test item	187.5	+	5.5	32.3	1.8	#
Test item	250.0	+	##	6.5	#	
Test item	375.0	+	##	9.3	#	

\# culture was not continued since a minimum of only four analysable concentrations is required
culture was not continued due to exceedingly severe cytotoxicity
data not used in evaluation due to unacceptable cytotoxicity

T-test: Exp I +S9 P < 0.05 at 31.3 µg/mL; Exp II -S9 P < 0.05 at 7.8, 15.6, and 23.4 µg/mL; Exp II +S9 P < 0.05 at 15.6, 31.3, 62.5 and 125.0 µg/mL.

Experiment I

Cytotoxic effects indicated by an adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 11.7 µg/mL and above without metabolic activation and at 125.0 µg/mL and above with metabolic activation.

The mean mutant colony numbers exceeded the 95% control limit at the concentration of 15.6 µg/mL without metabolic activation (HCD 95% control limit: 30.2), and at the lowest concentration of 7.8 µg/mL up to the highest evaluated concentration of 125.0 µg/mL with metabolic activation (HCD 95% control limit: 29.4). The data at 23.4 µg/mL were not taken into consideration since in culture II the relative adjusted cloning efficiency I was below the recommended limit of 10%. At this concentration, the mean mutant colony number exceeded the 95% control limit in the parallel culture (50.5 x 10^6 mutant colonies).

In the absence of metabolic activation a t-test evaluating the mean data of both parallel cultures showed no statistically significant increase in MF. A linear regression analysis (least squares) was performed to assess a possible concentration dependent increase of mutant frequencies. This analyses showed no concentration related trend in the mean mutant frequency in the absence of S9 in Experiment I. However, the highest dosage (23.4 µg/mL) was not included in this linear regression analysis due to too low cloning efficiency in one of the parallel cultures.

In the presence of metabolic activation a t-test evaluating the mean data of both parallel cultures showed a significant increase versus the corresponding solvent control at 31.3 µg/ml. The linear regression analyses showed no concentration related trend in the mean mutant frequency in the presence of S9 in Experiment I.

Experiment II

To further investigate the results of Experiment I, a second experiment was performed in the absence and presence of metabolic activation.

The mean mutant colony numbers exceeded the 95% control limit at 7.8, 15.6, and 23.4 µg/mL in the absence of metabolic activation. The t-test, indicated statistically significant higher mutation frequencies at these test concentrations. A linear regression analyses however, showed no concentration related trend in the mean mutant frequency.

In the presence of metabolic activation the 95% control limit was exceeded at 15.6 µg/mL and above. The t-test, indicated statistically significant higher mutation frequencies for all the evaluated test concentrations. A linear regression analyses however, showed no concentration related trend in the mean mutant frequency.

Discussion

EMS (300 µg/mL) and DMBA (2.3 µg/mL) were used as positive controls and showed a distinct increase in induced mutant colonies in both experiments.

Within both experiments there was notable inter-culture variability in MF. In many cases an increase in MF above the 95% control limit (HCD MF of 30.2 and 29.4 without and with metabolic activation, respectively) in one culture was not seen in the parallel culture under the same conditions. In the

absence of S9, neither Experiment I nor II clearly met the conditions for a positive response in this assay (refer to 'Evaluation criteria'), and despite concentrations being used in Experiment II at up to twice that of Experiment I (31.3 vs 15.6 µg/mL) no similar increase in MF was observed. Hence, the increases in MF observed without a consistent concentration related trend are considered to be of questionable biological significance. Therefore, in the absence of metabolic activiation, the results are considered equivocal.

In the presence of S9, neither Experiment I nor II met the conditions for a positive response in this assay, in the two experiments no concentration related trend was noted. Hence, the apparent increases in MF are also considered to be of questionable biological significance. In the experiments conducted in the presence of S9 an examination of the individual culture data indicates the responses in MF seen are not concentration related within cultures, or consistently reproducible between cultures. This may be taken to demonstrate a lack of a true mutagenic response in the presence of S9. It should also be noted that the cells used in these experiments had a spontaneous mutation frequency that was at the upper limit of the historical control limit and in one case exceeded the upper limit (Experiment I, culture I). In this case, it is apparent that the historical control range may not be entirely relevant for the evaluation of the toxicological significance of the cultures exposed to the test item. Based on the OECD-criteria (OECD guideline 476, 2016) a test may be considered as clearly negative if: a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control, b) there is no concentration-related increase when evaluated with an appropriate trend test, and c) all results are inside the distribution of the historical negative control data. Although for the experiments conducted in the presence of S9 only criterion b is fulfilled, based on the points discussed above (no clear dose-response, no consistent results between cultures and no strong increase compared to the concurrent controls) the test results may indicate a negative response.

CONCLUSIONS

The test substance R182281 was examined in two independent experiments in the absence and presence of metabolic activation. Although increases in mean MF were observed the data in the absence of S9 do not fully meet the requirements for a clear positive response and are thus considered equivocal. In the presence of S9 the collected data may indicate a negative response in this assay.

(Chang S, 2017)

B.6.8.1 – 6.4.4 DNA-repair assay in vitro with SDS-3701 - study 4

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: DNA-repair assay (differential killing) with bacteria, with and without metabolic activation

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
B: *S. typh.* TA1978 (repair competent) TA1538 (repair deficient by uvrB mutation)	Differential killing due to difference in DNA-repair competence	*	*	rat liver	Aroclor 1254	2-20 plate/plate vehicle: dimethylsulphoxide (DMSO)	Banzer, 1977
Test substance: SDS-3701, 4-hydroxy-2,5,6-trichloroisophthalonitrile, 99%, white amorphous powder GLP - statement: no No guideline available *: inconclusive							

Conclusions

The test substance did not cause differences in killing-zone diameter between two *Salmonella typhimurium* strains which were supposed to differ only in excision-repair competence. However, due to the limitations which were touched upon hereunder, this result cannot be accepted as evidence for the absence of repairable DNA damage caused by the test substance.

Guidelines and limitations

The test filter paper discs with test compounds did not cause growth inhibition in both tester strains. Thus the amounts tested may not have been high enough to reveal a difference between the tester strains in sensitivity to the lethality of the test substance. According to the reviewer, this precludes a conclusion on the ability of the test substance to cause repairable DNA damage. An adequate test should be carried out at dose levels which cause some killing of the repair competent strain. This is exemplified by the results obtained with the positive-control compounds 6-aminoanthracene (with activation) and 4-nitroquinoline-N-oxide (without activation). These compounds caused inhibition zones in both strain, while the ability to cause repair damage was demonstrated by clear differences between de diameters of the inhibition zones observed on the plates with the two strains.

B.6.8.1 – 6.4.5 Chromosome aberrations in CHO cells with SDS-3701 - study 5

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: chromosome aberrations in mammalian tissue-culture cells.

Indicator cells	Endpoint	Res. - act.	Res. + act.	Activation		Dose range	Reference
				Tissue	Inducer		
Chinese hamster ovary cells (CHO-K_1)	chromosomal aberrations	+	+	rat liver	Aroclor 1254	*initial assay* 16.3-260 ML/ml (20 h, -S9) 65-520 ML/ml (4 h, +S9) *indep. repeat assay* 160-260 ML/ml (20 h, -S9) 260-520 ML/ml (4 h, +S9) *44-hour harvest time* 16.3-260 ML/ml (20 h, -S9) 260-520 ML/ml (4 h, +S9)	Mizens, Laveglia, and Curry, 1994
Test substance: SDS-3701, 4-hydroxy-2,5,6-trichloroisophthalonitrile, 99.2%, white amorphous powder GLP - statement: yes According to OECD 473: yes							

Conclusions

The test substance was found to induce chromosomal aberrations in Chinese hamster ovary cells in the absence and the presence of liver homogenate from Aroclor-rats.

Guidelines and limitations

The study was carried out in compliance with OECD 473. No methodological limitations were identified.

B.6.8.1 – 6.4.6 In vitro forward-mutation assay in mouse BABL/3T3 cell with SDS-3701 - study 6

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: *In vitro* forward-mutation assay with mammalian tissue-culture cells, with and without metabolic activation.

Indicator cells	Endpoint	Res. - act.	Res. + act.	Activation		Dose range	Reference
				Species	Inducer		
mouse BALB/3T3, Clone A31	point mutations (ouabain res.)	*	*	rat liver	Aroclor 1254	30 ML/ml vehicle: acetone	Banzer and Kouri, 1977
Test substance: SDS-3701, 4-hydroxy-2,5,6-trichloroisophthalonitrile, 99%, white amorphous powder GLP - statement: no According to OECD 476: no *: inconclusive							

Conclusions

No significant increases of ouabain-resistant colonies were observed. However, this result cannot be accepted as evidence for the absence of mutagenic effects in the test system, due to the limitations listed below.

Guidelines and limitations

The study has not been carried out in compliance with OECD 476. The following deviations of this guideline were identified: the test was not repeated, the number of concentrations tested was to low (1 instead of 4) and no toxicity was observed at the concentration tested.

Genotoxicity SDS-3701 – in vivo

B.6.8.1 – 6.4.7 In vivo micronucleus test with SDS-3701 - study 7

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: micronucleus test.

Species	Endpoint	Result	Dose range	Reference
mouse	micronuclei in bone-marrow erythrocytes	*	6.5 mg/kg bw/day, for 5 days, by gavage dissolved in a solution of 10% dimethylsulphoxide (DMSO) in distilled water.	Legator, 1974
Test substance: SDS-3701, 4-hydroxy-2,5,6-trichloroisophthalonitrile, 99+%, white powder GLP-statement: no According to OECD 474: no *: inconclusive				

Conclusions

The treatment of mice with the test substance did not result in an increase of micronuclei in mouse bone-marrow erythrocytes. However, in view of the limitations listed hereunder, this outcome presents no evidence for the absence of *in vivo* mutagenicity of the test substance in mice.

Guidelines and limitations

The study was not carried out in compliance with OECD 474, since 1) only a summary report was available, 2) no information was provided about the sex of the animals, 3) only 1 dose was tested, 4) it was not indicated whether this dose caused cytotoxicity or represented the maximum tolerated dose, 5) the bone marrow was only collected once for scoring micronuclei, 6) the bone marrow was collected 3-4 h after the last treatment (which is at least 8-9 h too early), 7) no information was provided on the ratio of polychromatic to normochromatic erythrocytes and 8) the Annex II summary contains data not found in the original study report.

These and other deviations and limitations make the study unacceptable for evaluation.

B.6.8.1 – 6.4.8a In vivo micronucleus test with SDS-3701 - study 8a

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/05. Buskens, C.A.F. (2004), Micronucleous test in marrow cells of the mouse with SDS-3701, testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands. Report No. 402446. Unpublished. (Syngenta File Number R182281_10025)

GUIDELINES: OECD 474 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

Acceptability: The study was performed in accordance with OECD guideline 474 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

Following a dose finding study, 5 male and 5 female mice were dosed with 115 or 250 mg/kg bw and ten mice per sex with 500 mg/kg bw SDS-3701 in corn oil. Negative controls received vehicle only, while positive controls (five of each sex) received 50 mg/kg bw cyclophosphamide. Following administration the animals were allowed food and water *ad libitum*.

Twenty four (vehicle control, 115, 250 and 500 mg/kg bw) or 48 hours (500 mg/kg and positive control) after dosing, animals were killed by cervical dislocation.

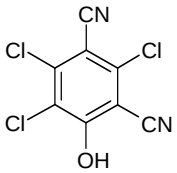
Animals were killed after 24h, positive controls were killed 48 h after dosing by cervical dislocation.

In a second experiment (due to mortality in the high dose group of the first experiment) five mice per sex were dosed with solvent or cyclophosphamid and ten mice per sex with 500 mg/kg bw SDS-3701 in corn oil. These animals were killed after 48h, solvent controls were killed 24 h after dosing by cervical dislocation.

Both femurs were dissected out and bone marrow smears were prepared. One smear per femur were fixed in methanol, defatted in xylene and Giemsa-stained. The stained smears were examined by light microscopy. The number of micronucleated polychromatic erythrocytes was counted in 2000 polychromatic erythrocytes. The ratio polychromatic to normochromatic erythrocytes was determined by counting and differentiating the first 1000 erythrocytes at the same time. Micronuclei were only counted in polychromatic erythrocytes.

Test Material: SDS-3701
4-hydroxy-2,5,6-trichloroisophtalonitrile; 4-OH-2,5,6-trichloro-1,3-dicyanobenzene

Description: White Powder
Lot/Batch#: Batch 51955-15-21
Purity: 99%



Vehicle and/or positive control: Corn oil, Cyclophosphamide 50 mg/kg bw

Results

Range Finding Test:

Table 6.8.1 – 6.4.8a-1: Preliminary Toxicity and Micronucleus Test – Mortality Data

Phase	Nominal concentration (mg/kg bw)	Mortality ratio		
		male	females	Combined
Preliminary toxicity test	**2000**	1/1	1/1	2/2
	1000	1/1	1/1	2/2
	500	0/3	0/3	0/6
Micronucleus test	**Solvent control**	0/5	0/5	0/10
	500	3/10	4/10	7/20
	250	0/5	0/5	0/10
	115	0/5	0/5	0/10
	Cyclophosphamide 50 mg/kg bw	0/5	0/5	0/10
Micronucleus test 1A	**Solvent control**	0/5	0/5	0/10
	500	5/10	5/10	10/20
	Cyclophosphamide 50 mg/kg bw	0/5	0/5	0/10

The animals treated at 500 mg/kg showed clinical signs indicative of systemic exposure to the test material. The mean micronucleated cell count for all dose groups of SDS-3701 was essentially comparable with the concurrent vehicle control group, at any of the three sampling times. Cyclophosphamid caused large significant increases in the frequency of micronucleated polychromatic erythrocytes.
The ratio of normochromatic to polychromatic erythrocytes was not influenced. However, the highest tolerable dose was employed as demonstrated by the number of mortalities at 500 mg/kg bw.

Table 6.8.1 – 6.4.8a-2: Summary of Micronucleus Results in Male Mice

Experiment	Treatment	Sampling time	Micronuclei per 2000 normochromatic erythrocytes	Ratio polychromatic to normochromatic erythrocytes
Experiment 1	**Solvent**	**24**	1.6	0.96
	115 mg/kg bw SDS-3701	**24**	1.4	0.95
	250 mg/kg bw SDS-3701	**24**	1.6	0.96
	500 mg/kg bw SDS-3701	**24**	1.0	0.93
	50mg/kg bw Cyclophosphamide	**48**	**27.2***	0.53
Experiment 1a	**Solvent**	**24**	1.6	0.94
	500 mg/kg bw SDS-3701	**48**	0.8	0.96
	50mg/kg bw Cyclophosphamide	**48**	**28.2***	0.56

*P< 0.01

Table 6.8.1 – 6.4.8a-3: Summary of Micronucleus Results in Female Mice

Experiment	Treatment	Sampling time	Micronuclei per 2000 normochromatic erythrocytes	Ratio polychromatic to normochromatic erythrocytes
Experiment 1	**Solvent**	**24**	1.4	0.98
	115 mg/kg bw SDS-3701	**24**	1.8	0.98
	250 mg/kg bw SDS-3701	**24**	1.2	0.99
	500 mg/kg bw SDS-3701	**24**	1.6	0.87
	50mg/kg bw Cyclophosphamide	**48**	**16.0***	0.63
Experiment 1a	**Solvent**	**24**	1.8	0.94
	500 mg/kg bw SDS-3701	**48**	2.3	0.91
	50mg/kg bw Cyclophosphamide	**48**	**22.0***	0.66

Cconclusions
SDS-3701 did not induce micronucleated polychromatic erythrocytes up to a dose of 500 mg per kg bw. Validity of the test performed was shown with a vehicle treated control group with no effects, a cyclophosphamide treated positive control group with a marked response.

B.6.8.1 – 6.4.8b Proof of exposure study for mouse micronucleus assay SDS-3701 - study 8b

Data requirement 2.25

Applicant to provide evidence of tissue exposure in the in vivo MN study with SDS-3701 – study 8.

See also 2(89, 101)
See reporting table 2(71)

NL (August 2017): The applicant provided a proof of exposure study in the mouse to support the mouse micronucleus assay (Buskens, 2004). This study is shown below. The RMS agrees with the conclusion that R182281 is systemically available and thus may reach the bone marrow.

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/11 Dunton, J. (2016) R182281 - Oral (Gavage) Proof of Exposure Study in the Mouse. Sequani Limited, Bromyard Road, Ledbury, Herefordshire, HR8 1LH, United Kingdom. Laboratory Report No. BFI0570. Issue date: 19 December 2016. Unpublished. (Syngenta File No. R182281_10063).

Guidelines: Proof of exposure to support a mouse micronucleus test

GLP: A signed and dated GLP statement was provided.

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

TEST MATERIAL (PURITY): R182281 (98.7 % w/w)
SYNONYMS: CSCA105253, SDS-3701

SPONSOR(S): Syngenta Ltd, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom. Oxon Italia S.p.A., Via Sempione 195, 20016 Pero, Milan, Italy. Arysta LifeScience SAS, BP 80 – Route d-Artix, 64150 Noguères, France.

EXECUTIVE SUMMARY

The purpose of this study was to demonstrate proof of exposure in the mouse after oral (gavage) administration of R182281 using a validated method in plasma (1) and blood (2). A proof of exposure experiment was required to support a previously conducted regulatory mouse micronucleus study (3) in order to demonstrate that the bone marrow was exposed to R182281. The study design was chosen to use the same species, route of exposure and vehicle, as well as the same concentration as used in the top dose of the *in vivo* micronucleus study.

In a Crl:CD-1 mouse proof of exposure study, three males were dosed once by oral gavage with R182281 at 250 mg/kg body weight. Blood samples were taken 1, 4 and 24 hours after dosing. Both blood and plasma were analysed for the presence of R182281. The vehicle was corn oil. There were no deaths and no clinical signs were observed throughout the study. All animals were killed and discarded following their final blood sample. Exposure to R182281 was confirmed quantitatively in all plasma and qualitatively in all blood samples.

In conclusion, systemic exposure to R182281 was demonstrated in male Crl:CD-1 mice following a single oral (gavage) administration of R182281 at 250 mg/kg, which was well tolerated in all animals. Proof of exposure was demonstrated by detectable blood and plasma concentrations of R182281 in male Crl:CD-1 mice with circulating concentrations in plasma ≥ 169 µg/mL.

MATERIALS AND METHODS

Materials:

Test Material: R182281

Description: White to off-white powder

Lot/Batch number: P2

Purity: 98.7 % w/w

CAS#: Not available

Stability of test compound: 31 January 2018

Control Materials:

Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	Corn oil	**Final Volume:** 10 mL/kg	**Route:** oral
Positive control :	N/A	**Final Doses:** N/A	**Route:** N/A

Test Animals:

Species	Mouse
Strain	Crl:CD-1
Age/weight at dosing	Six to seven weeks/28 to 30 g
Source	Charles River (UK) Limited, Margate, Kent, CT9 4LT, England.
Housing	3/cage
Acclimatisation period	5 days
Diet	*ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 19-23°C Humidity: 40-70% Photoperiod: 12 hours dark/12 hours light

Test compound administration:

Dose Level	**Final Volume**	**Route**
250 mg/kg bw	10 mL/kg	Oral gavage

Study Design and Methods:

In-life dates: Start: 06 October 2016 End: 24 November 2016

Experimental Design

Group	**Number of males**	**Dose level (mg/kg) R182281**	**Dose concentration (mg/mL)**

1	3	250	25

Animals were examined twice daily for mortality and morbidity and were given a detailed clinical examination. On the day of dosing, animals were observed before, shortly after and about 1, 4 and 24 hours after dosing. All animals were weighed on the day of dosing and were killed by exposure to carbon dioxide gas in a rising concentration following their final blood sample and discarded.

Blood sampling: Blood samples (100 µL) were taken from the tail vein, into tubes containing K_2EDTA anticoagulant. All animals were sampled 1, 4 and 24 hours after dosing. The plasma samples and blood samples were shipped on dry ice to the Principal Investigator for analysis.

Bioanalysis: Concentrations of R182281 in blood:acidified acetonitrile and plasma:acidified acetonitrile were determined using validated (plasma) or qualified (blood) LC-MS/MS methods.

RESULTS AND DISCUSSION

There were no deaths and no clinical signs were observed throughout the study.

Exposure to R182281 was confirmed quantitatively in all plasma and qualitatively in all blood samples (Tables B.6.8.1 - 6.4.8b-1/2). The blood analysis was performed qualitatively due to difficulties in validating a reliable method of quantitative analysis in mouse blood:acidified acetonitrile. Since bone marrow is well perfused, exposure of the marrow to the test item was indirectly demonstrated by the presence of measurable quantities of test item in the plasma and blood.

Table B.6.8.1 - 6.4.8b-1: Sample Results for R182281 in Mouse Plasma

Animal Number	Time point (hour)	Concentration (ng/mL)
1	1	271000
1	4	297000
1	24	315000
2	1	287000
2	4	265000
2	24	246000
3	1	169000
3	4	201000
3	24	284000

Note: all concentrations values are per mL of plasma

Lower limit of quantification (LLOQ) was 80 ng/mL in plasma

Table B.6.8.1 - 6.4.8b-2: Sample Results for R182281 in Mouse Blood

Animal Number	Time point (hour)	Concentration (ng/mL)
1	1	248000

Animal Number	Time point (hour)	Concentration (ng/mL)
1	4	308000
1	24	392000
2	1	404000
2	4	326000
2	24	335000
3	1	160000
3	4	218000
3	24	378000

Note: all concentrations values are per mL of blood

Lower limit of quantification (LLOQ) was 80 ng/mL in blood

CONCLUSIONS: Systemic exposure to R182281 was demonstrated in male Crl:CD-1 mice following a single oral (gavage) administration of R182281 at 250 mg/kg, which was well tolerated in all animals. Proof of exposure was demonstrated by detectable blood and plasma concentrations of R182281 in male Crl:CD-1 mice with circulating concentrations in plasma ≥ 169 µg/mL.

B.6.8.1 – 6.4.9 In vivo chromosome aberration test with SDS-3701 - study 9

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: *in vivo* chromosome aberrations

Species	Endpoint	Result	Dose range	Reference/notifier
Chinese hamster	chromosomal aberrations in bone-marrow cells	-	Four groups of males (n=5) and females (n= 5) were treated with a single dose of 0, 125, 250 or 500 mg/kg bw[1]. Sampling time: 6, 24 and 48 hours after treatment. Vehicle: aqueous 1% methylcellulose Positive control: cyclophosphamide	Mizens and Laveglia, 1995
Test substance: SDS-3701, 4-hydroxy-2,3,6-trichloroisophthalonitrile, 99.2 %, white powder GLP statement: yes According to OECD 475: no				

[1] In a dose-range finding study, four groups of two males and two females were given a single oral dose of 432, 720, 1200 or 2000 mg/kg bw. Moderate to severe clinical signs and mortality were observed at dose levels of 720 mg/kg bw and above.

Acceptability

The study has not been carried out according to OECD guideline 475, the most important deviations being that no information was provided on the mitotic indices and only 50 cells were analysed per animal. On the other hand, two sexes have been investigated. Based on these considerations, the study is considered acceptable for the overall toxicological evaluation of the test substance.

Conclusions

The test substance did not induce an increased incidence of chromosome aberrations at either the 24 or 48 hours sampling times. A statistically significant increase in the incidence of chromosome aberrations was observed at 250 mg/kg bw at the 6 hour sampling time. However, since this positive response was not dose-related and only observed at the 6 hour sampling time, the rapporteur concludes that the results cannot be regarded as a proof for clastogenicity of the test substance.

B.6.8.1 – 6.4.10 In vitro cell transformation test in rat embryo cells with SDS-3701 - study 10

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: cell-transformation test.

Cells	Endpoint	Result	Dose range	Reference
Fischer rat embryo cells F1706 P95 H4536 P97	cell transformation (morphol. transf., formation of colonies in soft agar, and tumourigenicity in rats)	H4536: - F1706: equivocal	0.1-10 ML/ml	Price, Schechtmann, and Killeen, 1978
Test substance: SDS-3701, 2,5,6-trichloroisophthalonitrile, 99+%, white powder GLP-statement: no No guideline available				

Conclusions

The test substance was negative for all three criteria in the H4536 cells. Treatment of the F1705 cells yielded late tumours in the rats. This effect is attributed by the authors of the study report to the large number of subcultures the cells went through. Thus, it is concluded that the test substance was not positive under the conditions of the test. However, the limitations listed hereunder underline the provisional character of this conclusion.

Guidelines and limitations

No guidelines are available for this type of cell-transformation test.

The preliminary toxicity test was only carried out with one of the cell lines (F1706), which implies that the highest concentration tested for cell transformation with the other cell line (H4536) may have been too low. In view of the results of the preliminary toxicity test, the highest concentration was also rather low for F1706.

The large number of subcultures the F1706 cells went through before the test started make it impossible to use the occurrence of late tumours as an independent test criterion. Furthermore, the high number of subcultures did not lead to late tumours in case of the solvent controls, which suggests an effect of the test-substance.

B.6.8.1 – 6.4.11 Dominant lethal test in mouse with SDS-3701 - study 11

Previous evaluation	In DAR (2000)
Evaluation RMS	Original study report not available to RMS

Study design and results

Type of study: dominant lethal test.

Species	Endpoint	Result	Dose range	Reference
mouse, males n = 10 mating schedule: 2 females/male per week for 8 weeks	dominant lethality	*	first study: 6.5 mg/kg bw/day; follow-up: 1 and 3 mg/kg bw/day; 5 days; gavage; dissolved in a solution of 10% dimethylsulphoxide (DMSO) in distilled water.	Legator, 1975
Test substance: SDS-3701, 4-hydroxy-2,5,6-trichloroisophthalonitrile, 99+%, white powder GLP-statement: no According to OECD 478: no * inconclusive				

Conclusions

In the first study the number of resorptions per pregnant female was significantly increased in the high dose group as compared to the negative control group in mating week 3. The biological significance of this increase is equivocal, since the average number of resorptions per pregnant female varied strongly between the various mating weeks both in the negative control and the SDS-3701 treated group. The results of the follow-up study did not reveal any treatment related effects.

Guidelines and limitations

The study has been performed prior to OECD guideline 478 became effective. The study is not suitable for evaluation among others because of a rather limited study design in combination with a low fertility index, especially in some of the mating weeks, and a large variability among observations between the mating weeks in both the negative control and the SDS-3701 treated group. In addition the report does not make clear whether the dose levels examined have been significantly high for a meaningful dominant lethal test.

B.6.8.1 – 6.4.12 Dominant lethal test in rat with SDS-3701 - study 12

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: dominant lethal test.

Species	Endpoint	Result	Dose range	Reference
rats, males (Sprague Dawley) n = 10 mating schedule: 2 females/male	dominant lethality	-	acute study: 0,2,4,8 mg/kg bw subacute study: 0,2,4,8 mg/kg bw/day, for five days, gavage treatment vehicle: 0.5% solution of hydroxypropylcellulose in distilled water	Hastings and Jessup, 1975
Test substance: SDS-3701, 99+%, white crystalline powder GLP-statement: no According to OECD 478: no				

Conclusions

The test substance did not induce dominant lethality in rats.

Guidelines and limitations

The study has been performed prior to OECD guideline 478 became effective. The highest dose level examined was rather low for this type of test. Apart from soft stools in animals of the 8 mg/kg/day group during the 5-day dosing period and for several days thereafter, no treatment-related effects were observed.

B.6.8.1 – 6.4.13 In vivo UDS test in rat with SDS-3701 - study 13

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/06 Honarvar, N. (2005), In vivo unscheduled DNA synthesis in primary rat hepatocytes with SDS-3701, testing facility: RCC Cytotest Cell Research GmbH, Rossdorf, Germany. Report No. 887200. Unpublished. (Syngenta File Number R182281_10024)

GUIDELINES: OECD 486 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

Acceptability: The study was performed in accordance with OECD guideline 486 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

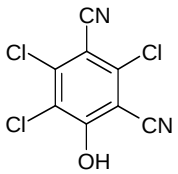
Study design

Initially four pre-experiments were performed with 2 male and 2 female rats receiving a single oral doses of 250, 500, 750 and 1000 mg/kg bw. SDS-3701 was tested in an unscheduled DNA synthesis assay in rat primary hepatocytes in vivo at dose level of 250 and 500 mg/kg bw (3 male rates per dose level). A vehicle control and two positive controls were included in the study. Hepatocytes were isolated 2h or 16 h after and viability tested by trypan blue dye exclusion.

At least three cultures were established from each animal using Williams E medium supplemented with L-glutamine, insulin and fetal calf serum. Cells were allowed to attach for 1.5 h, fresh medium with ^{3}H-TdR (5µCi/mL) added for 4h and finally the cells were fixed autoradiography was performed for 14 d. The number of silver grains was counted automatically. At least two slides per animal and 50 cells per slide were evaluated.

Materials

Test Material: SDS-3701
4-hydroxy-2,5,6-trichloroisophtalonitrile;
4-OH-2,5,6-trichloro-1,3-dicyanobenzene



Description: White Powder
Lot/Batch#: Batch 51955-15-21
Purity: 99%

Vehicle and/or positive control: Polyethylene glycol 400 (PEG 400) N,N'-Dimethylhydrazine dihydrochloride (DMH) and 2-Acetylaminofluorene (AAF)

Results

Based on the results of the preliminary studies, in which an increased incidene in clinical signs was observed at 750 and 1000 mg/kg bw, a maximum dose of 500 mg/kg bw was selected for the main

study. Since the observed clinical signs were not gender specific, the main experiment was performed using male rats only. In the main experiment, animals treated with 250 or 500 mg/kg bw showed ruffled fur and reduction of spontaneous activity 1- 16h following treatment. One animal died 16 h post treatment. In Table 5.8.1-10, the results of the UDS test are given.

Table 6.8.1 – 6.4.13-1: Effect of SDS-3701 on Unscheduled DNA Synthesis

Test substance	Dose level (mg/kg bw)	Preparation interval 2h		Preparation interval 16h	
		Viability (range %)	Cells in repair (mean,%)	Viability (range %)	Cells in repair (mean,%)
Corn oil	**10 mL/kg bw**	73-79	1.0	70-77	1.7
SDS-3701	**250**	76-86	2.7	81-85	2.0
	500	74-83	1.0	75-82	4.0
DMH	**80**	71-84	57.7		
2-AAF	**80**			78-79	69.7

DMH Dimethylhydrazine hydrochloride
2-AAF: 2-Acetylaminofluorene

Conclusions

The results indicate that SDS-3701 does not cause a significant increase in the mean number of incorporated ^{3}H-thymidine at dose levels of 250 and 500 mg/kg bw at preparation intervals of 2h or 16 h.. Therefore, it is concluded that the test substance is considered negative in the UDS in vivo rat hepatocyte assay no DNA damage in hepatocytes was induced.

Genotoxicity SDS-46851 – in vitro

B.6.8.1 – 6.4.14 Reverse mutation assay in bacteria with SDS-46851- study 14

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: reverse mutation assay, with bacteria, with and without metabolic activation; plate incorporation assay

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
B: *S. typh.* TA98 TA100 TA1535 TA1538 TA1537	 point mut. point mut. point mut. point mut. point mut.	 - - - - -	 - - - - -	rat liver	Aroclor 1254	39-3900 plate/plate vehicle: dimethylsulphoxide (DMSO)	Godek, Jones, and Killeen,1985
Test substance: SDS-46851, 2,4,5-trichloro-3-carboxybenzamide, 99.4%, white powder GLP - statement: yes According to OECD 471: no							

Conclusions

The test substance was not found to be mutagenic when tested in the *Salmonella*/microsome test in the presence and the absence of a liver homogenate from Aroclor-treated rats.

Guidelines and limitations

The study has not been carried out according to OECD 471; the study was only performed once; this is deemed acceptable in view of the unambiguous results.

B.6.8.1 – 6.4.15 Reverse mutation assay in bacteria with SDS-46851- study 15

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: reverse mutation assay, with bacteria, with and without metabolic activation; plate incorporation assay

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
B: *S. typh.* TA98	point mut.	-	-	rat kidney	Aroclor 1254	100-10.000 plate/plate vehicle: acetone	Haworth, Jones, and Killeen,1985
TA100	point mut.	-	-				
TA1535	point mut.	-	-				
TA1538	point mut.	-	-				
TA1537	point mut.	-	-				
Test substance: SDS-46851, 2,5,6-trichloro-3-carboxybenzamide, 99.4%, tan powder GLP - statement: yes According to OECD 471: no							

Conclusions

The test substance was not found to be mutagenic when tested in the *Salmonella*/microsome test in the presence and the absence of a liver homogenate from Aroclor-treated rats.

Guidelines and limitations

The study has not been carried out according to OECD 471; the study was only performed once; this is deemed acceptable in view of the unambiguous results.

B.6.8.1 – 6.4.16 Reverse mutation assay in bacteria with SDS46851 - study 16

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/43 Verspeek-Rip, C.M. (2004a), Evaluation of the mutagenic activity of SDS 46851 in the *Salmonella typhimurium* reverse mutation assay and the *Escherichia coli* reverse mutation assay (with independent repeat), testing facility: Notox B.V, 5231 DD's-Hertogenbosch, The Netherlands, published: no, report No. 402503 (Syngenta File Number R611965_10018)

GUIDELINES: Corresponding to OECD 471 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

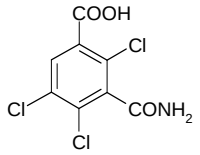
Acceptability: There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA1535 and TA1537 of *Salmonella typhimurium* and WP_2uvrA of *E.coli* were exposed to SDS 46851, using dimethyl sulfoxide as a vehicle at concentrations up to 5000 µg /plate, with and without S-9 activation.
The test article was tested at six dose levels along with appropriate vehicle and positive controls on the tester strains employed in the presence and absence of S9-mix. All dose levels, vehicle and positive controls were plated in triplicate.

Test Material: SDS-46851

2, 4,5-trichloro-isophthalamic acid, 3-carbamyl-2,4,5-trichlorobenzoic, 3-carboxy-2,5,6-trichlorobenzamide



Description: White Powder

Lot/Batch#: Batch 52031-13-23

Purity: 99%

Control Materials

Negative: Vehicle (Dimethyl sulfoxide)

Solvent: DMSO at 0.1 mL/plate

Results

No cytotoxicity was observed in a dose range finding assay.
On basis of the preliminary cytotoxicity test a mutation assay was performed in the presence of rat liver S9 at dose levels of 3 - 5000 µg SDS-46851/plate.
Triplicate plates per strain, dose and condition were used. No positive responses were observed with any of the strains used, in the presence as well as in the absence of microsomal enzymes. The results are summarized below.

Table 6.8.1 – 6.4.16-1: Mutagenic Response of SDS-46851, in Different *Salmonella* and *E.coli* Strains

SDS-46851 µg/plate	Average revertants per plate									
Strain:	TA98		TA100		TA1535		TA1537		WP_2uvrA	
S9-mix:	-	+	-	+	-	+	-	+	-	+
Vehicle*	27	39	116	113	7	9	5	5	8	12
3	nt	nt	102	112	nt	nt	nt	nt	11	17
10	nt	nt	105	115	nt	nt	nt	nt	13	13
33	nt	nt	105	110	nt	nt	nt	nt	13	13
100	24	25	94	106	7	6	5	5	12	17
333	29	30	107	113	8	10	3	5	14	15
1000	30	29	106	109	9	6	4	8	9	10
3330	26	34	96	108	7	5	5	4	12	15
5000	21	31	118	11	9	11	3	4	13	13
Pos. control**	709	578	540	596	1479	153	317	274	475	249

* DMSO

** 2-nitrofluorene	10 g/plate -	TA98 (-)
2-aminoanthracene	1-10 µg/plate	TA 98(+), TA100 (+), TA1535 (+), TA1537 (+), TA1538 (+)
Sodium azide	5 µg/plate -	TA 1535 (-)
9-Aminoacridine	60 µg/plate	TA1537 (-)
Methylmethansulfonate	10 µg/plate	TA100 (-)
4-Nitroquinoline-N-oxide	10 µg/plate	WP_2uvrA (-)

nt not tested

In a second independent experiment these results were confirmed

Conclusion

SDS-46851 was not mutagenic when tested on S. *typhimurium* strains TA98, TA100, TA1535 or TA1537 and E.coli strain WP_2uvrA, with or without S9-mix activation.

B.6.8.1 – 6.4.17 *In* vitro siter-chromatid exchange assay with SDS-46851- study 17

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: *in vitro* sister-chromatid exchange assay, with and without metabolic activation

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
Chinese Hamster Ovary (CHO-K1-BH4)	sister-chromatid exchanges	-	-	rat liver	Aroclor 1254	200-2000 ML/ml (5 h) vehicle: dimethylsulphoxide (DMSO)	Jones, Killeen, and SanSebastian, 1985
Test substance: SDS-46851, 2,5,6-trichloro-3-carboxybenzamide, 99.4%, beige powder GLP - statement: yes According to OECD 479: no							

Conclusions

The test substance was not found to be mutagenic when tested in the *in vitro* SCE test with Chinese Hamster Ovary cells, in the presence and the absence of a liver homogenate from Aroclor-treated rats.

Guidelines and limitations

The study has not been carried out according to OECD 479. Important deviations are:

- the study was only performed once; this is deemed acceptable in view of the unambiguous results;
- no toxicity was reported for the highest doses of the preliminary toxicity test; the highest dose tested for SCE induction was determined by acidity of the tissue-culture medium caused by the test substance; however, it is unclear whether higher doses were not possible due to this effect.

B.6.8.1 – 6.4.18 *In* vitro TK forward mutation assay with SDS-46851- study 18

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: L5178Y TK+/- forward mutation assay, with and without metabolic activation

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
L5178Y mouse lymphoma	thymidine kinase deficiency	-	-	rat liver	Aroclor 1242 and 1254	75-1000 ML/ml (4 h) dimethylsulphoxide (DMSO)	Jones and Sernau, 1985
Test substance: SDS-46851, 2,5,6-trichloro-3-carboxybenzamide, >98%, white powder GLP - statement: yes According to OECD 476: no							

Conclusions

The test substance was not found to be mutagenic when tested *in vitro* for forward mutations in the TK locus of L5178Y mouse lymphoma cells, in the presence and the absence of a liver homogenate from Aroclor-treated rats.

Guidelines and limitations

The study has not been carried out in compliance with OECD 479 because the highest dose tested only slightly affected the viability of the cells. According to the authors, higher doses might have lead to false positive results due to increased osmolality or acidity. This justification is acceptable.
The report refers to a positive study which was not present in the dossier.

B.6.8.1 – 6.4.19 In vitro cell mutation assay in mouse lymphoma cells with SDS46851 - study 19

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/44 Verspeek-Rip, C.M. (2005b), Evaluation of the mutagenic activity of SDS 46851 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat), testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands, published: no, report No. 402492. (Syngenta File Number R611965_10016)

GUIDELINES: OECD 476 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

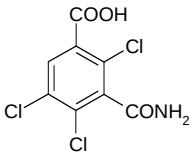
Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The test substance SDS-46851 was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and presence of an S9-activation system. Test substance doses up to 2660 µg/mL in the absence and in the presence of S9 (8% in the first test and 12% in the second test).

Test Material: SDS-46851

2, 4,5-trichloro-isophthalamic acid, 3-carbamyl-2,4,5-trichlorobenzoic, 3-carboxy-2,5,6-trichlorobenzamide



Description: White Powder

Lot/Batch#: Batch 52031-13-23

Purity: 99%

Control Materials

Solvent: Dimethyl sulfoxide

Solvent control: 0.8% DMSO in culture medium

Positive controls: Non-activation

Methyl methane-sulfonate (MMS) at final concentrations of 15 µg/mL

S9-activation

Cyclophosphamide (CP) at final concentrations of 10 µg/mL

Results

The cytotoxicity was determined by counting the cells after exposure and by measuring the relative suspension growth and cloning efficiency of the L5178Y cells 24 and 48 hours after treatment – compared to negative control and the relative total growth (RTG: product of RSG and the relative colony-forming ability ["cloning efficiency"] 48 hours after treatment). Cell toxicity became obvious at concentrations from 100 µg/mL and 333 µg/mL onwards in non-activated and in S9-activated systems, respectively.

Table 6.8.1 – 6.4.19-1: Cytotoxicity of SDS 46851 in Mouse Lymphoma L5178Y Cells

Concentration Chlorothalonil (µg/mL)	% relative survival (relative suspension growth)		
	Absence of S9		Presence of S9
Duration of treatment	3h	24h	3h
0	100	100	100
33	102	108	93
100	98	104	77
333	117	88	93
1000	101	77	88
2660	81	35	60

The results of the mutagenicity assay (experiment 1, treatment time 3 h) are summarized in Table 5.8-1.62. No increase in mutant frequency at 1 - 2660 µg/mL was observed in the absence nor in the presence of S9.A second experiment with a treatment duration of 24 h and the inclusion of 12 % S9 mix gave similarly negative results.

Both positive control compounds fulfilled the requirements for a valid test.

Table 6.8.1 – 6.4.19-2: Effects of (3h Treatment) on Gene Mutations at the TK-Locus of Mouse Lymphoma L5178Y Cells in the Absence or Presence of S9-Mix (%)

Concentration SDS-46851 (µg/mL)	Absence of S9-mix		Presence of S9-mix	
	Mutation frequency $x10^{-6}$	Survival (%)	Mutation frequency $x10^{6}$	Survival (%)
0	69	100	66	100
1	65	105	56	102
3	60	100	59	111
10	62	93	54	110
33	65	94	54	108
100	64	87	67	108
333	52	108	52	112
1000	68	97	63	117
2660	62	82	64	89
MMS (15 µg/mL)	1644	83		
CP (10 µg/mL)			2179	32

Conclusion

Based on the overall results of this study it is concluded, that the test substance SDS-46851 is not mutagenic at the TK-locus of mouse lymphoma L5178Y cells.

B.6.8.1 – 6.4.20 UDS test with primary rat hepatocytes with SDS-46851- study 20

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: unscheduled DNA in primary mammalian cells

Cells	Endpoint	Result	Dose range	Reference
primary rat hepatocytes	DNA repair (unscheduled DNA synthesis), measured as the incorporation of radioactive thymidine in nuclei	-	2.4-240 well/well (18-20 h) vehicle: ethanol (95%)	Jones, Killeen, and Ignatoski, 1985
Test substance: SDS-46851, 2,4,5-trichloro-3-carboxybenzamide, >99,4%, white powder GLP-statement: yes According to OECD 482: no				

Conclusions

The test substance did not induce unscheduled DNA synthesis in primary rat hepatocytes.

Guidelines and limitations

The study did not comply with OECD 482, as no independent repeat was performed. However, in view of the unambiguous outcome, this is deemed acceptable.

Genotoxicity SDS-46851 – in vivo

B.6.8.1 – 6.4.21 *In* vivo micronucleus assay with SDS-46851- study 21

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: micronucleus test.

Species	Endpoint	Result	Dose range	Reference
Initial test				
mouse	micronuclei in bone-marrow erythrocytes	+/-	500-5000 mg/kg bw, single oral dose, gavage vehicle: 0.5% hydroxy-propyl methyl cellulose in distilled water	Siou, Mizens, and Killeen, 1985
Repeat test				
		-	5000 mg/kg bw, single oral dose, gavage vehicle: 0.5% hydroxy-propyl methyl cellulose in distilled water	
Test substance: SDS-46851, 2,5,6-trichloro-3-carboxybenzamide, >92.3%, crystalline beige powder GLP-statement: yes According to OECD 474: no				

Conclusions

The treatment of mice with the test substance did result in slight, irregular increases of the number of micronuclei in the initial test. The repeat test did not show the test substance to induce micronuclei. In view of the limitations discussed hereunder, this outcome presents only limited evidence for the absence of *in vivo* mutagenicity of the test substance in mice.

Guidelines and limitations

The following considerations make clear that the study does not comply with OECD 474.
The slides from the 24-h bone-marrow sampling of the initial test showed poor staining and unexpected low responses for the positive control, i.e. urethane, while no positive-control slides were prepared for 48h and 72 h. These shortcomings make the initial test unacceptable for evaluation, because they preclude a proper evaluation of the sensitivity of the animals. This implies that negative results obtained with the properly stained slides of the initial test (bone-marrow sampling after 48h and 72 h), cannot be accepted as an indication for the absence of clastogenicity, and should, therefore, not be taken into account when the results of the repeat test are evaluated.
The reviewer concludes that the repeat test has to be regarded as a fully independent study, which should thus be evaluated fully independently of the initial study.
The repeat test only included the 5000-mg/kg dose and bone marrow samples taken at 24 h. In contrast, OECD 474 requires three bone-marrow samplings for an independent test. In addition, the repeat test cannot be accepted to verify the initial test, as it includes only one dose, while OECD 474 requires three for a verifying test.
In view of the significant, albeit small and irregular (not dose dependent) increases encountered in the initial test (notwithstanding the staining problem), the repeat test should have included the same dosages and sampling times as the initial test. In addition, the value of the repeat test is limited due to the absence of a decrease of the ratio between the numbers of poly- and normochromatic erythrocytes.
Therefore, the results of the repeat test provide only limited evidence for the absence of *in vivo* mutagenicity in mice, which need to be confirmed.

B.6.8.1 – 6.4.22 Micronucleus test in mouse with SDS-46851 - study 22

Previous evaluation	In addendum 14 DAR (April 2004)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: mouse micronucleus test

Species	Endpoint	Result	Dose range	Reference
mouse, CD-1 5/males/dose	micronuclei (bone marrow erythrocytes)	-	2000 mg/kg bw/d (limit dose), single dose IP; sacrifice at 24 and 48 h after dosing vehicle: 0.5% w/v hydroxypropyl methyl cellulose	Fox, 2002
Test substance: **R611965** (SDS 46851 or 3-carbamyl-2,4,5-trichlorobenzoic acid), batch no. P2, purity 99.2% w/w, Clinical signs of toxicity were observed at dose level 2000 mg/kg bw/d, but no specifications were given. The ratio PCE/NCE in test and control animals was fully comparable. GLP statement: yes According to OECD 474: yes				

Dose range finding

An experiment was performed to determine the maximum tolerated dose (MTD), based on patterns of lethalities or severe toxicity observed over a four-day observation period following a single oral or IP dose. Male rats received orally by gavage 800, 1250 and 2000 mg/kg of R611965 (2 animals/dose), 6 males received 50, 320, and 2000 mg/kg (2 animals/dose) IP and 6 rats received 2000 mg/kg IP (3 rats/sex). It was stated no significant adverse reactions up to 2000 mg/kg bw/d both oral and IP were

observed, however, data were not presented. Based on this data it was stated that there is no substantial difference in toxicity between the sexes and therefore only males were used in the micronucleus test.

Acceptability

The study is considered acceptable. Since neither in males nor in females treatment-related adverse effects were seen in the dose range finding experiment. Although, due to the lack of toxicokinetic and toxicodynamic information increase in incidence of micronucleated polychromatic erythrocytes in females cannot be ruled out.

Conclusions

The test substance (R611965) did not increase the incidence of micronucleated polychromatic erythrocytes in male mouse bone marrow cells. Though, due to the lack of toxicokinetic and toxicodynamic information increase in incidence of micronucleated polychromatic erythrocytes in females cannot be ruled out and therefore, this study can only be used as being indicative.

B.6.8.1 – 6.4.23a In vivo micronucleus test in mice with SDS46851 - study 23

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/45 Buskens, C.A.F. (2004a), Micronucleus test in bone marrow cells of the mouse with SDS 46851, testing facility: Notox B.V, 5231 DD 's-Hertogenbosch, The Netherlands, published: no, report No. 402481. (Syngenta File Number R611965_10017)

GUIDELINES: OECD 474 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

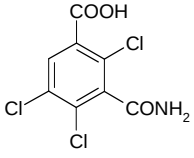
Acceptability: The study was performed in accordance with OECD guideline 474 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

Following a dose finding study, 5 male and 5 female NMRI mice were treated by gavage at a dose level of 2000 mg SDS-46851 per kg bodyweight. A negative control group was treated with the vehicle, a positive control group received Cyclophosphamide (50 mg/kg bw).Twentyfour or 48 hours after dosing, animals were killed by cervical dislocation and bone marrow smears were analysed microscopically by counting micronuclei in 2000 polychromatic erythrocytes per animal. The ratio polychromatic to normochromatic erythrocytes was determined by counting and differentiating the first 1000 erythrocytes at the same time. Micronuclei were only counted in polychromatic erythrocytes.

Test Material: SDS-46851

2, 4,5-trichloro-isophthalamic acid,



	3-carbamyl-2,4,5-trichlorobenzoic, 3-carboxy-2,5,6-trichlorobenzamide
Description:	White Powder
Lot/Batch#:	Batch 52031-13-23
Purity:	99%

Vehicle and/or positive control: Corn oil, Cyclophosphamide 50 mg/kg bw.

Results

No mortalities occurred and no clinical signs of toxicity were observed.

The mean micronucleated cell count for all dose groups of SDS-46851 were essentially comparable with the concurrent vehicle control group, at any of the three sampling times.

Cyclophosphamide caused large significant increases in the frequency of micronucleated polychromatic erythrocytes

The ratio of normochromatic to polychromatic erythrocytes was not influenced

Table 6.8.1 – 6.4.23-1: Summary of Micronucleus Results in Mice

Treatment	Sampling time	Micronuclei per 2000 normochromatic erythrocytes	Ratio polychromatic to normochromatic Erythrocytes
Solvent	**24**	0.8	1.13
2000 mg/kg bw SDS-46851	**24**	0.2	0.78
2000 mg/kg bw SDS-46851	**48**	0.4	1.06
50mg/kg bw CP	**48**	**59.8***	0.24

Conclusion

In an in vivo micronucleus test SDS-46851 did not induce micronucleated polychromatic erythrocytes at a dose of 2000 mg per kg bw. Validity of the test performed was shown with a cyclophosphamide treated positive control group with a marked response.

B.6.8.1 – 6.4.23b Proof of exposure - In vivo micronucleus test in mice with SDS46851

Data requirement 2.26

Applicant to provide evidence of tissue exposure in the in vivo MN study with SDS46851 – study 23.

See also 2(89)

See reporting table 2(72)

NL (August 2017): The RMS agrees with the statement provided by the applicant.

The notifier provided the following statement:

" R611965 (SDS-46851) was investigated in three mouse micronucleus studies. The *in vivo* bone marrow micronucleus assay is a widely used *in vivo* genotoxicity test. In the case that a negative

result is obtained evidence must be presented that the compound of interest was systemically available to the bone marrow. As the bone marrow is a well-perfused tissue, concentrations of compound-related material(s) in blood or plasma are generally similar to those observed in bone marrow. Therefore, proof of exposure can be obtained by measurement of the compound of interest either in blood or plasma.

In the three studies on R611965, although no direct blood concentration data was taken, the weight of evidence supports that the bone marrow was exposed to circulating R611965. In one of the studies (Fox 2002), R611965 was dosed intraperitoneally (*i.p.*). Intraperitoneal administration has the advantage that the compound will by-pass the GIT, effectively giving 100% absorption. Furthermore, all other chlorothalonil metabolites tested in the mouse MNA were systemically available following oral administration. This includes both upstream metabolites *e.g.* R613636 and closely related analogues e.g. R182281, R417888, SYN548708, SYN548764, R611968 and SYN507900. In rat, following oral administration of R611965, approximately 20% of an orally administered was recovered in urine (Ho *et al*, 1990). Likewise from the metabolism study of Punler *et al (*2013), following oral administration of chlorothalonil, R611965 was recovered in urine and bile.

Together, this data demonstrates that R611965 was negative in three MMNA studies. The *i.p.* administration would ensure that *ca.* 100% of the administered dose would have reached the liver. Once in the liver R611965 is unlikely to have been exhaustively metabolised and would therefore have been systemically available, based on the fate of all other chlorothalonil metabolites in mouse. This is further supported by rat, in which R611965 was a downstream metabolite of chlorothalonil excreted in urine. Also, following oral administration of R611965 to rat the dose was systemically available with *ca*. 20% of the dose in urine. “

Genotoxicity R417888 – in vitro

B.6.8.1 – 6.4.24 Reverse mutation assay in bacteria with R417888- study 24

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: reverse mutation assay, with bacteria, with and without metabolic activation; plate incorporation assay

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
B: *S. typh.*				rat liver	phenobarbital/beta-naphthoflavone	100-5000 µg/plate	Callander, 2000
TA98	point mut.	-	-			vehicle: dimethylsulphoxide (DMSO)	
TA100	point mut.	-	-				
TA1535	point mut.	-	-				
TA1537	point mut.	-	-				
B: *E. coli*							
WP2P	point mut.	-	-				
WP2P *uvrA*	point mut.	-	-				
Test substance: R417888, 2-amido-3,5,6-trichloro-4-cyanobenzene sulfonic acid, 97%, white solid GLP - statement: yes According to OECD 471/472: yes							

Conclusions

The test substance was not found to be mutagenic when tested in the *Salmonella/E. coli* microsome test in the presence and the absence of a liver homogenate from phenobarbital/beta-naphthoflavone-treated rats.

Guidelines and limitations

The study was carried out according to OECD 471/472. Two independent experiments were conducted, the second experiment using the pre-incubation protocol.

B.6.8.1 – 6.4.25 *In vitro* reverse mutation assay with SDS-417888 - study 25

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/15 Verspeek-Rip, C.M. (2005a), Evaluation of the mutagenic activity of VIS 01 in the *Salmonella typhimurium* reverse mutation assay and the Escherichia coli reverse mutation assay (with independent repeat), testing facility: Notox B.V, 5231 DD's-Hertogenbosch, The Netherlands. Report No. 402536. Unpublished. (Syngenta File Number R417888_10025)

GUIDELINES: Corresponding to OECD 471 (1997)

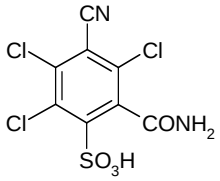
GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

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

Study design

In a reverse gene mutation assay in bacteria, strains TA98, TA100, TA1535 and TA1537 of *Salmonella typhimurium* and WP_2uvrA of *E.coli* were exposed to VIS 01, using dimethyl sulfoxide as a vehicle at concentrations up to 5000 µg /plate, with and without S-9 activation.
The test article was tested at six dose levels along with appropriate vehicle and positive controls on the tester strains employed in the presence and absence of S9-mix. All dose levels, vehicle and positive controls were plated in triplicate.

Test Material: VIS 01, 1-cyano-1, 5, 6-trichloro-3-amido-benzene-sulphonate



Description: White powder
Lot/Batch#: Batch 52168-11-16
Purity: 99.5%

Control Materials

Negative:	Vehicle (dimethyl sulfoxide)		
Solvent:	DMSO at 0.1 mL/plate		
Positive controls:	Without S9		
	2-nitrofluorene	10 g/plate -	TA98 - TA1538
	Sodium azide	5 µg/plate -	TA 1535
	9-Aminoacridine	60 µg/plate	TA1537
	Methylmethansulfonate	10 µg/plate	TA100
	4-Nitroquinoline-N-oxide	10 µg/plate	WP_2uvrA
	With S9		
	2-aminoanthracene	1-10 µg/plate	TA 98, TA100, TA1535, TA1537, WP_2uvrA

Results

No cytotoxicity was observed in a dose range finding assay.
On basis of the preliminary cytotoxicity test a mutation assay was performed in the presence of rat liver S9 at dose levels of 3 - 5000 mg VIS 01.
Triplicate plates per strain, dose and condition were used. No positive responses were observed with any of the strains used, in the presence as well as in the absence of microsomal enzymes. The results are summarized in Table 6.8.1 – 6.4.25-1.

Table 6.8.1 – 6.4.25-1: Mutagenic Response of VIS 01, in Different *Salmonella* and *E.Coli* Strains

VIS 01 µg/plate	Average revertants per plate									
Strain:	TA98		TA100		TA1535		TA1537		WP_2uvrA	
S9-mix:	-	+	-	+	-	+	-	+	-	+
Vehicle*	17	20	118	142	19	14	4	9	9	10
3	nt	nt	141	136	nt	nt	nt	nt	11	11
10	nt	nt	123	132	nt	nt	nt	nt	12	10
33	nt	nt	136	148	nt	nt	nt	nt	13	13
100	14	24	132	133	10	11	5	6	11	11
333	17	25	122	140	13	11	5	8	12	13
1000	16	24	121	133	20	15	6	5	13	14
3330	17	21	132	149	19	12	7	5	7	15
5000	18	22	109	121	21	17	6	8	9	12
Pos. control**	1044	926	1050	1181	1410	197	239	453	706	241

* DMSO

** 2-nitrofluorene	10 g/plate -	TA98 (-)
2-aminoanthracene	1-10 µg/plate	TA 98(+), TA100 (+), TA1535 (+), TA1537 (+), TA1538 (+)
Sodium azide	5 µg/plate -	TA 1535 (-)
9-Aminoacridine	60 µg/plate	TA1537 (-)
Methylmethansulfonate	10 µg/plate	TA100 (-)
4-Nitroquinoline-N-oxide	10 µg/plate	WP_2uvrA (-)

nt not tested

In a second independent experiment these results were confirmed.

Conclusions

VIS 01 was not mutagenic when tested on S. *typhimurium* strains TA98, TA100, TA1535 or TA1537 and *E.coli* strain WP_2uvrA, with or without S9-mix activation.

B.6.8.1 – 6.4.26 *In vitro* reverse mutation assay with SDS-417888 - study 26

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/20 Sokolowski A. (2007): Salmonella Typhirium and Escherichia Coli Reverse Mutation Assays with R417888, RCC – CCR, Rossdorf, Germany. Unpublished report number 1030808. (Syngenta File Number R417888_10020)

GUIDELINES: "Ninth Addendum to OECD Guidelines for testing of Chemicals", Section 4, number 471: "Bacterial Reverse----Mutation Test", adopted July 21, 1997 "Commission Directive 200/32/EC, L1362000, Annex 4D", May 19, 2000
GLP: This study was performed in accordance with the principles of Good Laboratory Practises.
Acceptability: There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design
The experiment was performed to assess the potential of the test item R417888 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, TA98, TA 100 and *Escherichia coli* strain WP2uvrA in a reverse mutation assay.
The *S. typhimurium* histidine (his) and the *E. coli* tryptophan (trp) reversion system measures $his^- \rightarrow his^+$ and $trp^- \rightarrow trp^+$ reversions, respectively. The S. typhimurium and Escherichia coli strains are constructed to differentiate between base pair (TA 1535, TA 100, and WP2 uvrA) and frameshift (TA 1537, TA 98) mutations.
The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item 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
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.

Test Material: R417888
Description: White hygroscopic solid
Lot/Batch #: BJQ-impurity 329-001
Purity: 95.0%
Stability of the compound: Expiration Date: December 05, 2008

Control Materials:
Negative: Untreated and solvent controls
Solvent/final Concentration: DMSO
Positive: Non activation (-S9):
TA1535, TA100: sodium azide – 10 µg/plate
TA1537, TA98 : 4-nitro-o-phenylene-diamine – 10 µg/plate in TA 98, 50 µg/plate in TA 1537
WP2 uvrA: methylmethane sulfonate – 3 µl/plate
Activation (+S9):
All the strains: 2-aminoanthracene – 2.5 µg/plate (TA 1535, TA 1537, TA 98, TA 100), 10 µg/plate (WP2 uvraA).

Results

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 five tester strains was observed following treatment with R417888 at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no ten.dency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. In Table 6.8.1 – 6.4.26-1 and -2 the results of experiment I and II, respectively, are given.

Table 6.8.1 – 6.4.26-1: Preliminary Cytotoxicity Assay and Experiment I

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2 uvrA
Without Activation	DMSO		15 ± 1	17 ± 6	36 ± 5	137 ± 2	52 ± 13
	Untreated		15 ± 2	18 ± 4	39 ± 1	150 ± 13	61 ± 5
	R417888	3 µg	16 ± 4	14 ± 5	28 ± 10	141 ± 5	50 ± 15
		10 µg	17 ± 2	18 ± 2	34 ± 9	137 ± 13	48 ± 3
		33 µg	15 ± 8	19 ± 3	38 ± 2	146 ± 15	57 ± 5
		100 µg	17 ± 4	17 ± 4	33 ± 3	149 ± 2	59 ± 8
		333 µg	17 ± 3	16 ± 5	35 ± 7	140 ± 11	48 ± 5
		1000 µg	19 ± 4	12 ± 3	30 ± 3	151 ± 10	50 ± 5
		2500 µg	22 ± 4	17 ± 3	40 ± 4	133 ± 16	47 ± 9
		5000 µg	22 ± 1	17 ± 3	33 ± 9	140 ± 4	46 ± 6
	NaN_3	10 µg	2181 ± 120			2468 ± 58	
	4-NOPD	10 µg			400 ± 36		
	4-NOPD	50 µg		97 ± 3			
	MMS	3.0 µL					1481 ± 86
With Activation	DMSO		23 ± 7	18 ± 6	43 ± 1	189 ± 10	65 ± 8
	Untreated		20 ± 1	20 ± 6	49 ± 7	189 ± 6	66 ± 12
	R417888	3 µg	22 ± 6	20 ± 6	46 ± 5	181 ± 13	57 ± 9
		10 µg	24 ± 6	14 ± 6	46 ± 3	172 ± 22	62 ± 6
		33 µg	20 ± 4	18 ± 4	40 ± 4	177 ± 4	66 ± 3
		100 µg	20 ± 1	20 ± 4	43 ± 2	183 ± 21	63 ± 5
		333 µg	21 ± 2	23 ± 5	42 ± 6	179 ± 4	51 ± 2
		1000 µg	28 ± 10	20 ± 13	38 ± 3	166 ± 4	56 ± 14
		2500 µg	22 ± 5	18 ± 2	39 ± 5	177 ± 18	68 ± 5
		5000 µg	21 ± 5	20 ± 4	42 ± 11	180 ± 3	68 ± 7
	2-AA	2.5 µg	449 ± 12	294 ± 14	1938 ± 145	3139 ± 254	

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2 uvrA
	2-AA	10.0 µg					275 ± 59

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 – 6.4.26-2: Experiment II

Metabolic Activation	Test Group	Dose Level (µg/plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	WP2 uvrA
Without Activation	DMSO		18 ± 5	12 ± 3	27 ± 6	139 ± 11	49 ± 8
	Untreated		24 ± 4	16 ± 4	30 ± 3	154 ± 17	47 ± 10
	R417888	33 µg	16 ± 4	17 ± 4	30 ± 10	134 ± 4	43 ± 6
		100 µg	20 ± 5	15 ± 3	32 ± 11	144 ± 12	53 ± 11
		333 µg	22 ± 4	13 ± 4	34 ± 2	145 ± 13	45 ± 6
		1000 µg	16 ± 4	14 ± 4	25 ± 4	147 ± 13	46 ± 3
		2500 µg	21 ± 8	9 ± 3	27 ± 7	140 ± 3	50 ± 6
		5000 µg	15 ± 8	10 ± 5	29 ± 13	138 ± 17	40 ± 4
	NaN3	10 µg	2090 ± 76			2514 ± 111	
	4-NOPD	10 µg			527 ± 21		
	4-NOPD	50 µg		153 ± 7			
	MMS	3.0 µL					524 ± 20
With Activation	DMSO		23 ± 4	20 ± 2	44 ± 7	174 ± 23	64 ± 6
	Untreated		30 ± 6	16 ± 7	44 ± 9	191 ± 13	49 ± 13
	R417888	33 µg	22 ± 1	21 ± 9	43 ± 13	176 ± 11	73 ± 6
		100 µg	24 ± 8	15 ± 1	30 ± 5	181 ± 1	71 ± 11
		333 µg	24 ± 5	19 ± 3	37 ± 3	191 ± 23	76 ± 6
		1000 µg	25 ± 8	19 ± 7	39 ± 5	170 ± 10	69 ± 3
		2500 µg	22 ± 5	19 ± 3	35 ± 2	194 ± 23	66 ± 8
		5000 µg	27 ± 10	23 ± 1	43 ± 7	185 ± 5	77 ± 10
	2-AA	2.5 µg	290 ± 23	164 ± 12	1332 ± 8	1585 ± 66	
	2-AA	10.0 µg					257 ± 6

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusions

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 five tester strains was observed following treatment with R417888 at any dose 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.

R417888 was not mutagenic when tested on S. *typhimurium* strains TA98, TA100, TA1535 or TA1537 and E.coli strain WP_2uvrA, with or without S9-mix activation.

B.6.8.1 – 6.4.27 In vitro chromosome aberration study with R417888- study 27

Previous evaluation	In addendum 14 DAR (April 2004)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: mammalian cells *in vitro*, cytogenic assay

Indicator cells	Endpoint	Res. -act.	Res. +act.	Activation		Dose range	Reference
				Tissue	Inducer		
human blood lymphocytes	chromosome aberration	+	-	rat liver	phenobar-bital and β-naphtho-flavone mixture	250, 2000, 3770 μg/ml (3770 μg/ml is equivalent to the limit conc. of 10 mM) solvent: DMSO	Fox, 2000a

Test substance: **R417888**, batch no. P8 (WRC#16882-46-01), purity 97% w/w, white solid
Cytotoxicity observed at dose level: 2000 μg/ml (exp 2 and 3 -S9) and 3770 μg/ml (all exp -S9)
Precipitation observed at dose level: not indicated
GLP statement: yes
According to OECD 473: yes
Within this study 4 experiments were performed:
Exp 1: +/-S9, 3 h treatment
Exp 2: +S9, 3 h treatment, -S9, 20 h treatment
Exp 3 and 4: -S9, 20 h treatment
A single sampling time, 20 hours after the start of treatment was used in all experiments.

Acceptability

The study is considered acceptable.

Conclusions

The test substance (R417888) induced a dose-related statistically significant increase in the percentage of aberrant cells excluding gaps in human blood lymphocytes when exposed to 3770 µg/ml R417888 in the absence of S9 for 20 hours (exp 2 and 4). When exposed to 2000 µg/ml (-S9) a significant induction of cells with chromosome aberrations was observed (exp 2 and 4), though this induction lay just within the top historical control range. When exposed to R417888 in the presence of S9 no aberrations were observed.

B.6.8.1 – 6.4.28 *in vitro* chromosome aberration study with SDS-417888 - study 28

Previous evaluation	In addendum 14 DAR (April 2004)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: mammalian cells *in vitro*, cytogenic assay

Indicator cells	Endpoint	Res. -act.	Res. +act.	Activation		Dose range	Reference
				Tissue	Inducer		
human blood lymphocytes	chromosome aberration	-	-	rat liver	phenobar-bital and β-naphtho-flavone mixture	3 h treatment: 250, 2000, 3770 μg/ml (3770 μg/ml is equivalent to the limit conc. of 10 mM) 20 h treatment: 250, 1000, 2000 μg/ml solvent: DMSO	Fox, 2000b

Test substance: **R417888**, batch no. ASW01787-01R, purity 99% w/w, white solid
Cytotoxicity observed at dose level: 2000 μg/ml (both exp -S9, exp 2 +S9) and 3770 μg/ml (both exp -/+S9)
Precipitation observed at dose level: not indicated
GLP statement: yes
According to OECD 473: yes

Indicator cells	Endpoint	Res. -act.	Res. +act.	Activation		Dose range	Reference
				Tissue	Inducer		
Within this study 2 experiments were performed: Exp 1: +/-S9, 3 h treatment Exp 2: +S9, 3 h treatment, -S9, 20 h treatment A single sampling time, 20 hours after the start of treatment was used in both experiments.							

Acceptability

The study is considered acceptable.

Conclusions

The test substance (R417888) did not induce clastogenic effects in human blood lymphocytes with or without S9.

B.6.8.1 – 6.4.29 *in vitro* chromosome aberration study with SDS-417888 - study 29

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/21 Kunz S. (2007): Chromosome Aberration Test in Human Lymphocytes in vitro with R417888. RCC-CCR, in den Leppsteinsweisen 19, 64380 Rossdorf, Germany. Unpublished Report number 1030810. (Syngenta File Number R417888_10022)

GUIDELINES: OECD 473 (1997) and EC method "Mutagenicity – In vitro Mammalian Chromosome Aberration Test" (2000)
GLP: This study was performed in accordance with the principles of Good Laboratory Practises.
Acceptability: The study was performed in accordance with OECD guideline 473 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The test item R417888, suspended in DMSO, was assessed for its potential to induce structural chromosomal aberrations in human lymphocytes *in vitro* in one experiment. The following study design was performed:

	Without S9 mix		With S9 mix
Exposure period	4 hrs	22 hrs	4 hrs
Recovery	18 hrs	—	18 hrs
Preparation interval	22 hrs	22 hrs	22 hrs

In each experimental group two parallel cultures were analysed. Per culture 100 metaphase plates were scored for structural chromosomal aberrations, except for the highest evaluated concentration after continuous treatment, in the absence of metabolic activation, where only 50 metaphase plates were scored.
The highest applied concentration in this study (2200 µg/mL of the test item, approx. 6.7 mM) was chosen with regard to the ability to formulate a homogeneous suspension of the test item in DMSO.
The chosen treatment concentrations were: 0 – 718.4 – 1257.1 – 2200 µg/ml.
The exposure periods were 4 hours with and without S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test item. In each experimental group two parallel cultures were analysed. 100 metaphase plates per culture were scored for structural chromosomal aberrations, except for the highest evaluated concentration after continuous treatment, in the absence of metabolic activation, where only 50 metaphase plates were scored due to strong clastogenic effects. 1000 cells were counted per culture for determination of mitotic index.

Test Material: R417888 (2-amido-3,5,6-trichloro-4-cyanobenzenesulfonic acid)
Description: White solid
Lot/Batch: BJQ-impurity 329-001
Purity: 95.0% (by RP-HPLC)
Stability of the compound: Expiration Date: December 05, 2008
Vehicle and/or positive control: DMSO (E.MERCK, D-64293 Darmstadt; purity 99.5%)

Results

The proliferation index of the lymphocytes in solvent control cultures in the 22 hrs preparation interval with and without S9 mix (4 hrs treatment; 1.02 and 1.08, respectively), in the 22 hours preparation interval without S9 mix (continuous treatment; 1.03), was checked by analysing the proportion of mitotic cells in the 1st, 2nd and 3rd metaphase (M1, M1+, M2 and M3) indicating that the lymphocytes divided about 1.5 times within the early preparation interval.

Test group	S9 mix	Preparation interval (hrs)	Proliferation index*	Cell cycle phase			
				M1	M1+	M2	M3
Solvent control**	-	22	1.08	89	6	5	0
Solvent control**	-	22 (continuous)	1.03	95	4	1	0
Solvent control**	+	22	1.02	97	3	0	0

* Proliferation index was determined in 100 metaphase plates from each test group.
** DMSO 0.5 % (v/v)

M1 = cells in first division metaphase after start of treatment; both sister-chromatids of each chromosome are stained uniformly dark
M1+ = cells between first and second metaphase after start of treatment; cells contain some differentially stained chromosome regions and chromosomes with both sister chromatids stained uniformly dark
M2 = cells in second division metaphase after start of treatment; each chromosome is differentially stained (with one chromatid darkly stained and its sister chromatid lightly stained)
M3 = cells in third division metaphase after start of treatment; cells contain some differentially stained chromosomes and chromosomes with both sister chromatids stained uniformly lightly.

In this study, after 4 hrs treatment, in the absence as well as in the presence of S9 mix, no biologically relevant cytotoxicity indicated by clearly reduced mitotic indices could be observed. After continuous treatment in the absence of S9 mix, cytotoxicity was observed at the highest evaluated concentration 1257.1 µg/mL (49.2 % of control). Cytotoxicity of R417888 is presented in Table 6.8.1 – 6.4.29-1.

Table 6.8.1 – 6.4.29-1 Cytotoxicity of R417888 in cultures of human lymphocytes

Concentration (µg/mL)	**Exposure time**	**Preparation interval**	**Mitotic cells per 1000 cells***	**% of solvent control**
Without S9 mix				
Solvent control	4 hrs	22 hrs	20.5	100.0
14.3	4 hrs	22 hrs	n.d.	n.d.
25.0	4 hrs	22 hrs	n.d.	n.d.
43.8	4 hrs	22 hrs	n.d.	n.d.
76.6	4 hrs	22 hrs	n.d.	n.d.
134.0	4 hrs	22 hrs	18.6	90.5
234.6	4 hrs	22 hrs	16.3	79.5
410.5	4 hrs	22 hrs	19.6	95.4
718.4	4 hrs	22 hrs	25.0	121.7
1257.1	4 hrs	22 hrs	16.0	77.8
2200.0	4 hrs	22 hrs	14.7	71.7
Solvent control	22 hrs	22 hrs	15.2	100.0
14.3	22 hrs	22 hrs	n.d.	n.d.
25.0	22 hrs	22 hrs	n.d.	n.d.
43.8	22 hrs	22 hrs	n.d.	n.d.
76.6	22 hrs	22 hrs	n.d.	n.d.
134.0	22 hrs	22 hrs	15.6	102.6
234.6	22 hrs	22 hrs	22.4	147.5
410.5	22 hrs	22 hrs	18.7	123.4
718.4	22 hrs	22 hrs	11.5	75.6
1257.1	22 hrs	22 hrs	7.5	49.2
2200.0	22 hrs	22 hrs	1.2	7.6

Concentration (µg/mL)	Exposure time	Preparation interval	Mitotic cells per 1000 cells*	% of solvent control
With S9 mix				
Solvent control	4 hrs	22 hrs	12.1	100.0
14.3	4 hrs	22 hrs	n.d.	n.d.
25.0	4 hrs	22 hrs	n.d.	n.d.
43.8	4 hrs	22 hrs	n.d.	n.d.
76.6	4 hrs	22 hrs	n.d.	n.d.
134.0	4 hrs	22 hrs	13.3	109.9
234.6	4 hrs	22 hrs	15.7	129.3
410.5	4 hrs	22 hrs	15.6	128.5
718.4	4 hrs	22 hrs	14.4	119.0
1257.1	4 hrs	22 hrs	13.1	107.9
2200.0	4 hrs	22 hrs	15.7	129.8

The table (6.8.1 – 6.4.29-2) below shows the occurrence of polyploid metaphases. No biologically relevant increase in the rate of polyploid metaphases was found after treatment with the test item (0.0 - 0.2 %) as compared to the rates of the solvent controls (0.0 – 0.2 %).

Table 6.8.1 – 6.4.29-2 Number of Polyploid Cells and Mitotic Index

Treatment	Conc.	S9	Exposure	Polyploid cells*				Mitotic indices**			
group	per mL	mix	period/	Culture		Total	%	Absolute		Mean	%***
			Recovery	1	2			1	2		
Solv. control#	0.5 %	-	4 / 18 hrs	0	1	1	0.2	20.9	20.1	20.5	100.0
Pos. control##	825.0µg	-	4 / 18 hrs	0	0	0	0.0	12.3	13.4	12.9	62.7
Test item	718.4µg	-	4 / 18 hrs	0	1	1	0.2	26.8	23.1	25.0	121.7
″	1257.1µg	-	4 / 18 hrs	1	0	1	0.2	14.0	17.9	16.0	77.8
″	2200.0µg	-	4 / 18 hrs	0	0	0	0.0	14.4	15.0	14.7	71.7
Solv. control#	0.5 %	+	4 / 18 hrs	0	0	0	0.0	11.7	12.5	12.1	100.0
Pos. control###	37.5 µg	+	4 / 18 hrs	0	0	0	0.0	6.1	3.8	5.0	40.9
Test item	718.4µg	+	4 / 18 hrs	0	0	0	0.0	13.8	15.0	14.4	119.0
″	1257.1µg	+	4 / 18 hrs	0	0	0	0.0	12.7	13.4	13.1	107.9
″	2200.0µg	+	4 / 18 hrs	0	1	1	0.2	16.3	15.1	15.7	129.8
Solv. control#	0.5 %	-	22 / 0 hrs	0	0	0	0.0	15.1	15.2	15.2	100.0
Pos. control##	660.0µg	-	22 / 0 hrs	0	0	0	0.0	6.6	8.8	7.7	50.8
Test item	410.5µg	-	22 / 0 hrs	0	0	0	0.0	18.4	19.0	18.7	123.4
″	718.4µg	-	22 / 0 hrs	1	0	1	0.2	10.5	12.4	11.5	75.6
″	1257.1µg	-	22 / 0 hrs	0	0	0	0.0	8.1	6.8	7.5	49.2

* The number of polyploid cells was determined of each test group in a sample of 250 cells per culture
** The mitotic index was determined in a sample of 1000 cells per culture of each test group in %
*** For the positive control groups and the test item groups, the relative values of the mitotic index are related to the solvent controls
# DMSO
EMS
CPA

In Table 6.8.1 – 6.4.29-3 the scorings for structural chromosome aberrations are given.
In the absence of S9 mix, statistically significant and biologically relevant increases in cells carrying chromosomal aberrations were observed after 4 hrs and 22 hrs treatment with the test item. The values were increased in a dose-dependent manner (2.0 %, 3.5 %, 14.0 %, and 2.5 %, 20.0 %, 58.0 % aberrant cells, excluding gaps, respectively) and at least the highest evaluated concentrations clearly exceeded the range of the laboratory's historical control data of 0.0 – 4.0 % aberrant cells, excluding gaps). Further, the number of cells carrying exchanges was distinctly increased, giving additional evidence for a clastogenic potential.

In the presence of S9 mix, neither a statistically significant nor a biologically relevant increase in cells carrying chromosomal aberrations was observed. The values after treatment with the test item (1.0 –

2.0 % aberrant cells, excluding gaps) were slightly above the value of the solvent control (0.5 % aberrant cells, excluding gaps) and clearly within the range of the laboratory's historical control data of 0.0 – 4.0 % aberrant cells, excluding gaps.

Table 6.8.1 – 6.4.29-3 Summary of structural chromosome aberrations

Preparation interval		Test item concentration in µg/mL	Polyploid cells in %	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	excl. gaps*	with exchanges
Exposure period 4 hrs without S9 mix							
22 hrs		Solvent control[1]	0.2	100.0	1.5	1.5	0.0
		Positive control[2]	0.0	62.7	14.0	**13.5**[s]	3.0
		718.4	0.2	121.7	3.5	2.0	1.0
		1257.1	0.2	77.8	3.5	3.5	0.5
		2200.0	0.0	71.7	14.5	**14.0**[s]	2.5
Exposure period 22 hrs without S9 mix							
22 hrs		Solvent control[1]	0.0	100.0	0.0	0.0	0.0
		Positive control[3]	0.0	50.8	25.0	**23.0**[s]	9.0
		410.5	0.0	123.4	2.5	**2.5**[s]	0.0
		718.4	0.2	75.6	20.5	**20.0**[s]	1.0
		1257.1[#]	0.0	49.2	58.0	**58.0**[s]	4.0
Exposure period 4 hrs with S9 mix							
22 hrs		Solvent control[1]	0.0	100.0	1.5	0.5	0.0
		Positive control[4]	0.0	40.9	22.0	**21.5**[s]	5.5
		718.4	0.0	119.0	1.5	1.0	0.0
		1257.1	0.0	107.9	2.0	2.0	0.5
		2200.0	0.2	129.8	2.0	2.0	0.0

* Including cells carrying exchanges
\# Evaluation of 50 metaphases per culture due to strong clastogenic effects
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 37.5 µg/mL

Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item R417888 induced structural chromosomal aberrations in human lymphocytes *in vitro* in the absence of metabolic activation. With metabolic activation, neither a statistically significant nor a biologically relevant increase in cells carrying chromosomal aberrations was observed.

B.6.8.1 – 6.4.30 Forward mutation assay in mouse lymphoma cells with R417888- study 30

Previous evaluation	In addendum 7 DAR (March 2001)
Evaluation RMS	No remarks on original assessment.

Study design and results

Type of study: L5178Y TK+/- forward mutation assay, with and without metabolic activation

Indicator cells	Endpoint	Res. - act.	Res.+ act.	Activation		Dose range	Reference
				Tissue	Inducer		
L5178Y mouse lymphoma	thymidine kinase deficiency	-	-	rat liver	phenobarbital/beta-naphthoflavone	exp 1: 500-3770 µg/ml (4 h) dimethylsulphoxide (DMSO)	Clay, 2000
		-	-			exp 2a: 500-2000 µg/ml (24 h)	
		-				exp 2b: 500-3770 µg/ml (4 h)	
						exp 3: 1000-2000 µg/ml (24 h)	
Test substance: R417888, 2-amido-3,5,6-trichloro-4-cyanobenzene sulfonic acid, 97%, white solid GLP - statement: yes According to OECD 476: yes							

Conclusions

The test substance was not found to be mutagenic when tested in vitro for forward mutations in the TK locus of L5178Y mouse lymphoma cells, in the presence and absence of a liver homogenate from phenobarbital/beta-naphthoflavone-treated rats.

Guidelines and limitations

The study was carried out in compliance with OECD 476 (1997). The highest dose tested in the first and second experiment (4 hours treatment) represents the limit dose of 10 mM for this type of study. The highest dose in the second experiment clearly affected the viability of the cells: 32-35% survival. Higher doses produced excessive toxicity. Treatment had no effect on pH or osmolality. Ethyl methanesulphonate and benzo(a)pyrene, used as positive controls, confirmed that the test system worked properly.

B.6.8.1 – 6.4.31 *In vitro* cell gene mutation assay with mouse lymphoma cells with SDS-417888 - study 31

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/16 Verspeek-Rip, C.M. (2006), Evaluation of the mutagenic activity of VIS 01 in an in vitro mammalian cell gene mutation test with L5178Y mouse lymphoma cells (with independent repeat), testing facility: Notox B.V, 5231 DD's-Hertogenbosch, The Netherlands. Report No. 402525. Unpublished. (Syngenta File Number R417888_10026)

GUIDELINES: OECD 476 (1997)

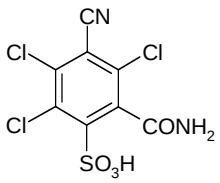
GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The test substance VIS 01 was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and presence of an S9-activation system. Test substance doses ranged from 10-3465 µg/mL in the absence of S9 and 3-100 µg/mL in the presence of S9.

Test Material: VIS 01, 1-cyano-1, 5, 6-trichloro-3-amido-benzene-sulphonate



Description: White powder
Lot/Batch#: Batch 52168-11-16
Purity: 99.5%

Results

The cytotoxicity was determined by counting the cells after exposure and by measuring the relative suspension growth and cloning efficiency of the L5178Y cells 24 and 48 hours after treatment –

compared to negative control and the relative total growth (RTG: product of RSG and the relative colony-forming ability ["cloning efficiency"] 48 hours after treatment). Cell toxicity became obvious at concentrations from 333 µg/mL onwards in S9-activated systems while no cytotoxicity was observed in the absence of S9. Cytotoxicity data are presented in Table 6.8.1 – 6.4.31-1.

Table 6.8.1 – 6.4.31-1: Cytotoxicity of VIS 01 in Mouse Lymphoma L5178Y Cells

Concentration VIS 01 (µg/mL)	% relative survival (relative suspension growth)			
	Absence of S9		Presence of S9	
	Experiment 1	Experiment 2	Experiment 1	Experiment 2
0	100	100	100	100
10	106		98	
33	104		104	
100	108		96	
333	108	99	70	78
1000	78	134	25	42
2000		110		18
3000		96		11

The results of the mutagenicity assay are summarized in Table 6.8.1 – 6.4.23-2.
In the absence of S9 mix VIS 01 did not induce an increase in mutant frequency at 10-3465 µg/mL. In the presence of S9 a dose related mutagenicity was noted at 333-1000 µg/mL. However, pronounced cytotoxicity was observed at these concentrations which may have caused this observation. Both positive control compounds fulfilled the requirements for a valid test.

Table 6.8.1 – 6.4.31-2: Effects of VIS 01 on Gene Mutations at the TK-Locus of Mouse Lymphoma L5178Y Cells in the Absence or Presence of S9-Mix

Concentration SDS-3701 (µg/mL)	Absence of S9-mix		Presence of S9-mix	
	Mutation frequency $x10^6$	Survival (%)	Mutation frequency $x10^6$	Survival (%)
0	109	100	118	100
3	nt	nt	148	101
10	125	113	220	113
33	94	115	175	103
100	106	96	163	81
333	127	122	308	83
560	96	69	624	59
1000	119	135	2096	31
2500	135	142		
3465	127	113		
MMS (15 µg/mL)	**1103**	**74**		
CP (10 µg/mL)			**3385**	**57**

CONCLUSIONS

Based on the overall results of this study it is concluded, that the test substance VIS 01 is mutagenic at the TK-locus of mouse lymphoma L5178Y cells in the presence of rat liver S9 mix at cytotoxic concentrations. No mutagenicity was observed in the absence of metabolic activation in mouse lymphoma L5178Y cells.

B.6.8.1 – 6.4.32 *In vitro* cell gene mutation assay with mouse lymphoma cells with SDS-417888 - study 32

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

	In the PPR Expert meeting (162 – session 2, September 2017), the results of this study were discussed and it was concluded that the response is considered equivocal after metabolic activation.

Report:	K-CA 5.8.1/22 Wollny H.E. (2007). Cell Mutation Assay at the Thymidine-Kinase Locus (TK+/-) in Mouse Lyphoma L5178Y Cells with R417888. RCC-CCR, Rossdorf, Germany. Unpublished report number 1030809. (Syngenta File Number R417888_10021)

GUIDELINES: Ninth Addendum to the OECD Guidelines for the Testing of Chemicals, February 1998, adopted July 21, 1997, Guideline No. 476 "In vitro Mammalian Cell Gene Mutation Test"
Commission Directive 2000/32/EC, L1362000, Annex 4E, dated May 19, 2000
GLP: This study was performed in accordance with the principles of Good Laboratory Practises.
Acceptability: The study was performed in accordance with OECD guideline 476. There were no deviations from the guideline considered to compromise the scientific validity of the study.

Study design
The test substance R417888 was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and presence of an S9-activation system. The highest concentration used in the pre-test was chosen with regard to the solubility of the test item in DMSO. Test item concentrations between 18.0 and 2300 µg/mL were used to evaluate toxicity in the presence (4 h treatment) and absence (4 h and 24 h treatment) of metabolic activation. The assay was performed in two independent experiments, using two parallel cultures each. Pulse treatment for 4 h was used in the first experiment with and without metabolic activation and in the second experiment with metabolic activation. Continuous treatment for 24 h was used in the second experiment without metabolic activation.
The main experiments were evaluated at the following concentrations:

Experiment I:
without S9 mix: 17.5; 35.0; 70.0; 105; and 140 µg/mL
with S9 mix: 600; 900; 1200; 1800; and 2300 µg/mL
Experiment II:
without S9 mix: 18.0; 36.0; 72.0; 144.0; and 216.0 µg/mL
with S9 mix: 75; 150; 300; 600; and 900 µg/mL

Test Material:	R417888
Description:	Hygroscopic solid
Lot/Batch#:	BJQ-impurity 329-001
Purity:	95.0%
Stability of the compound:	To be stored at room temperature, protected from light and moisture
Expiry date:	December 5, 2008
Solvent used:	DMSO (E.MERCK, D-64293 Darmstadt; purity 99.5%. The final concentration of DMSO in the culture medium was 0.5% (v/v)
Control Materials:	

Negative:	Tissue culture medium
Solvent:	DMSO
Positive - S9:	Methylmethane sulfonate (MMS), 13.0 to 19.5 µg/ml,
+ S9:	Cyclophosphamide (CPA), 3 to 6.0 µg/ml

Results

In the main experiments no precipitation occurred up to the maximum concentration with and without metabolic activation. In experiment I a slight turbidity was noted at the two highest concentration with metabolic activation.

Relevant toxic effects indicated by a relative cloning efficiency 1 or a relative total growth of less than 50 % of survival were observed in experiment I at 70 µg/mL and above in the absence and at 1800 µg/mL and above in the presence of metabolic activation following 4 h treatment. In experiment II toxicity as described above occurred at 216 µg/ml and above. The recommended toxic range of approximately 10 – 20 % of cloning efficiency 1 or relative total growth was covered with and without metabolic activation.

The data generated at 2300 µg/mL in experiment I with metabolic activation (culture I) were not considered valid based on exceedingly severe toxic effects (both parameters of toxicity fell below the 10 % limit). The striking difference in toxicity with and without metabolic activation and in between the two experiments with metabolic activation may indicate binding capacity of the test item to proteins or lipids present in the S9 fraction. Protein binding effects are further supported by the fact that higher concentrations are tolerated in the 24 h treatment compared to pulse treatment for 4 h. During long term exposure the concentration of horse serum is 15 % compared to 3 % during pulse treatment.

In the current study no substantial and reproducible dose dependent increase of the mutation frequency was observed up to the maximum concentration with and without metabolic activation. The mutation frequency exceeded the threshold of 126 above the corresponding solvent control at 1200 and 1800 µg/mL in the first culture of the first experiment with metabolic activation and at 216.0 µg/mL in the second culture of the second experiment without metabolic activation. However, both effects were judged as not reproduced and thus biologically irrelevant since no comparable effect occurred in the parallel cultures under identical conditions.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using SYSTAT® statistics software. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in all experimental parts. However, according to the IWGT recommendations, a significant trend is no criterion of a mutagenic result as long as the mutation frequencies do not exceed the historical control range (mean values of both parallel cultures). Therefore, the complete set of data is considered non-mutagenic.

In this study the range of the negative and solvent controls was from 38 up to 123 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 47 up to 215 mutant colonies per 10^6 cells.

MMS (19.5 µg/mL in experiment I and II, and 13 µg/mL in experiment II) and CPA (3.0 in experiment I and 3.0 and 4.8 µg/mL in experiment II) were used as positive controls and showed a distinct increase in induced total mutant colonies and an increase of the relative quantity of small versus large induced colonies. The positive control at 19.5 µg/mL in the second experiment without metabolic activation was

not considered valid since toxicity was exceedingly severe. The positive control at 13.0 µg/mL was valid since the relative cloning efficiency 1 or the relative total growth was at least 10 %. In Table 6.8.1 – 6.4.32-1 a summary of the results is presented.

Table 6.8.1 – 6.4.32-1 Summary of the Cell Mutation Assay

			relative	relative	mutant		relative	relative	mutant	
	conc. µg	S9	cloning	total	colonies/		cloning	total	colonies/	
	per mL	mix	efficiency 1	growth	10^6 cells	threshold	efficiency 1	growth	10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Neg. control with medium		-	100.0	100.0	105		100.0	100.0	117	
Solv. control with DMSO		-	100.0	100.0	65	191	100.0	100.0	64	190
Pos. control with MMS	19.5	-	78.9	41.7	271	191	38.2	24.8	294	190
Test item	8.8	-	79.7	culture was not continued#			135.1	culture was not continued#		
Test item	17.5	-	129.1	115.1	72	191	139.5	156.8	74	190
Test item	35.0	-	57.1	63.1	61	191	95.8	100.9	75	190
Test item	70.0	-	47.5	43.3	70	191	62.4	37.9	74	190
Test item	105.0	-	27.2	21.1	100	191	22.6	34.8	77	190
Test item	140.0	-	12.3	8.8	120	191	19.2	7.7	167	190
Test item	210.0	-	2.4	culture was not continued##			5.0	culture was not continued##		
Test item	280.0	-	0.0	culture was not continued##			0.9	culture was not continued##		
Neg. control with medium		+	100.0	100.0	38		100.0	100.0	61	
Solv. control with DMSO		+	100.0	100.0	49	175	100.0	100.0	105	231
Pos. control with CPA	3.0	+	59.3	30.3	234	175	64.5	37.5	388	231
Test item	75.0	+	104.7	culture was not continued#			114.6	culture was not continued#		
Test item	150.0	+	109.9	culture was not continued#			90.1	culture was not continued#		
Test item	300.0	+	90.1	culture was not continued#			85.7	culture was not continued#		
Test item	600.0	+	95.8	157.9	47	175	87.1	116.8	77	231
Test item	900.0	+	66.2	119.7	58	175	87.1	97.1	87	231
Test item	1200.0	+	53.7	33.2	185	175	54.1	73.6	79	231
Test item	1800.0	+	31.6	17.6	215	175	46.3	45.7	83	231
Test item	2300.0	+	**7.2**	**2.6**	**303**	**175**	24.4	27.7	146	231
Experiment II / 24 h treatment			culture I				culture II			
Neg. control with medium		-	100.0	100.0	96		100.0	100.0	123	
Solv. control with DMSO		-	100.0	100.0	100	226	100.0	100.0	72	198
Pos. control with MMS	13.0	-	11.4	6.3	951	226	3.0	10.6	1119	198
Pos. control with MMS	19.5	-	2.4	0.7	1805	226	1.5	0.4	7589	198
Test item	18.0	-	150.2	122.5	63	226	164.6	77.4	117	198
Test item	36.0	-	170.7	155.2	70	226	161.5	64.0	89	198
Test item	72.0	-	80.7	107.1	153	226	129.2	104.3	57	198
Test item	144.0	-	53.1	84.1	142	226	95.8	84.5	91	198
Test item	216.0	-	63.5	33.9	152	226	45.1	20.6	207	198
Test item	288.0	-	9.5	culture was not continued##			11.0	culture was not continued##		
Experiment II / 4 h treatment			culture I				culture II			
Neg. control with medium		+	100.0	100.0	48		100.0	100.0	49	
Solv. control with DMSO		+	100.0	100.0	87	213	100.0	100.0	94	220
Pos. control with CPA	3.0	+	33.7	25.5	160	213	49.3	32.3	204	220
Pos. control with CPA	4.8	+	14.5	5.7	302	213	20.3	7.5	555	220
Test item	75.0	+	92.4	49.3	47	213	108.1	127.6	71	220
Test item	150.0	+	91.0	62.0	102	213	91.4	55.4	179	220
Test item	300.0	+	80.8	79.8	68	213	91.4	102.3	87	220
Test item	600.0	+	50.4	53.3	70	213	53.0	68.6	74	220
Test item	900.0	+	19.8	12.9	109	213	22.6	19.7	202	220
Test item	1200.0	+	17.2	culture was not continued##			14.5	culture was not continued##		
Test item	1800.0	+	5.1	culture was not continued##			8.1	culture was not continued##		

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126

\# culture was not continued since a minimum of four concentrations is required by the guidelines

\## not determined, culture not continued due to exceedingly strong toxic effects

The values printed in bold are judged as invalid, since the acceptance criteria (page 20) are not met.

Conclusions

In conclusion it can be stated that under the experimental conditions reported the test item 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.

Data requirement 2.42a

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse lymphoma assay. The results will be discussed in an experts' meeting.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the Expert meeting, it was concluded that the response should be considered equivocal after metabolic activation and negative without metabolic activitation. Additional information is added below.

Response by notifier to the above data requirement:
"4 Hour exposure in the presence of metabolic activation Culture I: Mutant frequency (MF) increases obtained at 2300 µg/mL in Culture I are considered invalid and non-biologically relevant as the culture cytotoxicity exceeds acceptable limits (minimum RTG of 10%). In the parallel culture, at an acceptable cytotoxicity level, no relevant increase in induced MF was observed. This indicates the observed increase in MF in Culture I is cytotoxicity driven and as such may be considered biologically not relevant.
At concentrations of 1200 and 1800 µg/mL, with acceptable cytotoxicity, increases above the GEF were noted in Culture I. These however, were not seen in the parallel culture under the same treatment conditions and are therefore considered non-reproducible and to not meet the criteria for a positive response. Additionally the mean MF between Cultures I and II at these concentrations is respectively 132 and 149, well below the GEF threshold based on mean culture data of 203. Hence, and additionally, on this basis the isolated increases in MF in Culture I are considered not biologically relevant.

24 Hour treatment in the absence of metabolic activation: An isolated increase in MF was observed in Culture II at 216 µg/mL. This was marginally above the GEF but this increase was not observed in the parallel culture under the same conditions and further the mean Culture I and II MF data (180) are below the mean GEF (212). Hence this isolated increase is considered to be not biologically relevant.

In conclusion the isolated increases in MF observed are not considered to show any indication of a positive response in the assay and R417888 is considered to be negative in the *in vitro* mammalian gene mutation assay conducted in L5178y TK+/- cells. "

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
In the Expert meeting the results of this study were discussed. In culture 1, the dose-related increased MF from 1200 µg/mL (> GEF) with S9 was not reproduced in Culture 2 or in Experiment II, however, the cultures at 1200 µg/mL and 1800 µg/mL were not continued. Based on these findings it was concluded that the response should be considered equivocal after metabolic activation and negative without metabolic activitation.

Genotoxicity R417888 – in vivo

B.6.8.1 – 6.4.33a Micronucleus test in mouse with R417888- study 33

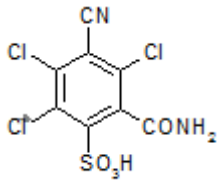
Previous evaluation	Submitted for the purpose of renewal, new data
Evaluation RMS	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/17 Meerts; Ir.I.A.T.M. (2005), Micronucleus test in bone marrow cells of the mouse with VIS 01, testing facility: Notox B.V, 5231 DD's-Hertogenbosch, The Netherlands. Report No. 402514. Unpublished. (Syngenta File Number R417888_10024)

GUIDELINES: OECD 474 (1997)
GLP: This study was performed in accordance with the principles of Good Laboratory Practices.
Acceptability: The study was performed in accordance with OECD guideline 474 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design
Following a dose finding study, NMRI mice (groups of 5 males) were treated by gavage at a dose level of 2000 mg VIS 01 per kg bodyweight. A negative control group was treated with the vehicle, a positive control group received Cyclophosphamide (50 mg/kg bw).
Twentyfour or 48 hours after dosing, animals were killed by cervical dislocation and bone marrow smears were analysed microscopically by counting micronuclei in 2000 polychromatic erythrocytes per animal.

Test Material: VIS 01, 1-cyano-1, 5, 6-trichloro-3-amido-benzene-sulphonate



Description: White powder

Lot/Batch#: Batch 52168-11-16
Purity: 99.5%
Vehicle and/or positive control: Physiological saline, Cyclophosphamide 50 mg/kg bw

Results

The mean micronucleated cell count for all dose groups of VIS 01 were essentially comparable with the concurrent vehicle control group, at any of the three sampling times.
Cyclophosphamide caused large significant increases in the frequency of micronucleated polychromatic erythrocytes. The ratio of normochromatic to polychromatic erythrocytes was not influenced. The resuls of the micronucleus test are given in Table 6.8.1 – 6.4.33-1.

Table 6.8.1 – 6.4.33-1: Summary of Micronucleus Results in Mice

Treatment	Sampling time	Micronuclei per 2000 normochromatic erythrocytes	Ratio polychromatic to normochromatic erythrocytes
Solvent	**24**	0.8	0.98
2000 mg/kg bw VIS 01	**24**	1.0	0.96
2000 mg/kg bw VIS 01	**48**	0.8	0.98
50mg/kg bw cyclophosphamide	**48**	**22.0***	**0.59***

*p<0.01

Conclusions

VIS 01 did not induce micronucleated polychromatic erythrocytes at a dose of 2000 mg per kg bw. Validity of the test performed was shown with a cyclophosphamide treated positive control group with a marked response.

B.6.8.1 – 6.4.33b Proof of exposure for in vivo micronucleus test in mouse with R417888 (SDS-46851)

Data requirement 2.27

Applicant to provide evidence of tissue exposure in the in vivo MN study with R417888 – study 33.

See also 2(89, 102)
See reporting table 2(73)

NL (August 2017): The applicant provided a proof of exposure study in the mouse to support the mouse micronucleus assay (Buskens, 2004a). This study is shown below. The RMS agrees with the conclusion that R417888 is systemically available and thus may reach the bone marrow.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the Expert meeting, the proof of bone marrow exposure in the mouse micronucleus assay was discussed. In this assay, no bone marrow toxicity was evident. Bone marrow exposure was demonstrated by detectable blood and plasma concentrations of R417888 in male Crl:CD-1 mice with circulating concentrations in plasma ≥ 8 µg/mL, though at 24 hours no exposure was detectable.

Based on the presence of the metabolite in blood and plasma at 4 hours post dosing, no concern for clastogenicity exists. However, no conclusion can be drawn on aneugenicity and therefore a data gap was recognized.

Report:	K-CA 5.8.1/30 Dunton, J. (2016) R417888 - Oral (Gavage) Proof of Exposure Study in the Mouse. Sequani Limited, Bromyard Road, Ledbury, Herefordshire, HR8 1LH, United Kingdom. Laboratory Report No. BFI0571. Issue date: February 2017. Unpublished. (Syngenta File No. R417888_10064).

STUDY TYPE: Proof of exposure to support a mouse micronucleus test

GLP: A signed and dated GLP statement was provided.

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

TEST MATERIAL (PURITY): R417888 (90.0 %)

SYNONYMS: CSCC890840, SDS-46851

SPONSOR: Syngenta Ltd, Jealott's Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom. Oxon Italia S.p.A., Via Sempione 195, 20016 Pero, Milan, Italy. Arysta LifeScience SAS, BP 80 – Route d-Artix, 64150 Noguères, France.

EXECUTIVE SUMMARY

The purpose of this study was to demonstrate proof of exposure in the mouse after oral (gavage) administration of R417888 using a validated method in plasma (1) and blood (2). A proof of exposure experiment was required to support a previously conducted regulatory mouse micronucleus study (3) in order to demonstrate that the bone marrow was exposed to R417888. The study design was chosen to use the same species, route of exposure and vehicle, as well as the same concentration as used in the top dose of the *in vivo* micronucleus study.

In a Crl:CD-1 mouse proof of exposure study, three males were dosed once by oral gavage with R417888 at 2000 mg/kg bw. Blood samples were taken 1, 4 and 24 hours after dosing. Both blood and plasma were analysed for the presence of R417888. The vehicle was 0.9 % (w/v) aqueous sodium chloride. There were no deaths and no clinical signs were observed throughout the study. All animals were killed and discarded following their final blood sample. Exposure to R417888 was confirmed quantitatively in all plasma samples and qualitatively in all blood samples taken at 1 and 4 hours after dosing.

In conclusion, systemic exposure to R417888 was demonstrated in male Crl:CD-1 mice following a single oral (gavage) administration of R417888 at 2000 mg/kg, which was well tolerated in all animals. Proof of exposure was demonstrated by detectable blood and plasma concentrations of R417888 in male Crl:CD-1 mice with circulating concentrations in plasma ≥ 8 µg/mL.

MATERIALS AND METHODS

Materials:

Test Material: R417888

Description: White to off-white powder

Lot/Batch number: KI-7232/7

Purity: 90.0 %, concentration was adjusted for purity

CAS#: Not available

Stability of test compound: 31 July 2017

Control Materials:

Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	0.9 % (w/v) aqueous sodium chloride	**Final Volume:** 10 mL/kg	**Route:** oral
Positive control :	N/A	**Final Doses:** N/A	**Route:** N/A

Test Animals:

Species	Mouse
Strain	Crl:CD-1
Age/weight at dosing	Six to seven weeks/29 to 33 g
Source	Charles River (UK) Limited, Margate, Kent, CT9 4LT, England.
Housing	3/cage
Acclimatisation period	5 days
Diet	*ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 19-23°C Humidity: 40-70% Photoperiod: 12 hours dark/12 hours light

Test compound administration:

Dose Level	**Final Volume**	**Route**
2000 mg/kg bw	10 mL/kg	Oral gavage

Study Design and Methods:

In-life dates: 06 October 2016 (animal arrival); 11 October 2016 (start of dosing)

Experimental Design

Group	Number of males	Dose level (mg/kg) R417888	Dose concentration (mg/mL)
1	3	2000	200

Animals were examined twice daily for mortality and morbidity and were given a detailed clinical examination. On the day of dosing, animals were observed before, shortly after and about 1, 4 and 24 hours after dosing. All animals were weighed on the day of dosing and were killed by exposure to carbon dioxide gas in a rising concentration following their final blood sample and discarded.

Blood sampling: Blood samples (100 µL) were taken from the tail vein, into tubes containing K_2EDTA anticoagulant. All animals were sampled 1, 4 and 24 hours after dosing. Immediately following collection of each sample, 20 µL of whole blood was accurately measured into a polypropylene tube containing 60 µL of acidified acetonitrile (1 % v/v formic acid in acetonitrile) (1:3 - whole blood:acidified acetonitrile). The samples were thoroughly mixed and then frozen on dry ice.
The residual blood was placed on a roller to mix and then held in ice until centrifuged (3000 g, 5 minutes, at approximately 4 °C) to derive plasma. Where possible, 30 µL of the plasma was aliquoted into tubes containing 90 µL of acidified acetonitrile (1 % v/v formic acid in acetonitrile)
(1:3 - plasma:acidified acetonitrile) within 30 minutes of sampling. Insufficient plasma was derived at the 4 hour timepoint for all animals and the 24 hour timepoint for Animals 4 and 6. For these samples, 20 µL of the resultant plasma was aliquoted into tubes containing 60 µL of acidified acetonitrile within 30 minutes of sampling. All samples were stored frozen (<- 70 °C) before being shipped on dry ice to the Principal Investigator for analysis.

Bioanalysis: Concentrations of R417888 in plasma:acidified acetonitrile were determined using a validated LC-MS/MS method.

RESULTS AND DISCUSSION

There were no deaths and no clinical signs were observed throughout the study.
Exposure to R417888 was confirmed quantitatively in all plasma and qualitatively in all blood samples taken 1 and 4 hours after dosing. Concentrations were measured in plasma at 1 and 4 hours post-dose and were ≥ 8 µg/mL. The 24 hour samples were below the lower limit of quantification for both blood and plasma.

Since bone marrow is well perfused, exposure of the marrow to the test item was indirectly demonstrated by the presence of measurable quantities of test item in the blood.

Table B.6.8.1 – 6.4.33b-1: Sample Results for R417888 in Mouse Plasma

Animal Number	Time point (hour)	Concentration (ng/mL)
4	1	12000
4	4	8000
4	24	BLQ
5	1	20200
5	4	9920
5	24	BLQ
6	1	17600
6	4	10800
6	24	BLQ

Note: all concentrations values are per mL of plasma

BLQ – Below the lower limit of quantification (LLOQ)

LLOQ was 80 ng/mL in plasma

Table B.6.8.1 – 6.4.33b-2: Sample Results for R417888 in Mouse Blood

Animal Number	Time point (hour)	Concentration (ng/mL)
4	1	8960
4	4	5360
4	24	BLQ
5	1	11100
5	4	5320
5	24	BLQ
6	1	10500
6	4	4080
6	24	BLQ

Note: all concentrations values are per mL of plasma

BLQ – Below the lower limit of quantification (LLOQ)

LLOQ was 80 ng/mL in blood

CONCLUSIONS: Systemic exposure to R417888 was demonstrated in male Crl:CD-1 mice following a single oral (gavage) administration of R417888 at 2000 mg/kg, which was well tolerated in all animals. Proof of exposure was demonstrated by detectable blood and plasma concentrations of R417888 in male Crl:CD-1 mice with circulating concentrations in plasma ≥ 8 µg/mL.

(Dunton, J. 2017)

B.6.8.1 – 6.4.34 In vivo UDS test with R417888- study 34

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Data requirement 2.28

Applicant to provide a technical position on the genotoxic potential of metabolite R417888 – study 34.

See experts' consultation in 2(91)
See reporting table 2(74)

NL (August 2017): The notifier did not provide a further argumentation. In three in vitro gene mutation asays a negative result was found. In a fourth study, it was concluded that R417888 is mutagenic at the TK-locus of mouse lymphoma L5178Y cells in the presence of metabolic activation, but not in the absence of metabolic activation. No acceptable in vivo follow up study is available for the positive response in one of the four in vitro gene mutations assays. However, as all bacterial reverse mutation assays and the other three mammalian gene mutation assays scored negative at similar and higher concentrations, R417888 is based on the weight of evidence considered non-mutagenic.

Report:	K-CA 5.8.1/18 Honarvar, N. (2006) In vivo unscheduled DNA synthesis in rat hepatocytes with VIS 01, facility: RCC Cytotest Cell Research GmbH, Rossdorf, Germany. Report No.921400. Unpublished. (Syngenta File Number R417888_10028)

GUIDELINES: OECD 486 (1997)

GLP: This study was performed in accordance with the principles of Good Laboratory Practices.

Acceptability: The study was performed in accordance with OECD guideline 486 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

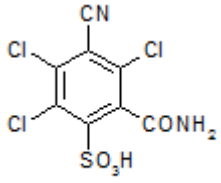
VIS 01 was tested in an unscheduled DNA Synthesis Assay in rat primary hepatocytes in vivo at dose levels of 1000 and 2000 mg/kg bw (4 males per dose level). Doses were based on a dose-range finding study with 2 animals per sex. A vehicle control and two positive controls were included in the main study. Hepatocytes were isolated 2 h or 16 h following treatment.

At least three cultures were established from each animal using Williams E medium supplemented with L-glutamine, insulin and fetal calf serum. Cells were allowed to attach for 1.5 h, fresh medium with ^{3}H-TdR (5µCi/mL) added for 4h and finally the cells were fixed autoradiography was performed for 14 d. The number of silver grains was counted automatically. At least two slides per animal and 50 cells per slide were evaluated.

Test Material: VIS 01, 1-cyano-1, 5, 6-trichloro-3-amido-benzene-sulphonate

Description: White powder
Lot/Batch#: Batch 52168-11-16

Purity: 99.5%



Vehicle and/or positive control: Polyethylene glycol 400 (PEG 400) N,N'-Dimethylhyrdazine dihydrochloride (DMH) and 2-Acetylamineoflurorene (AAF)

Results

In the main experiment, animals treated with 1000 or 2000 mg/kg bw showed ruffled fur 1- 4h following treatment. These symptoms subsided by 16 h after dosing. In Table 6.8.1 – 6.4.34-1, the results of the UDS test are given.

Table 6.8.1 – 6.4.34-1: Effect of VIS 01 on Unscheduled DNA Synthesis

Test substance	Dose level (mg/kg bw)	Preparation interval 2h		Preparation interval 16h	
		Viability (range %)	Cells in repair (mean,%)	Viability (range %)	Cells in repair (mean,%)
PEG 400	**10 mL/kg bw**	77-80	2	81-82	4
VIS 01	**1000**	81-83	4	76-78	1
	2000	81-86	3	71-76	5
DMH	**80**	72-78	37		
2-AAF	**80**			71-79	51

DMH Dimethylhydrazine hydrochloride
2-AAF: 2-Acetylaminofluorene

Conclusions

The results indicate that VIS 01 does not cause a significant increase in the mean number of incorporated ^{3}H-thymidine at dose levels of 1000 and 2000 mg/kg bw at preparation intervals of 2h or 16 h. Therefore, it is concluded that the test substance is considered negative in the UDS in vivo rat hepatocyte assay no DNA damage in hepatocytes was induced.

Genotoxicity SYN548708 – in vitro

B.6.8.1 – 6.4.35 Reverse mutation assay in bacteria with SYN548708 (R418503) - study 35

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/23 Sokolowski A (2015a) SYN548708 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1677301, issue date: 09 March 2015. Unpublished Syngenta File No. SYN548708_10000.

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.

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

Study design
This study was performed to investigate the potential of SYN548708 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 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:
Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II: 33; 100; 333; 1000; 2500; and 5000 µg/plate

Test Material:	SYN548708
Description:	White, solid
Lot/Batch number:	MES 373/1
Purity:	99.0 % (w/w)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	Recertification Date: 31 December 2016

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)

Results
The plates incubated with the test substance showed normal background growth in both experiments with and without metabolic activation
No cytotoxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in any of the bacterial strains tested either with or without metabolic activation.
No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548708 at any concentration, either in the presence or 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. They showed a distinct increase of induced revertant colonies.
The results of the Ames test are given in Table 6.8.1 – 6.4.35-1 and -2.

Table 6.8.1 – 6.4.35-1 Summary of results of experiment I

Study Name: 1677301
Experiment: 1677301 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1677301
Date Plated: 27/01/2015
Date Counted: 30/01/2015

Metabolic Activation	Test Group	Concentration Level (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	Deionised water		16 ± 4	11 ± 2	26 ± 9	180 ± 11	241 ± 11	378 ± 7
	Untreated		23 ± 2	13 ± 1	22 ± 3	195 ± 8	235 ± 17	361 ± 12
	SYN548708	3 µg	16 ± 2	12 ± 3	27 ± 8	181 ± 7	249 ± 11	385 ± 1
		10 µg	16 ± 1	12 ± 2	26 ± 3	198 ± 30	231 ± 11	353 ± 100
		33 µg	16 ± 4	12 ± 3	18 ± 1	186 ± 7	240 ± 21	340 ± 33
		100 µg	15 ± 4	10 ± 2	21 ± 3	193 ± 23	236 ± 44	379 ± 8
		333 µg	16 ± 4	11 ± 2	26 ± 6	205 ± 10	239 ± 12	328 ± 22
		1000 µg	17 ± 2	10 ± 3	19 ± 6	202 ± 11	206 ± 17	346 ± 43
		2500 µg	18 ± 5	10 ± 2	17 ± 2	205 ± 30	215 ± 32	360 ± 24
		5000 µg	18 ± 4	12 ± 4	23 ± 3	210 ± 16	220 ± 13	374 ± 34
	NaN3	10 µg	1372 ± 56			2238 ± 168		
	4-NOPD	10 µg			331 ± 15			
	4-NOPD	50 µg		73 ± 13				
	MMS	2.0 µL					4801 ± 340	4087 ± 739
With Activation	Deionised water		17 ± 5	13 ± 4	24 ± 8	199 ± 8	256 ± 12	399 ± 33
	Untreated		11 ± 4	13 ± 4	30 ± 8	195 ± 28	289 ± 11	401 ± 16
	SYN548708	3 µg	15 ± 4	13 ± 3	23 ± 7	198 ± 14	283 ± 18	409 ± 19
		10 µg	15 ± 3	14 ± 2	29 ± 2	189 ± 15	281 ± 10	412 ± 34
		33 µg	15 ± 2	11 ± 2	29 ± 4	198 ± 4	249 ± 38	432 ± 40
		100 µg	15 ± 3	15 ± 4	32 ± 4	185 ± 1	282 ± 25	422 ± 20
		333 µg	15 ± 3	15 ± 2	27 ± 5	188 ± 16	297 ± 67	403 ± 54
		1000 µg	13 ± 1	10 ± 2	31 ± 7	178 ± 38	237 ± 21	409 ± 16
		2500 µg	19 ± 3	9 ± 0	31 ± 8	201 ± 3	274 ± 24	422 ± 9
		5000 µg	16 ± 1	6 ± 1	31 ± 8	185 ± 14	256 ± 20	422 ± 19
	2-AA	2.5 µg	487 ± 34	127 ± 26	4017 ± 385	3740 ± 488		
	2-AA	10.0 µg					1498 ± 155	2270 ± 163

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 – 6.4.35-2 Summary of results of experiment II

Study Name: 1677301
Experiment: 1677301 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1677301
Date Plated: 10/02/2015
Date Counted: 13/02/2015

Metabolic Activation	Test Group	Concentration Level (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA 1535	TA 1537	TA 98	TA 100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	Deionised water		10 ± 2	12 ± 2	26 ± 6	181 ± 8	210 ± 4	392 ± 20
	Untreated		11 ± 4	9 ± 1	30 ± 10	201 ± 19	219 ± 7	379 ± 44
	SYN548708	33 µg	12 ± 2	12 ± 2	26 ± 3	225 ± 7	220 ± 8	388 ± 32
		100 µg	10 ± 2	10 ± 1	28 ± 8	228 ± 3	210 ± 14	353 ± 14
		333 µg	10 ± 3	10 ± 2	33 ± 6	206 ± 18	208 ± 15	362 ± 41
		1000 µg	12 ± 4	10 ± 0	29 ± 5	231 ± 3	208 ± 12	392 ± 25
		2500 µg	10 ± 0	11 ± 2	24 ± 2	220 ± 14	214 ± 22	352 ± 14
		5000 µg	8 ± 3	10 ± 3	24 ± 5	219 ± 8	213 ± 6	338 ± 25
	NaN3	10 µg	1253 ± 101			2111 ± 26		
	4-NOPD	10 µg			328 ± 21			
	4-NOPD	50 µg		79 ± 6				
	MMS	2.0 µL					3942 ± 216	3219 ± 141
With Activation	Deionised water		12 ± 3	11 ± 2	33 ± 9	200 ± 3	259 ± 24	441 ± 13
	Untreated		11 ± 2	21 ± 3	34 ± 10	201 ± 13	246 ± 16	426 ± 36
	SYN548708	33 µg	10 ± 1	10 ± 2	36 ± 2	224 ± 5	256 ± 8	440 ± 33
		100 µg	13 ± 1	13 ± 1	32 ± 12	228 ± 7	244 ± 8	447 ± 28
		333 µg	9 ± 1	10 ± 1	33 ± 15	202 ± 21	238 ± 23	426 ± 44
		1000 µg	12 ± 3	11 ± 3	33 ± 9	220 ± 6	237 ± 25	427 ± 16
		2500 µg	12 ± 4	13 ± 4	30 ± 3	210 ± 19	264 ± 7	445 ± 43
		5000 µg	13 ± 4	13 ± 4	34 ± 6	201 ± 24	253 ± 8	407 ± 8
	2-AA	2.5 µg	357 ± 41	171 ± 18	4196 ± 1080	3995 ± 98		
	2-AA	10.0 µg					1173 ± 120	2135 ± 151

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusion

During the described mutagenicity tests and under the experimental conditions reported, SYN548708 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN548708 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.36 In vitro chromosome aberration assay with SYN548708 (R418503) - study 36

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/24 Sokolowski, A. (2015a) SYN548708 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1677302, issue date: 30 July 2015. Unpublished. Syngenta File No. SYN548708_10004

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of SYN548708 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/β-naphthoflavone treated male rats).

In each treatment group two parallel cultures were analysed. At least 150 metaphases per culture were evaluated for structural chromosomal aberrations. 1000 cells per culture were counted for determination of mitotic index.

The highest applied concentration in this study (2000.0 µg/mL of the test substance) was chosen with respect to the current OECD Guideline 473 (2014). Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473 (2014).

Materials:

Test Material:	SYN548708
Description:	White solid
Lot/Batch number:	MES 373/1
Purity:	99 % w/w
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	Deionised water
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 770 µg/mL Presence of S9 mix: Cyclophosphamide 2.5 µg/mL

Absence of S9 mix	Experiment I	213.2, 373.2, 653.1 µg/mL
Presence of S9 mix	Experiment I	121.9, 213.2, 373.2 µg/mL

Results

In Experiment I, the exposure period was 4 hours with and without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance.

Since the test substance was considered to induce a significant increase in the frequency of cells with aberrations at one dose level, after 4 hours treatment in the absence of S9 mix, the second experiment was not evaluated for chromosome aberrations. Since the outcome of the first experiment is clear positive, the second experiment is not reported (performance and mitotic index) but the data are kept with the raw data and therefore archived.

The highest treatment concentration in this study, 2000.0 µg/mL was chosen with respect to the current OECD Guideline 473 (2014). No precipitation of the test substance in the culture medium was observed at the end of treatment.

The osmolarity and pH, measured with osmometer and pH meter were determined in the solvent control and the maximum concentration without metabolic activation: No relevant influence on the osmolarity or pH was observed.

In the absence and presence of S9 mix, concentrations showing clear cytotoxicity were not evaluable due to insufficient metaphases to score because the cells were in various stages of the cell cycle, even though only minor reductions of the MI were observed.

In the absence of S9 mix, one statistically significant increase in chromosomal aberrations was observed after treatment with 653.1 µg/mL (11.7 % aberrant cells, excluding gaps). In the presence of S9 mix, one statistically significant increase was observed after treatment with 373.2 µg/mL (4.8 % aberrant cells, excluding gaps). Both values clearly exceeded the two-fold standard deviation 95% control limit (2.6 % aberrant cells, excluding gaps) as well as the range of the laboratory historical solvent control data (without S9 mix: 0.0 – 3.0 % aberrant cells, excluding gaps; with S9 mix: 0.0 – 3.5 % aberrant cells, excluding gaps). However, the increase in chromosomal aberrations observed in Culture 1 in the presence of S9 mix was not reproduced in the second culture, even after the evaluation of a further 300 cells (7.0 versus 2.7 percent aberrant cells). The observation at the maximum dose level in the absence of S9-mix was of the ‘hockey-stick’ type, often associated with false positive responses. Therefore, this observation should be considered with caution, particularly as there was no correlation with the toxicity results (MI data).

Based on the shape of the dose response discussed above and following statistical advice it was considered a formal trend test was not appropriate in this instance. Since the test substance was considered to be clastogenic after 4 hours treatment in the absence of S9 mix the second experiment

was not evaluated for chromosome aberrations. 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 µg/mL) or CPA (2.5 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

Table 6.8.1 – 6.4.36-1 Summary of results of the chromosome aberration study with SYN 548708

Exp.	Preparation interval (h)	Test item concentration in µg/mL	Mitotic indices (% of control)	Aberrant cells (%)		
				incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	2.7	2.7	0.3
		Positive control[2]	231.2	13.0	**13.0**[S]	2.7
		213.2	107.3	1.7	1.7	0.0
		373.2	74.3	2.0	2.0	0.0
		653.1	89.0	11.7	**11.7**[S]	0.7
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	3.0	2.7	0.0
		Positive control[3]	48.8	8.3	**8.0**[S]	0.3
		121.9	70.0	3.3	2.7	0.0
		213.2	61.3	3.3	3.3	0.0
		373.2[#]	75.6	5.0	**4.8**[S]	0.5

* Including cells carrying exchanges
\# Evaluation of 300 metaphases per culture
S Aberration frequency statistically significant higher than corresponding control values
1 Deionised water 10.0 % (v/v)
2 EMS 770.0 µg/mL
3 CPA 2.5 µg/mL

Conclusion

In an in vitro chromosome aberration study with human lymphocytes SYN548708 induced structural chromosomal aberrations in human lymphocytes *in vitro* at the maximum dose level in the absence and presence of a metabolic activation system. The aberration frequency at the highest concentration was statistically significant increased and outside the laboratory historical solvent control data. However, there was no correlation with cytotoxicity and therefore, SYN548708 is considered to induce increased frequencies of cells with aberrations in this chromosome aberration test, when tested up to the highest evaluable concentrations. The RMS concludes that SYN548708 is clastogenic in this in vitro chromosome aberration test.

B.6.8.1 – 6.4.37 In vitro cell mutation assay with SYN548708 (R418503) - study 37

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/25 Wollny H (2015) SYN548708 - 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. 1677303 issue date: 04 May 2015. Unpublished. Syngenta File No. (SYN548708_10002).

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 473 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The test substance SYN548708 was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and presence of an S9-activation system. The maximum concentration of the pre-experiment was 4020 µg/mL, equal to approximately 10 mM, based on the molecular weight (401.11 g/mol) of the test substance. The maximum concentration of the main experiments was limited by cytotoxicity of the test item.

The assay was performed in two independent experiments, using two parallel cultures each. Experiments I and II were performed with and without metabolic activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations:

Experiment I:

without metabolic activation:	7.8; 15.5; 31.0; 46.5; and 62.0 µg/mL
with metabolic activation:	15.5; 31.0; 62.0; 93.0; and 124.0 µg/mL

Experiment II:

without metabolic activation:	10.0; 20.0; 30.0; 40.0; and 50.0 µg/mL
with metabolic activation:	10.0; 30.0; 60.0; 100.0; and 120.0 µg/mL

Test Material:	SYN548708
Description:	White, solid
Lot/Batch number:	MES 373/1
Molecular weight:	401.11 g/mol
Purity	99 % w/w (estimated error ± 2 %), concentration calculation not adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	Deionised water (10 %)
Positive control:	Absence of S9 mix: Methylmethanesulphonate, 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

The pre-experiment was performed in the presence and absence of metabolic activation with a treatment time of 4 hours. Test substance concentrations between 31.4 µg/mL and 4020 µg/mL were

used. The maximum concentration of the pre-experiment was equal to a molar concentration of approximately 10 mM.

Toxic effects leading to a RSG value below 50% were observed down to the lowest concentration without metabolic activation and at 125.6 µg/mL and above with metabolic activation. The test medium was checked for precipitation or phase separation at the end of the treatment period (4 hours) before the test substance was removed. Precipitation was noted at 1005.0 µg/mL and above with metabolic activation. There was no relevant shift of osmolarity and pH of the medium even at the maximum concentration of the test item (solvent control: 258 mOsm, pH 7.32 versus 286 mOsm and pH 7.34 at 4020 g/mL).

The concentrations used in the first experiment were selected based on cytotoxicity observed in the pre-experiment. The concentration range of the second experiment was based on cytotoxicity observed in the first experiment. The assay was performed in two independent experiments, using two parallel cultures each. Experiments I and II were performed with and without metabolic activation and a treatment period of 4 hours.

Relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in experiment I at 31.0 µg/mL and above without metabolic activation and at 124.0 µg/mL with metabolic activation. In experiment II cytotoxic effects occurred at 30.0 µg/mL and above without metabolic activation and at 60.0 µg/mL and above with metabolic activation. The recommended cytotoxic range of approximately 10-20% relative total growth was covered with and without metabolic activation. The data generated in the second experiment with metabolic activation at 120 µg/mL and above were not considered valid as both, the relative cloning efficiency 1 and the relative total growth fell short of the lower limit of 10% in both parallel cultures.

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 at acceptable levels of cytotoxicity with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was detected in the second culture of the first experiment with metabolic activation and in the first culture of the second experiment without metabolic activation. Since the mutation frequency did not exceed the threshold as indicated above, the statistical result is considered as biologically irrelevant fluctuation.

In this study the range of the solvent control values was from 90 up to 149 mutant colonies per 10^6 cells; the range of the group values treated with the test substance was from 70 up to 245 mutant colonies per 10^6 cells.

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 toxicity with at least one of the concentrations of the controls.

The results of the TK assay are given in Table 6.8.1 – 6.4.38-1.

Table 6.8.1 – 6.4.37-1 Summary of results of the TK assay with SYN 548708

	conc. µg per mL	S9 mix	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	99	225	100.0	100.0	149	275
Pos. control with MMS	19.5	-	56.7	39.0	305	225	163.5	33.4	471	275
Test item	1.9	-	112.1	culture was not continued*			206.2	culture was not continued*		
Test item	3.9	-	91.2	culture was not continued*			186.0	culture was not continued*		
Test item	7.8	-	92.5	88.7	106	225	168.2	56.5	87	275
Test item	15.5	-	79.8	49.6	152	225	92.6	66.5	122	275
Test item	31.0	-	34.9	14.9	138	225	23.8	23.5	192	275
Test item	46.5	-	14.3	8.4	159	225	8.9	9.7	245	275
Test item	62.0	-	13.0	6.3	114	225	4.0	3.7	172	275
Solv. control with water		+	100.0	100.0	134	260	100.0	100.0	91	217
Pos. control with CPA	3.0	+	90.4	63.1	225	260	70.1	92.1	181	217
Pos. control with CPA	4.5	+	54.3	38.0	408	260	70.1	52.0	302	217
Test item	3.9	+	95.8	culture was not continued*			158.5	culture was not continued*		
Test item	7.8	+	87.8	culture was not continued*			117.7	culture was not continued*		
Test item	15.5	+	122.0	106.9	111	260	167.9	108.5	105	217
Test item	31.0	+	105.9	114.0	101	260	155.6	126.0	70	217
Test item	62.0	+	91.7	86.9	85	260	110.8	125.0	84	217
Test item	93.0	+	53.3	47.7	165	260	107.6	53.9	152	217
Test item	124.0	+	12.9	14.6	230	260	35.3	18.3	203	217
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	90	216	100.0	100.0	91	217
Pos. control with MMS	19.5	-	44.3	21.3	311	216	55.5	18.1	353	217
Test item	2.5	-	83.0	culture was not continued*			82.2	culture was not continued*		
Test item	5.0	-	75.1	culture was not continued*			96.6	culture was not continued*		
Test item	10.0	-	109.0	85.9	101	216	111.6	114.9	72	217
Test item	20.0	-	58.9	41.6	134	216	84.8	60.5	83	217
Test item	30.0	-	37.8	29.0	107	216	64.9	33.9	130	217
Test item	40.0	-	34.7	13.5	131	216	29.1	14.2	140	217
Test item	50.0	-	18.7	7.7	197	216	27.6	12.2	108	217
Solv. control with water		+	100.0	100.0	93	219	100.0	100.0	92	218
Pos. control with CPA	3.0	+	69.0	53.9	143	219	61.6	55.5	183	218
Pos. control with CPA	4.5	+	38.3	29.0	341	219	42.9	35.6	313	218
Test item	10.0	+	90.4	83.1	112	219	73.1	161.1	94	218
Test item	30.0	+	84.2	84.0	129	219	65.2	107.1	108	218
Test item	60.0	+	53.0	44.4	71	219	59.9	31.9	127	218
Test item	100.0	+	22.5	6.0	96	219	20.1	7.5	118	218
Test item	120.0	+	**7.9**	**2.4**	**220**	**219**	**9.3**	**3.0**	**228**	**218**
Test item	140.0	+	4.3	culture was not continued**			3.4	culture was not continued**		
Test item	160.0	+	1.7	culture was not continued**			2.4	culture was not continued**		

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126

\# culture was not continued since a minimum of only four analysable concentrations is required

\## culture was not continued due to exceedingly severe cytotoxic effects

The values printed in bold are judged as invalid, since the acceptance criteria (page 23) are not met (relative cloning efficiency 1 and RTG < 10% in both parallel cultures).

Conclusion

In the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y the test substance SYN548708 did not induce mutations in the absence and presence of metabolic activation. Therefore, SYN548708 is considered to be non-mutagenic in this mouse lymphoma assay.

Genotoxicity SYN548708 – in vivo

B.6.8.1 – 6.4.38a In vivo micronucleus test with SYN548708 (R418503) - study 38

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/26 Dunton, J. (2015) SYN548708 – Oral (Gavage) Mouse Micronucleus Test. Sequani Ltd. Sequani Report No. BFI0373, issue date: 19 November 2015. Unpublished. Syngenta File No. SYN548708_10006.

Guidelines: Mouse 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.

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

Study design

SYN548708 was tested to evaluate its potential to cause damage to chromosomes or cell division apparatus, or to cause cell cycle interference, leading to micronucleus formation in polychromatic erythrocytes in the bone marrow of young adult mice.

In all phases, the dosing of the vehicle and test item was by oral (gavage) administration, on two consecutive occasions, approximately 24 hours apart.

In the dose-sighting phase, three groups of two male mice were given SYN548708 as a suspension in 0.5 % w/v aqueous carboxymethylcellulose with 0.1 % v/v Tween 80 at 500, 1250 or 2000 mg/kg/day, in order to determine the maximum tolerated dose (MTD).

In the range-finding phase, a group of three male and three female mice were SYN548708 at 2000 mg/kg/day.

For the main study phase, three groups, each of six male mice were dosed with 500, 1000 or 2000 mg/kg/day SYN548708. A group of six male mice (negative Controls) was dosed with the vehicle alone and a positive Control group, also of six male mice, was given a single 4 mg/kg intraperitoneal dose of Mitomycin C (MMC).Bone marrow was harvested from all range-finder and main study animals approximately 24 hours after the final dose administration and smears were prepared. The stained slides prepared for the main study were coded and 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei and the group frequencies were statistically analysed.

Test Material:	SYN548708
Description:	white powder
Lot/Batch number:	MES 373/1
Purity:	99 %
Stability of test compound:	Retest date : 31 December 2016

Control Materials:			
Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	0.5 % (w/v) aqueous carboxymethylcellulose with 0.1 % v/v Tween 80	**Final Volume:** 10 mL/kg	**Route:** oral
Positive control :	Mitomycin C	**Final Doses:** 4 mg/kg	**Route:** i.p.

Results

There were no test item related clinical signs following administration of SYN548708 at 1000 mg/kg/day or 2000 mg/kg/day, nor were there any clinical observations in Group 1 (negative Control) or Group 5 (positive Control). One male given 500 mg/kg/day SYN548708 had loose faeces after the second dose. There were no test item related clinical signs noted in any other animal.

There were no statistically significant increases in micronucleus frequency in male mice treated at any dose level of SYN548708, compared with the negative Control group.

There was no evidence of a statistically significant reduction in the PCE/NCE ratio in male mice treated with SYN548708, and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of SYN548708 to the bone marrow.

The animals dosed with MMC, the positive Control item, had statistically significant increases in the number of micronucleated cells compared to the concurrent Control group, which demonstrated that the test system was capable of detecting a known clastogen and that the scorers were capable of detecting micronuclei. There was a statistically significant decrease in the PCE/NCE ratio in the positive Control group, indicating toxicity to the bone marrow.

Conclusion

There were no statistically significant increases in micronucleus frequency in male mice treated at any dose level of SYN548708, compared with the negative Control group.

There was no evidence of a statistically significant reduction in the PCE/NCE ratio in male mice treated with SYN548708 and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of SYN548708 to the bone marrow.

There was no evidence of clastogenicity or aneugenicity following oral (gavage) administration of SYN548708 up to the OECD 474 limit dose of 2000 mg/kg/day. SYN548708 is considered to be neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

B.6.8.1 – 6.4.38b Proof of exposure - In vivo micronucleus test with SYN548708 (R418503)

Data requirement 2.29

Applicant to provide evidence of tissue exposure in the in vivo MN study with SYN548708 – study 38.

See also 2(89, 92)

See reporting table 2(75).

NL (August 2017): The applicant provided proof of exposure for the in vivo mouse micronucleus test (Dunton, 2015; study 38) by providing raw data on the presence of SYN548708 in blood samples. The RMS agrees with the conclusion that SYN548708 is systemically available and thus may reach the bone marrow.

The notifier provided the following evidence of tissue exposure in the in vivo MN study:
" The in vivo bone marrow micronucleus assay is a widely used in vivo genotoxicity test. In the case that a negative result is obtained evidence must be presented that the compound of interest was systemically available to the bone marrow. As the bone marrow is a well-perfused tissue, concentrations of compound-related material(s) in blood or plasma are generally similar to those observed in bone marrow. Therefore, proof of exposure can be obtained by measurement of the compound of interest either in blood or plasma.

Concern that the compound is not available for distribution into bone marrow because of irreversible binding to red blood cells can be addressed two ways. Either analyse for the compound in plasma or demonstrate that any binding to a matrix in whole blood (plasma or red blood cells) was reversible. The preparation of whole blood for analysis will yield reversibly bound compounds, but not those irreversibly bound and compound quantified will be that distributed in blood and plasma.
Blood samples were taken 1 and 4 hours following oral administration and again after the terminal blood sample approximately 24 hours after the second test item administration.
After sampling each blood sample was diluted with1 % (v/v) formic acid in acetonitrile [1:3 (v/v)]. Prior to analysis each sample was extracted using organic solvent. Following centrifugation, to pellet the protein, the resulting supernatant was analysed via mass spectrometry.

As shown in the table below, SYN548708 was freely available at concentrations similar to the 40 ng/mL low standard and quantifiable over several time points showing sustained exposure. It can therefore be concluded that SYN548708 was systemically available to allow bone marrow exposure.

SYN548708 Analytical Response and Retention Time Data

SYN548708		
Sample I.D.	Peak area counts	Retention time (min)
Blank	0.0	0.00
STD Low	1162.6	1.48
STD High	21822.5	1.47
Blank	0.0	0.00
RF1 71 M DAY 1 1h	1907.1	1.47
RF1 72 M DAY 1 1h	1142.2	1.48
RF1 73 M DAY 1 1h	223.1	1.47
RF1 71 M DAY 1 4h	1071.0	1.46
RF1 72 M DAY 1 4h	762.0	1.46
RF1 73 M DAY 1 4h	625.5	1.46
RF1 71 M DAY 1 TER	976.4	1.47
RF1 72 M DAY 1 TER	935.3	1.47
RF1 73 M DAY 1 TER	511.7	1.47
RF1 74 F DAY 1 1h	1107.8	1.48
RF1 75 F DAY 1 1h	512.9	1.46
RF1 76 F DAY 1 1h	756.3	1.47
RF1 74 F DAY 1 4h	668.6	1.47
RF1 75 F DAY 1 4h	0.0	0.00
RF1 76 F DAY 1 4h	972.3	1.47
RF1 74 F DAY 1 TER	520.8	1.46
RF1 75 F DAY 1 TER	431.6	1.46
RF1 76 F DAY 1 TER	817.5	1.46
Blank	0.0	0.00
STD Low	1173.0	1.46
STD High	23728.2	1.46
Blank	0.0	0.00

TER = terminal sample

"

Genotoxicity SYN548765 – in vitro

B.6.8.1 – 6.4.39 Reverse mutation assay in bacteria with SYN548765 (R419492) - study 39

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/27 Sokolowski A (2015b) SYN548765 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1678101, issue date: 16 April 2015. Unpublished Syngenta File No. SYN548765_10000.

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.

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

Study design

This study was performed to investigate the potential of SYN548765 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 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:

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

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

Test Material:	SYN548765
Description:	White, solid
Lot/Batch number:	MES 378/1
Purity:	92.0% w/w (estimated error ± 2%)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	28 February 2017
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)

Results

No precipitation of the test item occurred up to the highest investigated concentration.

The plates incubated with the test substance 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 any of the bacterial strains tested, neither with nor without metabolic activation. Only in experiment II minor cytotoxic effects were observed in strain TA 1535 without metabolic activation at 5000 µg/plate.

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548765 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 and all mutation rates were within the range of normal biological variability.

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

Table 6.8.1 – 6.4.39-1 Summary of results of experiment I

Study Name: 1678101
Experiment: 1678101 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1678101
Date Plated: 04/03/2015
Date Counted: 11/03/2015

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	Deionised water		9 ± 3	8 ± 1	25 ± 6	173 ± 11	214 ± 23	361 ± 9
	Untreated		12 ± 3	9 ± 1	20 ± 6	178 ± 8	222 ± 13	369 ± 29
	SYN548765	3 µg	10 ± 2	10 ± 3	26 ± 4	170 ± 10	225 ± 10	352 ± 19
		10 µg	8 ± 1	9 ± 2	19 ± 3	176 ± 5	224 ± 29	360 ± 40
		33 µg	12 ± 4	9 ± 1	28 ± 7	177 ± 21	243 ± 16	384 ± 18
		100 µg	11 ± 2	10 ± 0	26 ± 2	176 ± 16	230 ± 27	337 ± 22
		333 µg	10 ± 2	11 ± 3	29 ± 1	175 ± 7	198 ± 19	359 ± 23
		1000 µg	7 ± 3	10 ± 3	26 ± 2	190 ± 27	218 ± 29	340 ± 23
		2500 µg	12 ± 4	9 ± 2	30 ± 3	176 ± 13	218 ± 27	375 ± 47
		5000 µg	11 ± 2	10 ± 2	30 ± 3	174 ± 22	225 ± 15	366 ± 10
	NaN3	10 µg	1157 ± 55			2079 ± 86		
	4-NOPD	10 µg			286 ± 15			
	4-NOPD	50 µg		75 ± 10				
	MMS	2.0 µL					4054 ± 240	4056 ± 317
With Activation	Deionised water		11 ± 2	13 ± 3	36 ± 2	159 ± 4	246 ± 11	420 ± 59
	Untreated		10 ± 3	12 ± 3	38 ± 4	175 ± 21	258 ± 22	407 ± 31
	SYN548765	3 µg	9 ± 0	17 ± 6	43 ± 6	181 ± 31	246 ± 10	448 ± 11
		10 µg	11 ± 3	14 ± 3	40 ± 5	169 ± 41	240 ± 29	411 ± 11
		33 µg	13 ± 1	13 ± 1	34 ± 5	173 ± 27	245 ± 19	421 ± 35
		100 µg	13 ± 3	14 ± 1	37 ± 5	181 ± 15	248 ± 15	419 ± 4
		333 µg	12 ± 4	17 ± 3	37 ± 4	183 ± 12	258 ± 30	387 ± 29
		1000 µg	12 ± 2	17 ± 5	40 ± 6	156 ± 17	233 ± 11	400 ± 38
		2500 µg	11 ± 4	14 ± 2	36 ± 3	190 ± 1	244 ± 23	428 ± 21
		5000 µg	15 ± 5	17 ± 6	34 ± 8	175 ± 17	256 ± 15	425 ± 22
	2-AA	2.5 µg	399 ± 48	272 ± 19	3867 ± 55	4121 ± 257		
	2-AA	10.0 µg					1283 ± 107	2235 ± 137

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 – 6.4.39-2 Summary of results of experiment II

Study Name: 1678101
Experiment: 1678101 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1678101
Date Plated: 20/03/2015
Date Counted: 23/03/2015

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	Deionised water		11 ± 2	9 ± 2	19 ± 4	183 ± 4	192 ± 15	368 ± 13
	Untreated		10 ± 4	11 ± 1	21 ± 6	202 ± 11	224 ± 47	354 ± 22
	SYN548765	33 µg	12 ± 2	10 ± 2	23 ± 3	203 ± 2	187 ± 19	342 ± 15
		100 µg	12 ± 1	9 ± 4	13 ± 3	210 ± 4	178 ± 30	350 ± 27
		333 µg	10 ± 0	8 ± 3	16 ± 7	216 ± 6	208 ± 2	337 ± 13
		1000 µg	8 ± 1	8 ± 2	15 ± 1	220 ± 3	204 ± 24	352 ± 11
		2500 µg	12 ± 3	6 ± 0	13 ± 3	220 ± 15	168 ± 9	297 ± 24
		5000 µg	4 ± 1	4 ± 1	20 ± 4	224 ± 16	174 ± 28	328 ± 4
	NaN3	10 µg	1181 ± 62			1611 ± 120		
	4-NOPD	10 µg			267 ± 23			
	4-NOPD	50 µg		75 ± 17				
	MMS	2.0 µL					3494 ± 41	2674 ± 48
With Activation	Deionised water		10 ± 3	9 ± 2	33 ± 9	203 ± 9	233 ± 19	363 ± 47
	Untreated		11 ± 3	12 ± 3	30 ± 7	205 ± 9	248 ± 9	381 ± 10
	SYN548765	33 µg	10 ± 3	8 ± 3	24 ± 6	213 ± 7	225 ± 2	405 ± 14
		100 µg	9 ± 1	11 ± 3	34 ± 8	223 ± 9	237 ± 17	365 ± 40
		333 µg	9 ± 2	11 ± 3	26 ± 3	218 ± 12	218 ± 39	372 ± 26
		1000 µg	10 ± 2	11 ± 2	30 ± 7	210 ± 37	239 ± 27	364 ± 19
		2500 µg	9 ± 1	12 ± 2	29 ± 4	215 ± 13	226 ± 27	386 ± 16
		5000 µg	12 ± 2	10 ± 2	33 ± 4	183 ± 24	209 ± 37	382 ± 55
	2-AA	2.5 µg	387 ± 91	123 ± 9	4699 ± 399	3755 ± 361		
	2-AA	10.0 µg					1307 ± 65	2157 ± 114

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 reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli*, SYN548765 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN548765 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.40 In vitro chromosome aberration assay with SYN548765 (R419492) - study 40

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/27 Sokolowski, A. (2015c) SYN548765 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1678102, issue date: 29 July 2015. Unpublished. Syngenta File No. SYN548765_10004

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

GLP: Signed and dated GLP and Quality Assurance statements were provided.
Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of SYN548765 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/β-naphthoflavone treated male rats).
Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods were 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance.
In each treatment group two parallel cultures were analysed. At least 150 metaphases per culture were evaluated for structural chromosomal aberrations. 1000 cells per culture were counted for determination of mitotic index.
The highest applied concentration in this study (2174.0 µg/mL of the test substance) was chosen with regard to the purity (92 %) of the test substance and with respect to the current OECD Guideline 473 (2014).
Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473 (2014).

Test Material:	SYN548765
Description:	White solid
Lot/Batch number:	MES 378/1
Purity:	92 %
CAS#:	-
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	Deionised water 10%
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825.0 µg/mL (Exp. I), 660 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL

Results

The test substance SYN548765, dissolved in deionised water, was assessed for its potential to induce chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.
The highest treatment concentration in this study, 2174.0 µg/mL was chosen with regard to the purity (92 %) of the test substance and with respect to the current OECD Guideline 473 (2014).

No precipitation of the test substance in the culture medium was observed. The osmolarity and pH, measured with osmometer and pH meter were determined in the solvent control and the maximum concentration without metabolic activation: No relevant influence on the osmolarity or pH was observed. In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.
Neither with nor without metabolic activation a biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. However, in Experiment I with S9 mix at a concentration of 709.88 µg/mL 3.7 % aberrant cells, excluding gaps were found. This value is outside the two-fold 95 % control limit (2.6 % aberrant cells, excluding gaps) as well as above the historical control data range of 0.0 – 3.5 % aberrant cells, excluding gaps. Since there was neither statistical significance nor dose dependency observed, this finding was judged as biologically irrelevant. The increases in chromosomal aberrations observed in Experiment I without S9 mix at 709.88 µg/mL and above were judged as biologically irrelevant as they were not statistically significant and within the historical solvent control range (0.0 – 3.0 % aberrant cells, excluding gaps), even though they exceeded the two-fold 95 % control limit of the historical control data (2.6 % aberrant cells, excluding gaps).
In Experiment II in the absence of S9 mix at a concentration of 2174.00 µg/mL a statistically significant increase of metaphases with chromosomal aberrations was found (2.0 % aberrant cells, excluding gaps). This percentage lies within the two-fold 95 % control limit (2.3 % aberrant cells, excluding gaps) and the historical control data range of 0.0 – 3.0 % aberrant cells, excluding gaps and has to be judged as biologically irrelevant. No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (660.0 or 825.0 µg/mL) or CPA (15.0 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations in agreement with the laboratory control data.

Results of the in vitro chromosome aberration assay are given in Table 6.8.1 – 6.4.40.

Table 6.8.1 – 6.4.40 Summary of results of the chromosome aberration assay with SYN548765

Exp.	Preparation interval	Test item concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%)		
				incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	2.7	1.7	0.0
		Positive control[2]	84.5	11.0	**10.7**[S]	3.3
		709.88	81.3	3.3	3.0	0.0
		1242.29[#]	106.3	2.7	2.7	0.0
		2174.00	105.6	3.0	2.7	0.0
Exposure period 22 h without S9 mix						
II	22 h	Solvent control[1]	100.0	0.3	0.0	0.0
		Positive control[3]	51.4	17.3	**16.7**[S]	4.0
		709.88	80.3	1.7	1.0	0.0
		1242.29	85.2	1.3	0.7	0.3
		2174.00	85.9	2.0	**2.0**[S]	0.0
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	2.3	2.0	0.0
		Positive control[4]	47.4	22.7	**20.7**[S]	2.7
		709.88[#]	92.9	4.0	3.7	0.0
		1242.29	110.2	2.0	1.7	0.0
		2174.00	92.3	2.7	2.3	0.0
II	22 h	Solvent control[1]	100.0	1.0	1.0	0.0
		Positive control[4]	66.5	16.3	**16.3**[S]	2.0
		709.88	76.5	2.3	1.7	0.3
		1242.29	90.4	1.0	0.7	0.3
		2174.00	95.8	3.0	2.7	0.0

* Including cells carrying exchanges
S Aberration frequency statistically significant higher than corresponding control values
\# Evaluation of 300 metaphases per culture
1 Deionised water 10.0 % (v/v)
2 EMS 825.0 µg/mL
3 EMS 660.0 µg/mL
4 CPA 15.0 µg/mL

Conclusion

In an in vitro chromosome aberration study with human lymphocytes SYN548765did not induce structural chromosomal aberrations in human lymphocytes *in vitro*. Therefore, SYN548765 is considered to be non-clastogenic in this chromosome aberration test, when tested up to the highest applied concentration.

B.6.8.1 – 6.4.41 In vitro cell mutation assay in mouse lymphoma cells with SYN548765 (R419492) - study 41

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/28 Wollny H (2015a), SYN548765 - 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. 1678103 issue date: 29 July 2015. Unpublished. Syngenta File No. SYN548765_10002.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 476. There were no deviations from the guideline considered to compromise the scientific validity of the study.

Study design

The test substance SYN548765 was examined for its potential to induce gene mutations at the TK-locus of cultured mouse lymphoma L5178Y cells in both the absence and presence of an S9-activation system. The maximum concentration in the pre-experiment and in the main experiment, 4555.0 µg/mL was based on the purity (92% w/w) and molecular weight of the test item (419.1 g/mol). The maximum concentration of the main experiments was limited by cytotoxicity of the test item. The assay was performed in two independent experiments, using two parallel cultures each. Experiments I and II were performed with and without metabolic activation and a treatment period of 4 hours. The main experiments were evaluated at the following concentrations with and without metabolic activation: 284.7; 569.4; 1138.8; 2277.5; and 4555.0 µg/mL.

Test Material:	SYN548765
Description:	White, solid
Lot/Batch number:	MES 378/1
Molecular weight:	419.1 g/mol
Purity	92 % w/w (estimated error ± 2%), concentration calculation was adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	deionised water (10%)
Positive control:	Absence of S9 mix: Methylmethanesulphonate (MMS), 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

The pre-experiment was performed in the presence and absence of metabolic activation with a treatment time of 4 hours. Test substance concentrations between 35.6 µg/mL and 4555.0 µg/mL were used. The maximum concentration of the pre-experiment was equal to a molar concentration of approximately 10 mM.
No relevant toxic effects leading to a RSG value below 50% occurred up to the maximum concentration with and without metabolic activation. The concentrations used in both main experiments were selected based on the data generated in the pre-experiment. The maximum concentration again was 4555.0 µg/mL or approximately 10 mM. The individual concentrations were spaced by a factor of 2.0. To overcome problems with possible deviations in solubility or cytotoxicity, the main experiments were started with more than four concentrations.
The main study was performed to investigate the potential of SYN548765 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 metabolic activation and a treatment period of 4 hours.
The main experiments were evaluated at the following concentrations with and without metabolic activation: 284.7; 569.4; 1138.8; 2277.5; and 4555.0 µg/mL
No relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both parallel cultures occurred in experiments I and II.
No precipitation of the test item was noted at the end of treatment with and without metabolic activation.
No substantial or reproducible increase of the mutation frequency was noted in the main experiments with or without metabolic activation.
The threshold of 126 above the mutation frequency of the solvent control was slightly exceeded at 569.4 µg/mL in culture I of experiment I with metabolic activation (288 compared to 264 colonies per 10^6 cells). Another slight increase occurred at 1138.8 µg/mL in the first culture of experiment II with metabolic activation (217 compared to 212 colonies per 10^6 cells). However, both isolated increases were judged as biologically irrelevant as they were neither dose dependent nor reproduced in the parallel cultures under identical conditions.
A linear regression analysis (least squares) was performed to assess a possible concentration-dependent increase of mutant frequencies. No statistically significant trend occurred in any of the experiments.
In this study the range of the solvent control values was from 72 up to 144 mutant colonies per 10^6 cells; the range of the group values treated with the test substance was from 36 up to 288 mutant colonies per 10^6 cells. The viability of the solvent control of experiment I, culture 1 was 64% and thus 1% below the acceptable range. This deviation was judged as not relevant as it was very marginal.
MMS (19.5 µg/mL) and CPA (3.0 and 4.5 µg/mL) were used as positive control chemicals 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.

The results of the TK assay are given in Table 6.8.1 – 6.4.41.

Table 6.8.1 – 6.4.41 Summary of results of the TK assay with SYN 548708

	conc. µg per mL	S9 mix	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	72	198	100.0	100.0	95	221
Pos. control with MMS	19.5	-	98.2	25.0	391	198	88.8	29.6	309	221
Test item	142.3	-	105.7	culture was not continued#			139.8	culture was not continued#		
Test item	284.7	-	91.7	38.2	171	198	105.6	131.1	84	221
Test item	569.4	-	98.2	55.6	131	198	124.0	206.9	55	221
Test item	1138.8	-	84.5	75.5	78	198	116.4	149.5	75	221
Test item	2277.5	-	109.8	50.9	137	198	96.6	124.3	77	221
Test item	4555.0	-	71.4	29.6	112	198	87.4	71.8	100	221
Solv. control with water		+	100.0	100.0	138	264	100.0	100.0	78	204
Pos. control with CPA	3.0	+	65.1	81.9	168	264	70.7	55.7	136	204
Pos. control with CPA	4.5	+	90.5	51.4	329	264	56.8	42.9	289	204
Test item	142.3	+	110.4	culture was not continued#			89.8	culture was not continued#		
Test item	284.7	+	102.9	118.1	112	264	89.8	74.7	50	204
Test item	569.4	+	80.6	52.5	288	264	91.3	77.0	70	204
Test item	1138.8	+	110.4	134.2	166	264	96.4	83.8	36	204
Test item	2277.5	+	55.3	74.1	145	264	89.8	88.4	97	204
Test item	4555.0	+	102.9	79.2	150	264	64.7	65.3	70	204
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	83	209	100.0	100.0	83	209
Pos. control with MMS	19.5	-	89.1	54.4	302	209	67.1	32.1	302	209
Test item	142.3	-	127.9	culture was not continued#			84.3	culture was not continued#		
Test item	284.7	-	54.1	112.9	77	209	79.2	78.7	69	209
Test item	569.4	-	31.4	111.2	91	209	98.2	99.2	61	209
Test item	1138.8	-	40.6	139.6	91	209	96.5	74.4	84	209
Test item	2277.5	-	36.8	129.5	73	209	98.2	64.7	93	209
Test item	4555.0	-	27.1	96.7	104	209	105.8	90.9	51	209
Solvent control with water		+	100.0	100.0	86	212	100.0	100.0	144	270
Pos. control with CPA	3.0	+	45.1	13.9	1197	212	67.6	44.7	345	270
Pos. control with CPA	4.5	+	25.5	10.3	651	212	24.0	25.2	418	270
Test item	142.3	+	74.4	culture was not continued#			105.6	culture was not continued#		
Test item	284.7	+	64.2	82.0	149	212	111.8	103.7	144	270
Test item	569.4	+	70.7	62.1	105	212	126.8	80.7	122	270
Test item	1138.8	+	70.7	77.1	217	212	88.8	90.5	107	270
Test item	2277.5	+	62.2	125.1	71	212	132.9	71.6	112	270
Test item	4555.0	+	93.5	88.6	133	212	124.0	69.3	160	270

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126
# culture was not continued as a minimum of only four analysable concentrations is required

Conclusion
In the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y the test substance SYN548765 did not induce mutations in the absence and presence of metabolic activation. Therefore, SYN548765 is considered to be non-mutagenic in this mouse lymphoma assay.

Data requirement 2.42b

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse

lymphoma assay. The results will be discussed in an experts' meeting.

Response by notifier to the above data requirement:

"No increases in MF above the GEF were observed in the absence of metabolic activation. In Experiment I (569.4 µg/mL) and II (1138.8 µg/mL) with metabolic activation an isolated increase in MF in Culture I above the Culture I GEF was observed. This was not noted in the parallel culture under the same treatment conditions and so is considered to be not biologically relevant. Furthermore, the mean MF for Cultures I and II (Exp 1 179, GEF 234; Exp II 162 GEF 241) was well below the mean GEF and so do not meet the criteria for a positive response. These isolated non-reproducible increases are therefore of no biological significance and the study is considered to show no mutagenic effect. SYN548765 is considered to be negative in the in vitro mammalian gene mutation assay conducted in L5178y TK+/- cells."

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

In the Expert meeting, the results of this study were discussed and it was concluded that the response is considered negative.

Genotoxicity SYN548766 – in vitro

B.6.8.1 – 6.4.42 Reverse mutation assay in bacteria with SYN548766 (R471811) - study 42

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/29 Sokolowski, A (2015d) SYN548766 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1678001, issue date: 06 August 2015. Unpublished Syngenta File No. SYN548766_10002.

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.

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

Study design

This study was performed to investigate the potential of SYN548766 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 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:

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

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

Test Material:	SYN548766
Description:	White solid
Lot/Batch number:	MES 392/1
Purity:	95 % w/w (estimated error : ± 2%) concentration adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Recertification Date:	30 April 2017
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)

Results

No precipitation of the test substance occurred up to the highest investigated dose. The plates incubated with the test substance 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 any of the bacterial strains tested neither with nor without metabolic activation.

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548766 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 and all mutation rates were within the range of normal biological variability. Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

In experiment II, the data in the untreated control of strain WP2 pKM101 without S9 mix were slightly below the laboratory´s historical control data range. Since this deviation is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies and had no detrimental impact on the outcome of the study.

Results of Experiment I and II are given tables 6.8.1-6.4.42-1 and -2.

Table 6.8.1 – 6.4.42-1 Summary of results of the Ames test with SYN548766, experiment I

Study Name: 1678001 — Study Code: Harlan CCR 1678001
Experiment: 1678001 VV Plate — Date Plated: 26/06/2015
Assay Conditions: — Date Counted: 29/06/2015

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	Deionised water		11 ± 2	10 ± 3	29 ± 5	162 ± 20	227 ± 16	354 ± 13
	Untreated		9 ± 2	14 ± 6	21 ± 5	149 ± 27	222 ± 13	386 ± 12
	SYN548766	3 µg	11 ± 3	8 ± 2	28 ± 7	161 ± 17	235 ± 31	382 ± 18
		10 µg	9 ± 3	10 ± 4	20 ± 3	173 ± 8	226 ± 32	390 ± 30
		33 µg	10 ± 3	10 ± 2	25 ± 7	193 ± 9	209 ± 25	390 ± 25
		100 µg	10 ± 5	9 ± 2	24 ± 8	173 ± 7	201 ± 3	350 ± 28
		333 µg	10 ± 4	8 ± 1	31 ± 3	185 ± 19	224 ± 10	371 ± 32
		1000 µg	12 ± 3	11 ± 3	21 ± 3	188 ± 15	220 ± 31	371 ± 31
		2500 µg	11 ± 5	8 ± 4	26 ± 4	175 ± 10	208 ± 16	373 ± 17
		5000 µg	12 ± 5	7 ± 1	23 ± 4	170 ± 5	218 ± 0	380 ± 15
	NaN3	10 µg	1160 ± 51			2013 ± 62		
	4-NOPD	10 µg			387 ± 51			
	4-NOPD	50 µg		72 ± 11				
	MMS	2.0 µL					3780 ± 267	4571 ± 386
With Activation	Deionised water		12 ± 3	13 ± 3	32 ± 6	187 ± 19	240 ± 11	418 ± 18
	Untreated		10 ± 3	14 ± 0	37 ± 9	153 ± 19	271 ± 21	420 ± 44
	SYN548766	3 µg	11 ± 3	13 ± 4	40 ± 9	187 ± 14	278 ± 9	438 ± 27
		10 µg	12 ± 7	15 ± 2	38 ± 6	158 ± 28	261 ± 8	441 ± 35
		33 µg	14 ± 4	14 ± 2	29 ± 8	160 ± 3	252 ± 22	418 ± 21
		100 µg	13 ± 5	15 ± 2	37 ± 12	158 ± 11	234 ± 20	410 ± 60
		333 µg	9 ± 1	11 ± 4	35 ± 8	164 ± 21	256 ± 12	423 ± 37
		1000 µg	12 ± 3	14 ± 2	34 ± 6	166 ± 24	254 ± 7	374 ± 28
		2500 µg	10 ± 2	11 ± 3	35 ± 10	182 ± 10	250 ± 15	388 ± 64
		5000 µg	10 ± 3	13 ± 5	40 ± 5	146 ± 2	254 ± 43	407 ± 31
	2-AA	2.5 µg	393 ± 16	159 ± 23	4927 ± 109	4330 ± 338		
	2-AA	10.0 µg					1184 ± 55	2339 ± 94

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 – 6.4.42-2 Summary of results of the Ames test with SYN548766, experiment II

Study Name: 1678001 — Study Code: Harlan CCR 1678001
Experiment: 1678001 HV2 Pre — Date Plated: 07/07/2015
Assay Conditions: — Date Counted: 10/07/2015

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	Deionised water		14 ± 2	8 ± 1	20 ± 6	152 ± 9	194 ± 28	366 ± 26
	Untreated		12 ± 4	9 ± 5	27 ± 6	149 ± 6	209 ± 34	343 ± 6
	SYN548766	33 µg	14 ± 1	10 ± 4	26 ± 3	173 ± 20	208 ± 5	349 ± 32
		100 µg	14 ± 2	7 ± 3	20 ± 6	145 ± 19	197 ± 27	335 ± 26
		333 µg	14 ± 6	10 ± 5	25 ± 2	151 ± 17	207 ± 7	374 ± 19
		1000 µg	11 ± 4	12 ± 6	22 ± 6	146 ± 29	181 ± 27	346 ± 10
		2500 µg	13 ± 0	10 ± 2	24 ± 4	173 ± 23	193 ± 29	324 ± 6
		5000 µg	14 ± 4	11 ± 3	28 ± 3	147 ± 9	195 ± 3	337 ± 6
	NaN3	10 µg	1037 ± 89			1986 ± 214		
	4-NOPD	10 µg			558 ± 60			
	4-NOPD	50 µg		157 ± 24				
	MMS	2.0 µL					4035 ± 94	3047 ± 58
With Activation	Deionised water		16 ± 3	14 ± 6	34 ± 10	153 ± 14	268 ± 18	394 ± 33
	Untreated		17 ± 6	16 ± 1	32 ± 7	179 ± 17	255 ± 28	393 ± 10
	SYN548766	33 µg	14 ± 3	15 ± 6	36 ± 2	144 ± 17	271 ± 15	405 ± 11
		100 µg	16 ± 5	10 ± 2	28 ± 6	156 ± 16	259 ± 15	405 ± 34
		333 µg	18 ± 2	10 ± 3	36 ± 4	161 ± 24	248 ± 45	413 ± 50
		1000 µg	14 ± 3	15 ± 6	31 ± 9	173 ± 4	257 ± 44	403 ± 14
		2500 µg	13 ± 1	11 ± 2	28 ± 5	130 ± 4	239 ± 11	382 ± 24
		5000 µg	13 ± 2	9 ± 1	29 ± 4	158 ± 18	246 ± 8	377 ± 13
	2-AA	2.5 µg	352 ± 9	167 ± 30	4610 ± 130	3345 ± 604		
	2-AA	10.0 µg					1168 ± 161	2007 ± 96

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 reverse mutation assay with *Salmonella typhimurium* and *Escherichia coli* SYN548766 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN548766 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.43 In vitro chromosome aberration assay with SYN548766 (R471811) - study 43

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/30 Sokolowski, A. (2015e) SYN548766 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1678002, issue date: 10 August 2015. Unpublished. Syngenta File No. SYN548766_10004

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

GLP: Signed and dated GLP and Quality Assurance statements were provided.
Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of SYN548766 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/β-naphthoflavone treated male rats).
In each experimental group two parallel cultures were analysed. Per culture 150 metaphases were evaluated for structural chromosomal aberrations, except for the positive control in Experiment II without S9 mix, where only 50 metaphases were evaluated.
The highest applied concentration in this study (3890.0 µg/mL of the test substance, approx. 10 mM) was chosen with regard to the molecular weight and the purity (95 %) of the test substance and with respect to the current EPA and EU test guidelines meeting and exceeding the maximum concentration required by the OECD Guideline 473 (2014) and in agreement with OECD guideline 473 (1997).
Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473 (2014).

Materials:

Test Material:	SYN548766
Description:	White solid
Lot/Batch number:	MES 392/1
Purity:	95 % (concentrations corrected for purity)
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	Deionised water final concentration10%
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825.0 µg/mL (Exp. I), 550 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 2.5 µg/mL (exp. I) and 10 µg/mL (exp. II)

Results

The test substance SYN548766, dissolved in deionised water, was assessed for its potential to induce chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

The highest treatment concentration in this study, 3890.0 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the purity (95 %) of the test substance and with respect to the current OECD Guideline 473 (2014) and in agreement with OECD guideline 473 (1997).
No precipitation of the test substance in the culture medium was observed.
The osmolarity and pH were determined in the solvent control and the maximum concentration without metabolic activation: No relevant influence on the osmolarity or pH was observed.
In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.
Under all of the exposure conditions examined no statistically significant and biologically relevant increases in structural chromosomal aberrations were observed. In Experiment II at 3890.0 µg/mL with and without S9 a small increase in chromosomal aberrations (2.7 %, excluding gaps) outside the 95% control limit of the historical negative control data (with S9: 2.6 %, excluding gaps; without S9: 2.3 %, excluding gaps) was observed. However, these increases were within the solvent historical control range (0.0 – 3.0 %, excluding gaps), not statistically significant and therefore considered to be without biological relevance. Since no statistically significant increases in percentage aberrant cells were observed under any exposure condition a trend test was not considered necessary.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (825.0 or 550.0 µg/mL) or CPA (2.5 or 10.0 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations. The value of the positive control in experiment II without S9 mix exceeded the upper limit of the historical control. This effect indicates the sensitivity of the cells rather than compromising the assay.

The results of the in vitro chromosome aberration study are summarized in Table 6.8.1 – 6.4.43.

Table 6.8.1 – 6.4.43 Summary of results of the chromosome aberration assay with SYN548766, experiment II

Exp.	Preparation interval	Test item concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%)		
				incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	2.3	2.3	0.0
		Positive control[2]	76.3	11.0	**10.7**[S]	0.3
		1270.2	103.9	1.3	1.3	0.0
		2222.9	108.5	1.7	1.0	0.0
		3890.0	110.2	1.7	1.3	0.3
Exposure period 22 h without S9 mix						
II	22 h	Solvent control[1]	100.0	1.3	1.3	0.0
		Positive control[3#]	58.5	40.0	**40.0**[S]	7
		1270.2	86.2	1.0	1.0	0.3
		2222.9	88.1	2.3	1.7	0.0
		3890.0	91.5	3.3	2.7	0.0
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	2.7	2.7	0.0
		Positive control[4]	76.2	10.7	**10.7**[S]	0.7
		1270.2	96.7	0.3	0.3	0.0
		2222.9	105.6	2.7	2.3	0.0
		3890.0	91.7	0.7	0.7	0.0
II	22 h	Solvent control[1]	100.0	2.0	2.0	0.0
		Positive control[5]	57.4	19.3	**18.3**[S]	5.0
		1270.2	78.8	1.0	1.0	0.0
		2222.9	107.1	2.3	2.0	0.7
		3890.0	122.4	3.0	2.7	0.0

* Including cells carrying exchanges
\# evaluation of 50 cell per culture
S Aberration frequency statistically significant higher than corresponding control values
1 Deion. water 10.0% (v/v)
2 EMS 825.0 µg/mL
3 EMS 550.0 µg/mL
4 CPA 2.5 µg/mL
5 CPA 10.0 µg/mL

Conclusion

In an in vitro chromosome aberration study with human lymphocytes the test substance SYN548766 did not induce structural chromosomal aberrations in human lymphocytes in vitro. Therefore, SYN548766 is considered to be non-clastogenic in this chromosome aberration test.

B.6.8.1 – 6.4.44 In vitro cell mutation assay in mouse lymphoma cells with SYN548766 (R471811) - study 44

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/31 Wollny H (2015b), SYN548766 - 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. 1678003 issue date: 30 July 2015. Unpublished. Syngenta File No. SYN548766_10000.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 476. There were no deviations from the guideline considered to compromise the scientific validity of the study.

Study design

The study was performed to investigate the potential of SYN548766 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 metabolic activation and a treatment period of 4 hours.

The main experiments I and II were evaluated at the following concentrations:

without metabolic activation: 243.8; 487.5; 975.0; 1950.0; 3900.0 µg/mL

with metabolic activation: 243.8; 487.5; 975.0; 1950.0; 3900.0 µg/mL

The maximum concentration of the pre-experiment and the main experiments was 3900 µg/mL, equal to approximately 10 mM, based on the molecular weight (369.5 g/mol) and the purity (95%) of the test substance.

Test Material:	SYN548766
Description:	White, solid
Lot/Batch number:	MES 392/1
Molecular weight:	369.5 g/mol
Purity	95% w/w (estimated error ± 2%), concentration calculation was adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	deionised water (10%)
Positive control:	Absence of S9 mix: Methylmethanesulphonate (MMS), 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

Based on the results of the pre-test at least four adequate concentrations were chosen for the mutation experiment. No relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in experiment I and II 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 with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using the validated R Script LM.Rnw statistics software. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was detected in culture I of the first experiment without metabolic activation. Since the mutation frequency did not exceed the threshold as indicated above and the effect was not reproduced in the parallel culture, this was not considered biologically relevant.

In this study the range of the solvent control values was from 68 up to 111 mutant colonies per 10^6 cells; the range of the group values treated with the test substance was from 37 up to 177 mutant colonies per 10^6 cells.

The total suspension growth of the solvent control of the first experiment, culture II without metabolic activation exceeded the upper limit of the acceptance criteria (42.0 compared to an upper limit of 32). This deviation had no impact on the study data as it was minor and in contrast to a low proliferation rate, a high proliferation does not reduce the sensitivity of a cell population towards possible mutagenic effects during treatment and until selection. The total suspension growth of the parallel culture remained within the range of the acceptance criteria.

The viability slightly exceeded the upper limit of 120% in the second culture of experiment II without metabolic activation (121%). This deviation was judged as biologically irrelevant as it was very minor and the viability of the parallel culture remained well within the acceptable range.

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 toxicity with at least one of the concentrations of the controls.

The results of the TK assay are presented in Table 6.8.1-6.4.44.

Table 6.8.1 – 6.4.44 Summary of results of the TK assay with SYN548766

			relative	relative	mutant		relative	relative	mutant	
	conc. µg	S9	cloning	total	colonies/		cloning	total	colonies/	
	per mL	mix	efficiency 1	growth	10^6 cells	threshold	efficiency 1	growth	10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	76	202	100.0	100.0	79	205
Pos. control with MMS	19.5	-	64.0	29.8	329	202	50.2	16.6	406	205
Test item	121.9	-	100.0	culture was not continued#			86.9	culture was not continued#		
Test item	243.8	-	111.5	110.9	73	202	89.9	69.9	104	205
Test item	487.5	-	109.7	85.1	70	202	96.4	102.6	95	205
Test item	975.0	-	147.8	91.3	74	202	105.9	100.9	73	205
Test item	1950.0	-	101.5	99.8	86	202	103.8	51.3	131	205
Test item	3900.0	-	109.7	81.8	107	202	114.9	64.3	139	205
Solv. control with water		+	100.0	100.0	68	194	100.0	100.0	90	216
Pos. control with CPA	3.0	+	22.6	15.0	452	194	25.7	48.7	301	216
Pos. control with CPA	4.5	+	23.1	22.4	388	194	21.9	22.5	424	216
Test item	121.9	+	120.1	culture was not continued#			82.8	culture was not continued#		
Test item	243.8	+	100.0	88.9	68	194	91.5	173.5	51	216
Test item	487.5	+	77.2	91.5	81	194	70.9	149.6	58	216
Test item	975.0	+	90.3	92.0	71	194	56.3	145.9	37	216
Test item	1950.0	+	68.0	106.5	45	194	88.4	112.0	53	216
Test item	3900.0	+	57.2	78.4	67	194	80.2	127.9	75	216
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	111	237	100.0	100.0	79	205
Pos. control with MMS	19.5	-	59.9	32.4	397	237	56.8	37.5	252	205
Test item	121.9	-	100.0	culture was not continued#			68.3	culture was not continued#		
Test item	243.8	-	79.6	97.5	92	237	81.4	101.8	82	205
Test item	487.5	-	73.2	93.1	143	237	76.7	91.9	101	205
Test item	975.0	-	85.4	150.9	111	237	73.4	140.2	70	205
Test item	1950.0	-	78.2	88.3	177	237	61.9	106.5	80	205
Test item	3900.0	-	68.7	100.4	115	237	40.1	93.3	69	205
Solv. control with water		+	100.0	100.0	72	198	100.0	100.0	86	212
Pos. control with CPA	3.0	+	37.6	23.5	225	198	22.5	29.6	359	212
Pos. control with CPA	4.5	+	39.7	17.9	478	198	17.3	21.5	403	212
Test item	121.9	+	89.2	culture was not continued#			73.6	culture was not continued#		
Test item	243.8	+	86.7	86.5	76	198	88.9	121.6	64	212
Test item	487.5	+	109.1	91.6	103	198	81.3	95.8	93	212
Test item	975.0	+	114.0	99.8	72	198	85.7	69.5	120	212
Test item	1950.0	+	117.4	71.7	87	198	67.0	97.4	67	212
Test item	3900.0	+	78.6	79.6	120	198	71.3	97.1	78	212

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126

\# culture was not continued since a minimum of only four analysable concentrations is required

Conclusion

In the mouse lymphoma thymidine kinase locus assay test substance SYN548766 did not induce mutations using the cell line L5178Y in the absence and presence of metabolic activation. Therefore, SYN548766 is considered to be non-mutagenic in this mouse lymphoma assay.

Genotoxicity SYN548738 – in vitro

B.6.8.1 – 6.4.45 Reverse mutation assay in bacteria with SYN548738 (SYN548008) - study 45

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/32 Sokolowski A (2015f) SYN548738 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1677901, issue date: 16 April 2015. Unpublished Syngenta File No. SYN548738_10000.

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.

Acceptability: The study was performed in accordance with OECD guideline 471 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

This study was performed to investigate the potential of SYN548738 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 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:

Experiment I:	3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment II:	
All strains without S9 mix:	33; 100; 333; 1000; 2500; and 5000 µg/plate
All strains with S9 mix:	3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Test Material:	SYN548738
Description:	White, solid
Lot/Batch number:	MES 376/1
Purity:	93.0% w/w (estimated error ± 2%)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	28 February 2017

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)

Results

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 any of the bacterial strains tested neither with nor without metabolic activation.

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548738 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 and all mutation rates were within the range of normal biological variability. Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

In experiment I, the number of colonies did not quite reach the lower limit of our historical control data in the negative and solvent control of strain TA98 with S9 mix. These deviations are rather small, therefore this effect is judged to be based upon biological fluctuations and has no detrimental impact on the outcome of the study.

The results of the Ames test are given in Table 6.8.1 – 6.4.45-1 and -2.

Table 6.8.1 – 6.4.45-1 Summary of results of the Ames test SYN548738, experiment I

Study Name: 1677901
Experiment: 1677901 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1677901
Date Plated: 04/03/2015
Date Counted: 09/03/2015

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	Deionised water		10 ± 3	10 ± 1	19 ± 4	181 ± 12	215 ± 7	346 ± 18
	Untreated		9 ± 1	9 ± 2	30 ± 3	193 ± 16	227 ± 45	412 ± 20
	SYN548738	3 µg	11 ± 2	11 ± 3	21 ± 2	173 ± 9	202 ± 7	402 ± 36
		10 µg	11 ± 2	10 ± 3	17 ± 3	184 ± 14	205 ± 27	371 ± 20
		33 µg	14 ± 2	11 ± 3	23 ± 3	182 ± 19	188 ± 3	332 ± 13
		100 µg	12 ± 0	11 ± 2	22 ± 6	182 ± 27	170 ± 17	367 ± 21
		333 µg	12 ± 4	13 ± 3	24 ± 3	175 ± 11	214 ± 4	382 ± 26
		1000 µg	11 ± 1	11 ± 3	22 ± 5	167 ± 11	170 ± 33	319 ± 27
		2500 µg	12 ± 1	11 ± 1	23 ± 5	156 ± 12	182 ± 4	360 ± 16
		5000 µg	14 ± 2	8 ± 1	24 ± 2	170 ± 22	193 ± 13	396 ± 5
	NaN3	10 µg	1017 ± 42			2088 ± 206		
	4-NOPD	10 µg			259 ± 36			
	4-NOPD	50 µg		62 ± 7				
	MMS	2.0 µL					3706 ± 780	3584 ± 170
With Activation	Deionised water		12 ± 4	17 ± 3	15 ± 5 BM	157 ± 13	230 ± 7	381 ± 25
	Untreated		11 ± 1	22 ± 8	11 ± 3 BM	155 ± 14	263 ± 44	411 ± 3
	SYN548738	3 µg	10 ± 3	16 ± 3	15 ± 3 BM	144 ± 13	235 ± 13	384 ± 45
		10 µg	11 ± 3	17 ± 4	17 ± 2 BM	154 ± 6	238 ± 9	374 ± 18
		33 µg	11 ± 2	20 ± 6	15 ± 1 BM	149 ± 19	203 ± 14	384 ± 16
		100 µg	12 ± 4	14 ± 3	17 ± 1 BM	149 ± 9	204 ± 23	350 ± 21
		333 µg	14 ± 2	17 ± 4	16 ± 4 BM	148 ± 6	224 ± 13	437 ± 52
		1000 µg	14 ± 1 R	13 ± 6 R	16 ± 2 BMR	144 ± 9 R	223 ± 12 R	413 ± 25 R
		2500 µg	14 ± 2 R	13 ± 2 R	16 ± 2 BMR	133 ± 7 R	230 ± 21 R	385 ± 14 R
		5000 µg	14 ± 2 R	11 ± 2 R	16 ± 2 BMR	147 ± 18 R	211 ± 18 R	377 ± 49 R
	2-AA	2.5 µg	358 ± 31	226 ± 21	3728 ± 1200	3273 ± 133		
	2-AA	10.0 µg					1366 ± 95	2294 ± 215

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

R	Reduced background growth
B	Extensive bacterial growth
M	Manual count

Table 6.8.1 – 6.4.45-2 Summary of results of the Ames test SYN548738, experiment II

Study Name: 1677901 Study Code: Harlan CCR 1677901
Experiment: 1677901 HV2 Pre Date Plated: 18/03/2015
Assay Conditions: Date Counted: 25/03/2015

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	Deionised water		11 ± 2	11 ± 3	27 ± 2	182 ± 19	221 ± 40	355 ± 29
	Untreated		9 ± 3	11 ± 2	25 ± 2	193 ± 27	217 ± 10	333 ± 48
	SYN548738	33 µg	11 ± 4	12 ± 6	28 ± 6	191 ± 34	189 ± 30	356 ± 38
		100 µg	10 ± 2	10 ± 5	25 ± 5	175 ± 28	188 ± 5	353 ± 6
		333 µg	9 ± 3	11 ± 4	26 ± 3	190 ± 12	191 ± 19	360 ± 16
		1000 µg	11 ± 3	7 ± 2	31 ± 2	201 ± 16	223 ± 19	352 ± 45
		2500 µg	12 ± 2	6 ± 1	33 ± 4	204 ± 16	202 ± 15	367 ± 7
		5000 µg	7 ± 1	7 ± 1	28 ± 6	181 ± 20	233 ± 31	361 ± 16
	NaN3	10 µg	1101 ± 57			2148 ± 86		
	4-NOPD	10 µg			379 ± 21			
	4-NOPD	50 µg		84 ± 4				
	MMS	2.0 µL					4255 ± 156	2618 ± 196
With Activation	Deionised water		11 ± 2	15 ± 2	37 ± 6	195 ± 26	246 ± 10	381 ± 50
	Untreated		11 ± 2	13 ± 2	37 ± 6	179 ± 8	275 ± 12	404 ± 14
	SYN548738	3 µg	10 ± 2	16 ± 1	45 ± 2	169 ± 10	245 ± 10	378 ± 7
		10 µg	11 ± 3	14 ± 2	33 ± 2	185 ± 2	244 ± 10	359 ± 18
		33 µg	10 ± 3	15 ± 1	34 ± 6	180 ± 16	261 ± 18	357 ± 28
		100 µg	9 ± 2	11 ± 2	27 ± 3	171 ± 11	244 ± 11	413 ± 13
		333 µg	10 ± 3	15 ± 1	37 ± 6	193 ± 13	252 ± 23	388 ± 10
		1000 µg	11 ± 3	12 ± 3	33 ± 4	195 ± 22	227 ± 17	277 ± 18
		2500 µg	10 ± 3	17 ± 3	35 ± 10	176 ± 4	221 ± 41	327 ± 20
		5000 µg	10 ± 3	11 ± 2	34 ± 9	183 ± 16	204 ± 17	381 ± 66
	2-AA	2.5 µg	448 ± 11	134 ± 11	4748 ± 709	3210 ± 288		
	2-AA	10.0 µg					1155 ± 178	2600 ± 50

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusion

In the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay SYN548738 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN548738 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.46 In vitro chromosome aberration assay with SYN548738 (SYN548008) - study 46

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/33 Sokolowski, A. (2015g) SYN548738 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1677902, issue date: 29 July 2015. Unpublished. Syngenta File No. SYN548738_10002

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA PPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of SYN548738 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/β-naphthoflavone treated male rats). Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods were 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance.

In each treatment group two parallel cultures were analyzed. 150 metaphases per culture were evaluated for structural chromosomal aberrations. 1000 cells per culture were counted for determination of mitotic index. The highest applied concentration in this study (2150.0 µg/mL of the test substance) was chosen with regard to the purity (93 %) of the test substance and with respect to the current OECD Guideline 473 (2014).

Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473 (2014).

Test Material:	SYN548738
Description:	White solid
Lot/Batch number:	MES 376/1
Purity:	93 % (concentrations corrected for purity)
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:	
Negative:	-
Solvent control (final concentration):	Deionised water 10%
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825.0 µg/mL (Exp. I), 550 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL

Results

No precipitation of the test substance in the culture medium was observed. The osmolarity and pH, measured with osmometer and pH meter were determined in the solvent control and the maximum concentration without metabolic activation: No relevant influence on the osmolarity or pH was observed. In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In this study in the absence and presence of S9 mix, no statistically significant or biologically relevant increase in structural chromosomal aberrations was observed at the concentrations evaluated. The aberration rates of the cells after treatment with the test substance (0.7 – 3.0 % aberrant cells,

excluding gaps) did not exceed the solvent control values (1.0 – 3.3 % aberrant cells, excluding gaps) and were within the range of the laboratory historical solvent control data.
The chromosomal aberrations observed in Experiment II with S9 mix in the solvent control were outside the two-fold 95% control limits (2.6 %, excluding gaps), but were still inside the historical control range (0.0 – 3.5 %, excluding gaps) and therefore judged acceptable. The chromosomal aberrations induced in Experiment II with S9 mix at 702.04 µg/mL were outside of the two-fold 95% control limits (2.6 %, excluding gaps), but this increase was lower than in the solvent control and therefore judged as biological irrelevant.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (550.0 or 825.0 µg/mL) or CPA (15.0 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.
The results of the in vitro chromosome aberration study are presented in Table 6.8.1 – 6.4.46.

Table 6.8.1 – 6.4.46 Summary of results of the chromosome aberration assay with SYN548738

Exp.	Preparation interval	Test item concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%)		
				incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	1.0	1.0	0.0
		Positive control[2]	84.5	8.3	**7.3**[S]	1.0
		702.04	124.4	3.3	2.3	0.0
		1228.57	96.5	0.7	0.7	0.0
		2150.00	85.7	2.7	2.3	0.7
Exposure period 22 h without S9 mix						
II	22 h	Solvent control[1]	100.0	2.0	2.0	0.3
		Positive control[3]	51.8	18.3	**18.0**[S]	2.3
		702.04	95.2	1.3	1.0	0.0
		1288.57	92.6	0.7	0.7	0.0
		2150.00	87.3	0.7	0.7	0.0
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	1.3	1.0	0.0
		Positive control[4]	98.1	15.7	**15.3**[S]	2.3
		702.04	120.5	1.7	1.7	0.0
		1228.57	80.7	2.3	2.3	0.0
		2150.00	84.5	2.3	2.0	0.0
II	22 h	Solvent control[1]	100.0	4.0	3.3	0.3
		Positive control[4]	66.5	16.3	**15.7**[S]	1.7
		702.04	101.4	3.3	3.0	0.0
		1288.57	107.7	2.0	1.7	0.3
		2150.00	115.3	1.7	1.7	0.0

* Including cells carrying exchanges
S Aberration frequency statistically significant higher than corresponding control values
1 Deion. water 10.0 % (v/v)
2 EMS 825.0 µg/mL
3 EMS 550.0 µg/mL
4 CPA 15.0 µg/mL

Conclusion

In the in vitro chromosome aberration test, the test substance SYN548738 did not induce structural chromosomal aberrations in human lymphocytes *in vitro*. Therefore, SYN548738 is considered to be non-clastogenic in this chromosome aberration test, when tested up to the highest applied concentration.

B.6.8.1 – 6.4.47 In vitro cell mutation assay in mouse lymphoma cells with SYN548738 (SYN548008) - study 47

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/34 Sokolowski A. (2015h), SYN548738 - 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. 1677903 issue date: 07 August 2015. Unpublished. Syngenta File No. SYN548738_10004.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The study was performed to investigate the potential of SYN548738 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y. The assay was performed in three independent experiments, using two parallel cultures each. Experiments I and II were performed with and without liver microsomal activation with a treatment period of 4 hours. Experiment III was performed without metabolic activation only and a treatment period of 4 hours, in order to to assess the relevance of an isolated response in Experiment II Culture I without metabolic activation. The maximum concentration of the pre-experiment and the main experiments was 4700 µg/mL, equal to approximately 10 mM, based on the molecular weight (437.2 g/mol) and the purity (93%) of the test substance.

The main experiments were evaluated at the following concentrations:

Experiment I:	
without metabolic activation:	293.8; 587.5; 1175.0; 2350.0; and 4700.0 µg/mL
with metabolic activation:	293.8; 587.5; 1175.0; 2350.0; and 4700.0 µg/mL
Experiment II:	
without metabolic activation:	293.8; 587.5; 1175.0; 2350.0; and 4700.0 µg/mL
with metabolic activation:	293.8; 587.5; 1175.0; 2350.0; and 4700.0 µg/mL

Experiment III:

without metabolic activation:	587.5; 1175.0; 2350.0; 3525.0; and 4700.0 µg/mL

Test Material:	SYN548738
Description:	White, solid
Lot/Batch number:	MES 376/1
Molecular weight:	437.2 g/mol
Purity	93 % w/w, concentration calculation adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	deionised water (10 %)
Positive control:	Absence of S9 mix: Methylmethanesulphonate, 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

Based on the results of the pre-test at least four adequate concentrations were chosen for the mutation experiment. The highest concentration as specified in testing guidelines should be 10 mM, but not higher than 5 mg/mL, unless limited by the solubility or toxicity of the test substance. No toxic effect leading to a RSG value below 50% was observed up to the maximum concentration of 4700 µg/mL with and without metabolic activation. The maximum concentration was 4700 µg/mL of the test item equal to approximately 10 mM. The individual concentrations were spaced by a factor of 2. Experiment III was performed to verify data generated in the second experiment without metabolic activation. Following the expression phase of 48 hours the cultures at the lowest concentration in all parts of the main experiments were not continued as a minimum of only four analysable concentrations is required by the test guidelines.

Relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in experiment II at 4700 µg/mL without metabolic activation The data generated at this concentration were borderline acceptable with a RTG of 9.4% in culture I and 11.8% in culture II. The marked difference in cytotoxicity between both experiments without metabolic activation is possibly due to a different cell density during treatment. The cell density of the solvent control of experiment I without metabolic activation was 440000 and 413200 cells per mL in the parallel cultures. In the second experiment without metabolic activation the corresponding values were 258400 and 302400 cells per mL.

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 first experiment with and without metabolic activation and in the second experiment with metabolic activation. In the second experiment without metabolic activation, the threshold was exceeded in the first but not in the second culture at the highest concentration. To verify this isolated increase of the mutation frequency a third experiment was performed without metabolic activation. No increase of the mutation frequency was noted in the third experiment up to the maximum concentration. Thus the isolated increase noted in the second experiment without metabolic activation was deemed to be of no toxicological significance.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was detected in both cultures of the second experiment

without metabolic activation. Since the significant increase was not reproduced in the first or the third experiment without metabolic activation it was judged to be based on a toxicity artefact.
In this study the range of the solvent control values was from 53 up to 144 mutant colonies per 10^6 cells; the range of the group values treated with the test substance was from 60 up to 232 mutant colonies per 10^6 cells. The viability slightly exceeded the upper limit of 120% in the second culture of the second experiment without metabolic activation (124%). The data are acceptable as the deviation was minor and the viability of the parallel culture remained within the acceptable range (118%). In the first culture of the third experiment the viability fell just short of the lower limit of 65% (60%). The data are acceptable however, as the parallel culture showed a viability of 81%.
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 toxicity with at least one of the concentrations of the controls.

The results of the TK assay are presented in Table 6.8.1 – 6.4.47.

Table 6.8.1 – 6.4.47 Summary of results of the TK assay with SYN548738

	conc. µg per mL	S9 mix	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	109	235	100.0	100.0	93	219
Pos. control with MMS	19.5	-	69.0	38.7	349	235	80.5	35.7	409	219
Test item	146.9	-	101.7	culture was not continued#			105.1	culture was not continued#		
Test item	293.8	-	107.3	112.3	111	235	96.8	115.4	107	219
Test item	587.5	-	96.7	96.3	100	235	106.9	105.0	101	219
Test item	1175.0	-	95.1	109.9	125	235	93.8	102.6	121	219
Test item	2350.0	-	103.6	107.9	120	235	100.0	89.2	113	219
Test item	4700.0	-	96.7	134.8	90	235	98.4	81.9	102	219
Solv. control with water		+	100.0	100.0	137	263	100.0	100.0	88	214
Pos. control with CPA	3	+	54.8	58.6	179	263	84.8	46.6	182	214
Pos. control with CPA	4.5	+	48.9	51.8	332	263	64.6	39.6	336	214
Test item	146.9	+	108.7	culture was not continued#			119.7	culture was not continued#		
Test item	293.8	+	93.8	95.5	92	263	121.9	114.7	91	214
Test item	587.5	+	89.6	101.9	77	263	108.3	76.4	109	214
Test item	1175.0	+	96.8	136.5	81	263	104.8	103.6	95	214
Test item	2350.0	+	98.4	128.8	79	263	92.6	105.0	99	214
Test item	4700.0	+	95.3	157.5	88	263	97.0	110.8	82	214
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	53	179	100.0	100.0	78	204
Pos. control with MMS	19.5	-	55.7	14.0	529	179	66.1	14.9	610	204
Test item	146.9	-	63.3	culture was not continued#			101.4	culture was not continued#		
Test item	293.8	-	75.6	91.5	87	179	100.0	117.0	60	204
Test item	587.5	-	65.2	94.5	69	179	107.4	47.0	100	204
Test item	1175.0	-	81.7	58.5	81	179	109.0	56.6	80	204
Test item	2350.0	-	71.2	55.8	93	179	82.0	33.1	126	204
Test item	4700.0	-	41.9	9.4	232	179	27.9	11.8	141	204
Solvent control with water		+	100.0	100.0	131	257	100.0	100.0	111	237
Pos. control with CPA	3.0	+	103.1	23.0	896	257	47.2	15.6	770	237
Pos. control with CPA	4.5	+	13.6	19.0	446	257	36.4	18.1	339	237
Test item	146.9	+	101.5	culture was not continued#			63.8	culture was not continued#		
Test item	293.8	+	94.1	117.1	167	257	72.0	79.6	164	237
Test item	587.5	+	145.7	106.7	144	257	52.1	83.6	173	237
Test item	1175.0	+	137.7	86.7	165	257	50.6	82.4	133	237
Test item	2350.0	+	101.5	118.7	175	257	44.6	83.9	137	237
Test item	4700.0	+	139.6	114.0	119	257	53.6	82.6	118	237
Experiment III / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	144	270	100.0	100.0	80	206
Pos. control with MMS	19.5	-	27.3	10.4	1488	270	30.0	12.7	581	206
Test item	293.8	-	69.4	culture was not continued#			44.9	culture was not continued#		
Test item	587.5	-	75.0	139.0	139	270	48.2	68.8	103	206
Test item	1175.0	-	87.4	161.5	79	270	127.7	82.4	78	206
Test item	2350.0	-	75.0	148.2	79	270	44.2	46.6	126	206
Test item	3525.0	-	40.2	170.7	88	270	54.0	60.9	100	206
Test item	4700.0	-	61.4	117.1	121	270	54.8	69.8	94	206

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126

\# culture was not continued since a minimum of only four analysable concentrations is required

Conclusion

In the mouse lymphoma thymidine kinase locus assay test substance SYN548738 did not induce mutations using the cell line L5178Y in the absence and presence of metabolic activation. Therefore, SYN548738 is considered to be non-mutagenic in this mouse lymphoma assay.

Data requirement 2.42c

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse lymphoma assay. The results will be discussed in an experts' meeting.

Response by notifier to the above data requirement:

"A single isolated increase in MF was observed in Experiment II Culture I in the absence of metabolic activation. This was associated with an unacceptable level of cytotoxicity (RTG <10.0) and so is considered not biologically relevant. Additionally, no relevant increase in MF was observed in five other cultures across three experiments under the same treatment conditions. On this basis SYN548738 is considered inactive in this assays and the study is reported as negative. SYN548738 is considered to be negative in the in vitro mammalian gene mutation assay conducted in L5178y TK+/- cells. "

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

In the Expert meeting, the results of this study were discussed and it was concluded that the response is considered negative.

Genotoxicity SYN548580 – in vitro

B.6.8.1 – 6.4.48 Reverse mutation assay in bacteria with SYN548580 - study 48

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/35 Sokolowski A (2015i) SYN548580 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1677501, issue date: 09 March 2015. Unpublished Syngenta File No. SYN548580_10000.

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.

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

Study design

This study was performed to investigate the potential of SYN548580 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 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:

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

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

Test Material:	SYN548580
Description:	Off-White, solid
Lot/Batch number:	MES 374/1
Purity:	97.0 % (w/w)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	Recertification Date: 31 December 2016

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)

Results

No precipitation of the test item occurred up to the highest investigated dose. The plates incubated with the test substance 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 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 SYN548580 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 and all mutation rates were within the range of normal biological variability. Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

In experiment II the data in the untreated control of strain WP2 pKM101 without S9 mix were slightly under our historical control range. Since this deviation is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies.

Results are presented in Table 6.8.1 – 6.4.48-1 and -2.

Table 6.8.1 – 6.4.48-1 Summary of results of experiment I

Study Name: 1677501
Experiment: 1677501 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1677501
Date Plated: 28/01/2015
Date Counted: 02/02/2015

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	11 ± 2	24 ± 4	153 ± 5	198 ± 30	313 ± 5
	Untreated		10 ± 2	11 ± 2	25 ± 5	170 ± 7	217 ± 11	349 ± 11
	SYN548580	3 µg	10 ± 3	11 ± 3	29 ± 1	155 ± 9	198 ± 35	333 ± 27
		10 µg	11 ± 4	11 ± 1	26 ± 2	150 ± 5	194 ± 12	293 ± 46
		33 µg	9 ± 1	11 ± 4	21 ± 5	162 ± 23	193 ± 32	285 ± 19
		100 µg	10 ± 2	10 ± 3	26 ± 5	163 ± 18	176 ± 28	310 ± 26
		333 µg	8 ± 1	12 ± 3	20 ± 1	169 ± 23	235 ± 30	330 ± 9
		1000 µg	8 ± 1	10 ± 3	20 ± 8	171 ± 24	198 ± 3	350 ± 16
		2500 µg	8 ± 1	12 ± 3	29 ± 10	160 ± 1	177 ± 22	318 ± 4
		5000 µg	9 ± 3	7 ± 2	26 ± 2	155 ± 10	180 ± 6	366 ± 57
	NaN3	10 µg	1241 ± 44			2232 ± 366		
	4-NOPD	10 µg			306 ± 14			
	4-NOPD	50 µg		72 ± 4				
	MMS	2.0 µL					3749 ± 422	3585 ± 473
With Activation	DMSO		13 ± 4	18 ± 3	37 ± 3	128 ± 17	222 ± 2	348 ± 14
	Untreated		14 ± 3	20 ± 5	46 ± 4	181 ± 12	264 ± 40	344 ± 39
	SYN548580	3 µg	14 ± 5	19 ± 4	36 ± 5	134 ± 10	222 ± 27	378 ± 4
		10 µg	12 ± 4	15 ± 7	32 ± 7	148 ± 7	226 ± 7	326 ± 37
		33 µg	10 ± 4	20 ± 7	42 ± 12	122 ± 9	178 ± 12	356 ± 40
		100 µg	11 ± 2	18 ± 7	30 ± 4	131 ± 19	185 ± 47	347 ± 15
		333 µg	13 ± 4	22 ± 4	43 ± 10	143 ± 19	245 ± 42	359 ± 9
		1000 µg	13 ± 1	19 ± 2	33 ± 12	153 ± 17	223 ± 28	359 ± 12
		2500 µg	10 ± 3	21 ± 5	30 ± 7	132 ± 29	235 ± 74	343 ± 29
		5000 µg	12 ± 5	19 ± 3	35 ± 13	123 ± 13	218 ± 23	310 ± 13
	2-AA	2.5 µg	454 ± 31	204 ± 24	3134 ± 269	3539 ± 96		
	2-AA	10.0 µg					1430 ± 75	2229 ± 241

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 – 6.4.48-2 Summary of results of experiment II

Study Name: 1677501
Experiment: 1677501 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1677501
Date Plated: 10/02/2015
Date Counted: 13/02/2015

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 ± 2	8 ± 1	28 ± 2	163 ± 21	190 ± 26	360 ± 6
	Untreated		9 ± 1	11 ± 3	27 ± 7	202 ± 17	211 ± 12	392 ± 10
	SYN548580	33 µg	9 ± 2	9 ± 3	28 ± 5	158 ± 19	167 ± 15	338 ± 17
		100 µg	9 ± 2	8 ± 2	27 ± 9	162 ± 21	201 ± 9	330 ± 67
		333 µg	9 ± 2	9 ± 2	22 ± 2	170 ± 11	174 ± 8	374 ± 25
		1000 µg	8 ± 2	9 ± 1	30 ± 5	129 ± 13	186 ± 25	378 ± 33
		2500 µg	11 ± 3	7 ± 3	24 ± 5	138 ± 21	189 ± 16	384 ± 33
		5000 µg	10 ± 2	10 ± 2	31 ± 10	139 ± 17	167 ± 38	362 ± 16
	NaN3	10 µg	1202 ± 61			1800 ± 51		
	4-NOPD	10 µg			287 ± 47			
	4-NOPD	50 µg		86 ± 12				
	MMS	2.0 µL					3408 ± 379	2687 ± 132
With Activation	DMSO		10 ± 3	13 ± 1	35 ± 7	160 ± 37	221 ± 22	372 ± 12
	Untreated		13 ± 3	14 ± 4	40 ± 5	199 ± 7	268 ± 13	442 ± 1
	SYN548580	33 µg	10 ± 2	12 ± 1	32 ± 3	150 ± 8	207 ± 9	395 ± 26
		100 µg	13 ± 5	13 ± 2	28 ± 2	168 ± 22	234 ± 42	407 ± 31
		333 µg	11 ± 2	12 ± 2	31 ± 9	170 ± 13	201 ± 18	362 ± 33
		1000 µg	10 ± 2	12 ± 2	31 ± 8	133 ± 24	222 ± 4	398 ± 43
		2500 µg	12 ± 3	10 ± 3	33 ± 2	148 ± 7	225 ± 16	384 ± 13
		5000 µg	9 ± 3	11 ± 4	36 ± 8	155 ± 8	180 ± 22	396 ± 29
	2-AA	2.5 µg	343 ± 50	208 ± 19	2752 ± 469	3834 ± 644		
	2-AA	10.0 µg					1189 ± 46	1905 ± 220

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusion

In the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay test substance SYN548580 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN548580 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.49 In vitro chromosome aberration assay with SYN548580 - study 49

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/36 Sokolowski, A. (2015j) SYN548580 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1677502, issue date: 30 July 2015. Unpublished. Syngenta File No. SYN548580_10004

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of SYN548580 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/β-naphthoflavone treated male rats). Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods were 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance. In each treatment group two parallel cultures were analysed. 150 metaphases per culture were evaluated for structural chromosomal aberrations, except for the positive controls in. 1000 cells per culture were counted for determination of mitotic index.
The highest treatment concentration in this study, 2060.0 µg/mL was chosen with regard to the purity (97 %) of the test substance and with respect to the current OECD Guideline 473 (2014).
Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473 (2014).

Test Material:	SYN548580
Description:	Off-white solid
Lot/Batch number:	MES 374/1
Purity:	97 % w/w
CAS#:	-
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO 0.5%
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 770 µg/mL (Exp. I), 550 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL

Results

The pre-experiment is reported as the main experiment I since the criteria mentioned under Acceptability of the assay were met. Precipitation of the test substance in the culture medium was observed in Experiment I and II in the absence of S9 mix at 2060.0 µg/mL at the end of treatment indicating the maximum concentration tested under these conditions was also at the limit of solubility. The osmolarity and pH, measured with osmometer and pH meter were determined in the solvent control and the maximum concentration without and with metabolic activation: The pH was adjusted to physiological values using small amounts of 2 M NaOH. No relevant influence on the osmolarity was observed. 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 statistically significant or biologically relevant increase in structural chromosomal aberrations was observed at the concentrations evaluated. The aberration rates of the cells after treatment with the test substance in Experiment I with and without S9 mix and Experiment II with S9 mix (0.3 – 2.7 % aberrant cells, excluding gaps) were close to the solvent control values (1.0 – 2.3 % aberrant cells, excluding gaps) and within the range of the laboratory historical solvent control data (see Appendix I).
In Experiment I in the absence of S9 mix the value at 1177.1 µg/mL and in Experiment II in absence and presence of S9 mix the value at 1177.1 µg/mL and 2060.0 µg/mL; respectively, exceeded the two-fold standard deviation 95 % control limit of the historical control data (with and without S9 pulse treatment: 2.6 % aberrant cells, excluding gaps; without S9 continuous exposure: 2.3 % aberrant cells, excluding gaps). These findings were judged as biologically irrelevant since no statistically significant increase in relation to the concurrent controls were observed.
In Experiment II in the absence of S9 mix after continuous treatment with 1177.1 µg/mL one single increase in chromosomal aberrations (3.3 % aberrant cells, excluding gaps), slightly above the range of the laboratory historical solvent control data (0.0 – 3.0 % aberrant cells, excluding gaps) and outside the two-fold standard deviation 95 % control limits (2.3 %, excluding gaps) was observed. Since the value was not statistically significant and no apparent dose-dependency was observed, the finding was also regarded as biologically irrelevant.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (550.0 or 770.0 µg/mL) or CPA (2.5 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations in agreement with the laboratory control data.

The results of the chromosome aberration test are given in Table 6.8.1 – 6.4.49.

Table 6.8.1 – 6.4.49 Summary of results of the chromosome aberration test

Exp.	Preparation interval	Test item concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%) incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	1.7	1.0	0.0
		Positive control[2]	90.6	13.0	**13.0[S]**	2.7
		672.7	100.0	1.3	1.0	0.3
		1177.1	106.1	2.7	2.7	0.0
		2060.0[P]	104.7	2.3	1.7	0.0
Exposure period 22 h without S9 mix						
II	22 h	Solvent control[1]	100.0	2.7	2.3	0.0
		Positive control[3]	42.5	20.3	**20.3[S]**	5.3
		672.7	101.2	2.3	2.0	0.0
		1177.1	103.6	3.3	3.3	0.3
		2060.0[P]	84.1	1.3	1.0	0.0
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	1.7	1.7	0.0
		Positive control[4]	84.8	8.3	**8.0[S]**	0.3
		384.4	80.0	2.0	2.0	0.0
		672.7	93.9	0.3	0.3	0.0
		2060.0	111.5	1.0	1.0	0.0
II	22 h	Solvent control[1]	100.0	2.3	2.3	0.0
		Positive control[4]	44.3	10.3	**9.7[S]**	1.3
		672.7	90.8	1.0	0.3	0.0
		1177.1	103.0	2.3	1.7	0.0
		2060.0	93.4	3.0	2.7	0.7

* Including cells carrying exchanges
P Precipitation occurred at the end of treatment
S Aberration frequency statistically significant higher than corresponding control values
1 DMSO 0.5 % (v/v)
2 EMS 770.0 µg/mL
3 EMS 550.0 µg/mL
4 CPA 2.5 µg/mL

Conclusion

In the chromosome aberration test the test substance SYN548580 did not induce structural chromosomal aberrations in human lymphocytes *in vitro*. Therefore, SYN548580 is considered to be non-clastogenic in this chromosome aberration test, when tested up to the highest applied concentration.

B.6.8.1 – 6.4.50 In vitro cell mutation assay in mouse lymphoma cells with SYN548580 - study 50

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/37 Wollny H (2015c), SYN548580 - 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. 1677503, issue date: 10 April 2015. Unpublished. Syngenta File No. SYN548580_10002.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The study was performed to investigate the potential of SYN548580 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 metabolic activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations with and without metabolic activation: 182.5; 365.0; 730.0; 1460.0; and 2920 µg/mL

The maximum concentration of the pre-experiment was 2180 µg/mL, based on the solubility properties of the test substance in DMSO. As neither cytotoxicity nor precipitation was noted up to the maximum concentration, the concentration of DMSO in culture medium of the main experiments was raised to 1% to increase the maximum concentration of the test item to 2920 µg/mL or 10 mM.

Test Material:	SYN548580
Description:	Off-white, solid
Lot/Batch number:	MES 374/1
Molecular weight:	283.5 g/mol
Purity	97 % w/w (estimated error ± 2 %), concentration calculation not adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 % in the pre-experiment and 1.0% in the main experiments)
Positive control:	Absence of S9 mix: Methylmethanesulphonate (MMS), 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

Based on the results of the pre-test at least four adequate concentrations were chosen for the mutation experiment. No relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both parallel cultures occurred in experiments I and II. No precipitation of the test item was noted at the end of treatment with and without metabolic activation.

No substantial or reproducible dose dependent increase of the mutation frequency exceeding the threshold of 126 above the corresponding solvent control was observed with and without metabolic activation. The threshold of 126 above the corresponding solvent control was exceeded in the second culture of experiment I at 182.5, 1460, and 2920 µg/mL without metabolic activation. This increase however, was not reproduced in the parallel culture or in the second experiment without metabolic activation. Furthermore, the increase was not dose dependent, as indicated by lack of statistical significance in the applied trend test.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using RStudio (Version 0.98, RStudio Inc.). No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

In this study the range of the solvent controls was from 62 up to 156 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 53 up to 352 mutant colonies per 10^6 cells.

MMS (19.5 µg/mL) and CPA (3.0 and 4.5 µg/mL) were used as positive controls, which showed a distinct increase in induced total mutant colonies at acceptable levels of toxicity with at least one of the concentrations of the controls.

The relative cloning efficiency II (viability) slightly exceeded the upper limit of the acceptance criteria by 1% (121% versus a limit of 120%) in the first culture of the first experiment with metabolic activation and in the second culture of the second experiment with metabolic activation. This deviation was judged as irrelevant as it was only marginally above the acceptance criteria limit of 120%, and the viability of the parallel cultures remained well within the acceptable range.

The results of the TK assay are given in Table 6.8.1 – 6.4.50.

Table 6.8.1 – 6.4.50 Summary of results of the TK assay, Experiment I and II

			relative	relative	mutant		relative	relative	mutant	
	conc. µg	S9	cloning	total	colonies/		cloning	total	colonies/	
	per mL	mix	efficiency 1	growth	10^6 cells	threshold	efficiency 1	growth	10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	104	230	100.0	100.0	156	282
Pos. control with MMS	19.5	-	24.3	18.3	933	230	47.1	27.8	710	282
Test item	91.3	-	119.8	culture was not continued#			91.4	culture was not continued#		
Test item	182.5	-	136.5	63.1	193	230	101.5	65.9	290	282
Test item	365.0	-	94.4	128.2	119	230	83.8	69.1	186	282
Test item	730.0	-	86.6	86.0	126	230	94.2	77.0	185	282
Test item	1460.0	-	110.9	64.1	171	230	91.4	61.0	297	282
Test item	2920.0	-	95.7	82.6	105	230	115.2	59.1	352	282
Solv. control with DMSO		+	100.0	100.0	76	202	100.0	100.0	89	215
Pos. control with CPA	3.0	+	43.6	35.1	431	202	56.2	42.3	459	215
Pos. control with CPA	4.5	+	33.7	28.7	463	202	70.5	32.7	497	215
Test item	91.3	+	124.1	culture was not continued#			105.2	culture was not continued#		
Test item	182.5	+	73.4	99.8	76	202	124.2	102.4	69	215
Test item	365.0	+	47.0	63.7	116	202	115.0	157.0	53	215
Test item	730.0	+	30.3	57.0	130	202	108.9	82.7	111	215
Test item	1460.0	+	77.7	36.1	154	202	98.4	142.5	54	215
Test item	2920.0	+	67.4	60.5	95	202	105.2	82.2	89	215
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	149	275	100.0	100.0	92	218
Pos. control with MMS	19.5	-	61.2	22.3	492	275	70.2	25.7	377	218
Test item	91.3	-	85.7	culture was not continued#			98.3	culture was not continued#		
Test item	182.5	-	92.3	78.9	177	275	113.3	84.3	138	218
Test item	365.0	-	84.2	87.8	188	275	92.1	74.3	148	218
Test item	730.0	-	90.5	58.8	254	275	107.2	85.3	113	218
Test item	1460.0	-	94.1	89.5	209	275	98.3	74.0	126	218
Test item	2920.0	-	66.0	82.0	249	275	89.2	64.6	167	218
Solv. control with DMSO		+	100.0	100.0	85	211	100.0	100.0	62	188
Pos. control with CPA	3.0	+	79.6	65.9	470	211	79.3	38.3	384	188
Pos. control with CPA	4.5	+	77.2	42.1	517	211	71.7	29.1	444	188
Test item	91.3	+	105.6	culture was not continued#			106.9	culture was not continued#		
Test item	182.5	+	93.3	67.0	134	211	98.4	89.3	88	188
Test item	365.0	+	107.6	61.1	114	211	95.3	78.2	92	188
Test item	730.0	+	103.7	65.1	101	211	103.4	87.6	66	188
Test item	1460.0	+	101.8	52.0	120	211	101.7	68.8	98	188
Test item	2920.0	+	84.7	81.5	145	211	96.8	102.6	74	188

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126

\# culture was not continued as a minimum of only four analysable concentrations is required

CONCLUSION:

In the mouse lymphoma thymidine kinase locus assay the test substance SYN548580 did not induce mutations using the cell line L5178Y in the absence and presence of metabolic activation. Therefore, SYN548580 is considered to be non-mutagenic in this mouse lymphoma assay.

Data requirement 2.42d

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse

lymphoma assay. The results will be discussed in an experts' meeting.

Response by notifier to the above data requirement:

"In Experiment I Culture II, in the absence of metabolic activation, isolated increases in MF were observed at 1460 and 2920 µg/mL. These increases were not observed in the parallel culture or in Experiment II under the same treatment conditions and due to their lack of reproducibility are considered not biologically relevant. Furthermore, the mean MF (234 and 228) were below the Culture I and II mean GEF (256) and so do not meet the criteria for a positive response. Hence, for these reasons the isolated, non-reproducible increases in MF are considered not biologically relevant and SYN548580 does not show a mutagenic effect in this assay. SYN548580 is considered to be negative in the in vitro mammalian gene mutation assay conducted in L5178y TK+/- cells."

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

In the Expert meeting, the results of this study were discussed and it was concluded that the response is considered negative.

Genotoxicity SYN548764 – in vitro

Comment by RMS:

After the Ames, in vitro chromosome aberration, in vitro mouse lymphoma and in vivo micronucleus assays had been performed for metabolite SYN548764 (M11, SYN548581), the notifier discovered that the tested substance was not correctly synthesized and was actually the alternative isomer R417888 (M12). Therefore the studies presented below (B.6.8.1 – 6.4.51/54) should be regarded as additional data generated on R417888.

B.6.8.1 – 6.4.51 Reverse mutation assay in bacteria with SYN548764[3] - study 51

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

[3] Comment by RMS: After the Ames, in vitro chromosome aberration, in vitro mouse lymphoma and in vivo micronucleus assays had been performed for metabolite SYN548764 (M11, SYN548581), the notifier discovered that the tested substance was not correctly synthesized and was actually the alternative isomer R417888 (M12). Therefore the studies presented below should be regarded as additional data generated on R417888.

Report:	K-CA 5.8.1/38 Sokolowski (2015k) SYN548764 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1677401, issue date: 14 April 2015. Unpublished Syngenta File No. SYN548764_10000.

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.

Acceptability: The study was performed in accordance with OECD guideline 471 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The test substance SYN548764 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:

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

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

Test Material:	SYN548764
Description:	White, solid
Lot/Batch number:	MES 377/1
Purity:	95.0% w/w (estimated error ± 2%)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	28 February 2017
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)

Results

No precipitation of the test item occurred up to the highest investigated concentration.

The plates incubated with the test substance 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 any of the bacterial strains tested neither with nor without metabolic activation.

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN548764 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 and all mutation rates were within the range of normal biological variability.

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

The results of the study are given in Tables 6.8.1 – 6.4.51-1 and -2.

Table 6.8.1 – 6.4.51-1 Summary of results, Experiment I

Study Name: 1677401
Experiment: 1677401 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1677401
Date Plated: 03/03/2015
Date Counted: 06/03/2015

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	10 ± 2	20 ± 1	183 ± 13	193 ± 4	367 ± 16
	Untreated		15 ± 1	14 ± 2	31 ± 9	194 ± 17	222 ± 9	371 ± 19
	SYN548764	3 µg	9 ± 1	9 ± 0	24 ± 7	181 ± 4	164 ± 8	360 ± 11
		10 µg	11 ± 3	9 ± 0	21 ± 4	171 ± 3	172 ± 15	352 ± 29
		33 µg	10 ± 3	8 ± 2	19 ± 6	173 ± 6	164 ± 14	362 ± 18
		100 µg	9 ± 0	11 ± 4	23 ± 6	186 ± 7	182 ± 10	378 ± 39
		333 µg	9 ± 1	9 ± 3	26 ± 4	176 ± 15	184 ± 19	340 ± 12
		1000 µg	10 ± 2	8 ± 2	22 ± 2	158 ± 18	177 ± 6	367 ± 10
		2500 µg	8 ± 1	6 ± 2	23 ± 3	196 ± 23	171 ± 2	347 ± 22
		5000 µg	8 ± 1	6 ± 2	24 ± 3	185 ± 11	186 ± 35	356 ± 26
	NaN3	10 µg	1218 ± 132			1704 ± 129		
	4-NOPD	10 µg			283 ± 29			
	4-NOPD	50 µg		69 ± 9				
	MMS	2.0 µL					3597 ± 361	4059 ± 393
With Activation	DMSO		11 ± 2	14 ± 5	31 ± 4	140 ± 6	211 ± 11	371 ± 19
	Untreated		10 ± 3	19 ± 6	34 ± 7	163 ± 2	296 ± 30	396 ± 18
	SYN548764	3 µg	10 ± 3	14 ± 3	27 ± 3	135 ± 19	232 ± 5	391 ± 20
		10 µg	11 ± 5	14 ± 4	26 ± 6	127 ± 7	214 ± 13	406 ± 29
		33 µg	10 ± 4	12 ± 4	24 ± 2	129 ± 14	189 ± 18	400 ± 24
		100 µg	12 ± 2	12 ± 3	31 ± 4	125 ± 16	198 ± 27	388 ± 10
		333 µg	10 ± 3	13 ± 4	26 ± 9	156 ± 8	201 ± 15	385 ± 10
		1000 µg	9 ± 3	14 ± 4	34 ± 6	160 ± 17	207 ± 13	379 ± 18
		2500 µg	9 ± 4	9 ± 1	24 ± 3	158 ± 8	212 ± 32	364 ± 23
		5000 µg	11 ± 2	13 ± 4	25 ± 7	138 ± 7	198 ± 14	367 ± 36
	2-AA	2.5 µg	368 ± 15	202 ± 5	3609 ± 243	3645 ± 311		
	2-AA	10.0 µg					1269 ± 88	1712 ± 175

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 – 6.4.51-2 Summary of results, Experiment II

Study Name: 1677401
Experiment: 1677401 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1677401
Date Plated: 20/03/2015
Date Counted: 23/03/2015

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		12 ± 4	9 ± 1	20 ± 4	151 ± 19	197 ± 22	332 ± 26
	Untreated		12 ± 4	10 ± 1	18 ± 4	197 ± 7	218 ± 17	351 ± 10
	SYN548764	33 µg	10 ± 2	7 ± 2	21 ± 7	136 ± 13	167 ± 22	297 ± 17
		100 µg	10 ± 3	10 ± 4	22 ± 6	147 ± 22	164 ± 11	322 ± 53
		333 µg	10 ± 3	7 ± 1	20 ± 1	136 ± 34	162 ± 14	308 ± 26
		1000 µg	10 ± 2	7 ± 1	21 ± 7	132 ± 8	155 ± 24	278 ± 32
		2500 µg	11 ± 3	10 ± 2	19 ± 4	127 ± 3	153 ± 8	267 ± 28
		5000 µg	8 ± 4	8 ± 2	22 ± 7	111 ± 12	153 ± 11	297 ± 20
	NaN3	10 µg	1137 ± 81			1720 ± 156		
	4-NOPD	10 µg			318 ± 41			
	4-NOPD	50 µg		72 ± 12				
	MMS	2.0 µL					3218 ± 183	2531 ± 104
With Activation	DMSO		9 ± 1	11 ± 2	27 ± 6	154 ± 17	205 ± 10	347 ± 25
	Untreated		10 ± 2	10 ± 3	28 ± 1	201 ± 20	252 ± 31	377 ± 12
	SYN548764	33 µg	10 ± 3	14 ± 2	25 ± 7	132 ± 9	185 ± 10	353 ± 11
		100 µg	10 ± 3	13 ± 4	27 ± 6	143 ± 33	195 ± 12	353 ± 23
		333 µg	10 ± 2	12 ± 4	29 ± 6	145 ± 14	230 ± 19	364 ± 34
		1000 µg	10 ± 2	11 ± 3	35 ± 5	150 ± 15	196 ± 19	360 ± 42
		2500 µg	10 ± 3	9 ± 6	28 ± 4	140 ± 6	198 ± 20	319 ± 39
		5000 µg	9 ± 2	12 ± 1	32 ± 5	140 ± 18	220 ± 28	393 ± 83
	2-AA	2.5 µg	394 ± 55	126 ± 26	4207 ± 471	3766 ± 147		
	2-AA	10.0 µg					1228 ± 29	1860 ± 68

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusion

In the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay the test substance SYN548764 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN548764 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.52 In vitro chromosome aberration assay with SYN548764[4] - study 52

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/39 Sokolowski, A. (2015l) SYN548764 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1677402, issue date: 30 July 2015. Unpublished. Syngenta File No. SYN548764_10006

[4] Comment by RMS: After the Ames, in vitro chromosome aberration, in vitro mouse lymphoma and in vivo micronucleus assays had been performed for metabolite SYN548764 (M11, SYN548581), the notifier discovered that the tested substance was not correctly synthesized and was actually the alternative isomer R417888 (M12). Therefore the studies presented below should be regarded as additional data generated on R417888.

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of SYN548764 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/β-naphthoflavone treated male rats). Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods were 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance. In each treatment group two parallel cultures were analysed. At least 150 metaphases per culture were evaluated for structural chromosomal aberrations. 1000 cells per culture were counted for determination of mitotic index.

The highest treatment concentration in this study, 3700.0 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the purity (95 %) of the test substance and with respect to the current EPA and EU test guidelines meeting and exceeding the maximum concentration required by the OECD Guideline 473 (2014) and in agreement with OECD guideline 473 (1997).

Test Material:	SYN548764
Description:	White solid
Lot/Batch number:	MES 375/1
Purity:	95 % w/w
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	Deionised water
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825 µg/mL (Exp. I), 550 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL

Results

No precipitation of the test substance in the culture medium was observed. The osmolarity and pH, measured with osmometer and pH meter were determined in the solvent control and the maximum concentration without metabolic activation: No relevant influence on the osmolarity or pH was observed. 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 clear cytotoxicity was observed at the highest applied and evaluated concentration.

In Experiment I with and without metabolic activation neither a statistically significant nor a biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed at the concentrations evaluated. The aberration rates of the cells after treatment with the test substance (1.0 – 2.7 % aberrant cells, excluding gaps) exceeded the solvent control values (1.7 – 2.3 % aberrant cells, excluding gaps) and the two-fold 95 % control limits of the solvent control (2.6 %, without gaps), but were not statistically significant and within the range of the laboratory historical solvent control data (0 – 3.0% aberrant cells, excluding gaps) and therefore judges as biologically irrelevant.
In Experiment II without metabolic activation after treatment with 3700 µg/mL one statistically significant increase in chromosomal aberrations (6.0 % aberrant cells, excluding gaps), clearly exceeding the two-fold 95 % control limits of the solvent control (2.3 %, without gaps) as well as the range of the laboratory historical solvent control data (0.0 – 3.0 % aberrant cells, excluding gaps) was observed. Clear cytotoxicity (mean MI 39.7%) was observed only just short of the 40% MI value but judged to be acceptable, however one culture displayed more overt cytotoxicity (MI 32.4% of control). While these data may be considered relevant under the EPA and EU test guidelines which requires testing up to 5000 mg/mL or 10 mM, they are considered to be of limited biological relevance when judged against the most recent version of the OECD 473 (2014) test guideline for this assay. A trend test was not performed because the number of evaluated concentrations was too small to give a significant result. Also the shape of the dose response curve was indicative of a high concentration /cytotoxic response, and hence a trend test may be of limit relevance in assessing this dose response.
With metabolic activation one increase in chromosomal aberrations (3.8 % aberrant cells, excluding gaps), above the range of the laboratory historical solvent control data (0.0 – 3.0 % aberrant cells, excluding gaps) was observed after treatment with 2114.3 µg/mL. The value was also outside of the two-fold 95 % control limits (2.6 %, excluding gaps). However, the increase was not statistically significant when compared to the concurrent solvent control and not dose dependent it was judged as biologically irrelevant. The rate of chromosomal aberrations in the solvent control of Experiment II with S9 was slightly outside the two-fold 95 % control limits (2.6 %, without gaps), but still within the range of the historical solvent control (0.0 – 3.0 %, excluding gaps) and therefore the assay was considered valid.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (550.0 or 825.0 µg/mL) or CPA (15.0 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations in agreement with the laboratory control data. The resuls of the chromosome aberration test are given in Table 6.8.1 – 6.4.52.

Table 6.8.1 – 6.4.52 Summary of results

Exp.	Preparation interval	Test item concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%) incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 hrs without S9 mix						
I	22 h	Solvent control[1]	100.0	2.0	1.7	0.0
		Positive control[2]	65.7	8.3	**7.3**[S]	1.0
		1208.2	88.6	1.7	1.7	0.0
		2114.3	101.8	1.3	1.3	0.0
		3700.0	85.2	2.7	2.7	0.0
Exposure period 22 hrs without S9 mix						
II	22 h	Solvent control[1]	100.0	1.3	1.3	0.3
		Positive control[3]	44.5	18.3	**18.0**[S]	2.3
		1208.2	70.8	2.3	2.0	0.0
		2114.3	66.3	2.7	2.7	0.0
		3700.0	39.7	6.7	**6.0**[S]	0.0
Exposure period 4 hrs with S9 mix						
I	22 h	Solvent control[1]	100.0	2.3	2.3	0.0
		Positive control[4]	67.5	15.7	**15.3**[S]	2.3
		1208.2	97.9	2.0	1.3	0.0
		2114.3	106.8	1.0	1.0	0.0
		3700.0	112.0	1.7	1.0	0.0
II	22 h	Solvent control[1]	100.0	3.7	3.0	0.3
		Positive control[4]	65.3	16.3	**15.7**[S]	1.7
		1208.2	97.4	3.0	2.7	0.3
		2114.3[##]	94.0	4.5	3.8	0.2
		3700.0	99.6	3.0	2.3	0.3

* Including cells carrying exchanges
Evaluation of 300 metaphases per culture
S Aberration frequency statistically significant higher than corresponding control values
1 Deion. water 10.0 % (v/v)
2 EMS 825.0 µg/mL
3 EMS 550.0 µg/mL
4 CPA 15.0 µg/mL

Conclusion

In the chromosome aberration test the test substance SYN548764 induced structural chromosomal aberrations in human lymphocytes *in vitro* in the absence of a metabolic activation system at one high cytotoxic concentration. SYN548764 was tested up to 3700 µg/plate in accordance with 10 mM as required by the EPA (1998) and EU (2008) test guidelines, at higher concentration than required by the OECD 473 guideline (2014). Therefore, SYN548764 is considered to display equivocal clastogenic activity in this chromosome aberration test, when tested up to the highest applied concentration.

B.6.8.1 – 6.4.53 In vitro cell mutation assay in mouse lymphoma cells with SYN548764[5] - study 53

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/40 Wollny H (2015d) SYN548764 - 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. 1677403 issue date: 04 May 2015. Unpublished. Syngenta File No SYN548764_10002.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design

The study was performed to investigate the potential of SYN548764 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 metabolic activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations

Experiment I:	
without metabolic activation:	231.3; 462.5; 693.8; 925.0; and 1387.5 µg/mL
with metabolic activation:	231.3; 462.5; 925.0; 1850.0; and 3700.0 µg/mL
Experiment II:	
without metabolic activation:	400.0; 800.0; 1050.0; and 1400.0 µg/mL
with metabolic activation:	400.0; 800.0; 1600.0; 2400.0; and 3700.0 µg/mL

The maximum concentration of the test item in the pre-experiment and in both main experiments, 3700 µg/mL equal to approximately 10 mM, was based on the purity (95% w/w) and molecular weight of the test item (351.5 g/mol).

[5] Comment by RMS: After the Ames, in vitro chromosome aberration, in vitro mouse lymphoma and in vivo micronucleus assays had been performed for metabolite SYN548764 (M11, SYN548581), the notifier discovered that the tested substance was not correctly synthesized and was actually the alternative isomer R417888 (M12). Therefore the studies presented below should be regarded as additional data generated on R417888.

Test Material:	SYN548764
Description:	White, solid
Lot/Batch number:	MES 377/1
Molecular weight:	351.5 g/mol
Purity	95 % w/w (estimated error ± 2 %), concentration calculation adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	Deionised water (10 %)
Positive control:	Absence of S9 mix: Methylmethanesulphonate, 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

Based on the results of the pre-test at least four adequate concentrations were chosen for the mutation experiment. Precipitation occurred at the two highest concentrations in experiment II with metabolic activation. Relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in experiment I at 693.8 µg/mL and above without metabolic activation and in experiment II at 800.0 µg/mL and above without metabolic activation. The recommended cytotoxic level of approximately 10% -20% relative total growth was covered without metabolic activation.

No substantial or reproducible increase of the mutation frequency was noted in the main experiments with and without metabolic activation. The threshold of 126 above the mutation frequency of the solvent control was solely exceeded at the two highest concentrations of the first experiment, culture II without metabolic activation (276 and 264 compared to 250 colonies per 10^6 cells). However, the increase was not reproduced in the parallel culture under identical conditions -or in the second experiment without metabolic activation at comparable levels of cytotoxicity.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was solely detected in the second culture of the first experiment without metabolic activation. This trend was judged as biologically irrelevant as discussed above.

In this study the range of the solvent control values was from 65 up to 160 mutant colonies per 10^6 cells; the range of the group values treated with the test substance was from 59 up to 276 mutant colonies per 10^6 cells.

The cloning efficiency 2 slightly exceeded the upper limit of 120% in the solvent control of the second culture of the second experiment without metabolic activation. The deviation was judged as irrelevant as it was very minor (121% compared to an upper limit of 120%) and the cloning efficiency 2 of the parallel culture remained well within the acceptable range (106%).

MMS (19.5 µg/mL) and CPA (3.0 and 4.5 µg/mL) were used as positive control chemicals 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.

The resuls of the TK assay are given in Table 6.8.1 – 6.4.53.

Table 6.8.1 – 6.4.53 Summary of results

			relative	relative	mutant		relative	relative	mutant	
	conc. µg	S9	cloning	total	colonies/		cloning	total	colonies/	
	per mL	mix	efficiency 1	growth	10^6 cells	threshold	efficiency 1	growth	10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	160	286	100.0	100.0	124	250
Pos. control with MMS	19.5	-	45.5	23.6	850	286	77.9	31.8	558	250
Test item	115.6	-	91.9	culture was not continued#			82.7	culture was not continued#		
Test item	231.3	-	95.0	89.0	110	286	83.9	95.8	86	250
Test item	462.5	-	54.7	74.7	145	286	64.5	73.8	124	250
Test item	693.8	-	48.9	56.3	161	286	64.5	41.9	243	250
Test item	925.0	-	47.5	38.8	92	286	37.2	28.2	276	250
Test item	1387.5	-	11.6	12.7	123	286	11.1	7.3	264	250
Test item	1850.0	-	4.9	culture was not continued##			2.5	culture was not continued##		
Solv. control with water		+	100.0	100.0	103	229	100.0	100.0	111	237
Pos. control with CPA	3.0	+	101.4	91.9	293	229	100.0	46.1	279	237
Pos. control with CPA	4.5	+	101.4	95.4	333	229	62.8	32.4	503	237
Test item	115.6	+	127.3	culture was not continued#			86.0	culture was not continued#		
Test item	231.3	+	101.4	93.8	105	229	81.2	64.6	128	237
Test item	462.5	+	94.4	85.3	108	229	92.6	40.7	184	237
Test item	925.0	+	100.0	136.1	67	229	139.0	53.8	104	237
Test item	1850.0	+	129.1	93.5	119	229	111.9	69.0	122	237
Test item	3700.0	+	120.2	86.8	87	229	117.7	48.4	160	237
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with water		-	100.0	100.0	109	235	100.0	100.0	101	227
Pos. control with MMS	19.5	-	50.0	32.5	288	235	75.7	13.9	478	227
Test item	100.0	-	91.3	culture was not continued#			191.7	culture was not continued#		
Test item	200.0	-	80.0	76.0	95	235	188.5	53.6	96	227
Test item	400.0	-	61.0	36.6	125	235	116.9	52.5	111	227
Test item	800.0	-	17.2	7.0	149	235	12.4	5.3	189	227
Test item	1050.0	-	10.3	7.2	155	235	11.1	3.7	203	227
Test item	1400.0	-	42.8	23.2	135	235	55.8	12.8	123	227
Test item	1600.0	-	1.1	culture was not continued##			9.2	culture was not continued##		
Solvent control with water		+	100.0	100.0	65	191	100.0	100.0	95	221
Pos. control with CPA	3.0	+	46.9	27.4	241	191	33.7	20.4	494	221
Pos. control with CPA	4.5	+	31.6	14.7	731	191	21.3	12.1	748	221
Test item	200.0	+	103.6	culture was not continued#			79.7	culture was not continued#		
Test item	400.0	+	86.3	78.0	76	191	109.5	87.2	116	221
Test item	800.0	+	96.7	50.3	144	191	100.0	72.2	120	221
Test item	1600.0	+	86.3	58.0	96	191	70.8	63.5	144	221
Test item	2400.0 (P)	+	89.1	51.3	87	191	100.0	69.6	113	221
Test item	3700.0 (P)	+	72.0	62.8	59	191	93.4	48.4	100	221

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126
\# culture was not continued as a minimum of only four analysable concentrations is required
culture was not continued due to exceedingly severe cytotoxic effects
P = precipitation visible at the end of treatment

Conclusion
In the mouse lymphoma thymidine kinase locus assay the test substance SYN548764 did not induce mutations using the cell line L5178Y in the absence and presence of metabolic activation. Therefore, SYN548764 is considered to be non-mutagenic in this mouse lymphoma assay.

Data requirement 2.42e

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse lymphoma assay. The results will be discussed in an experts' meeting.

Response by notifier to the above data requirement:

"Two isolated increases in MF were observed in Experiment 1 Culture II in the absence of metabolic activation (925 and 1387.5 µg/mL). The RTG at 1387.5 µg/mL is considered to be excessively cytotoxic (RTG < 10%) and so this MF can be discounted as not biologically relevant. The MF observed at 925 µg/mL was not reproduced in the parallel culture under the same treatment conditions or in Experiment II at higher treatment concentrations (1050 and 1400 µg/mL). Furthermore, the mean Culture I and II MF in experiment I (184) was well below the mean Culture I and II GEF (268). For these reasons the isolated MF increase is considered to be not biologically relevant and SYN548764 is considered to give a negative response in this assay. SYN548764 is considered to be negative in the in vitro mammalian gene mutation assay conducted in L5178y TK+/- cells."

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

In the Expert meeting, the results of this study were discussed and it was concluded that the response is considered negative.

B.6.8.1 – 6.4.54a In vivo micronucleus test in mice with SYN548764[6] - study 54

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/42 Dunton, J. (2015a) SYN548764 – Oral (Gavage) Mouse Micronucleus Test. Sequani Ltd. Sequani Report No. BFI0404, issue date: 18 November 2015. Unpublished. Syngenta File No. SYN548764_10008.

GUIDELINES: Mouse 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.

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

Study design

[6] Comment by RMS: After the Ames, in vitro chromosome aberration, in vitro mouse lymphoma and in vivo micronucleus assays had been performed for metabolite SYN548764 (M11, SYN548581), the notifier discovered that the tested substance was not correctly synthesized and was actually the alternative isomer R417888 (M12). Therefore the studies presented below should be regarded as additional data generated on R417888.

SYN548764 was tested to evaluate its potential to cause damage to chromosomes or cell division apparatus, or to cause cell cycle interference, leading to micronucleus formation in polychromatic erythrocytes in the bone marrow of young adult mice.
In all phases, the dosing of the vehicle and test item was by oral (gavage) administration, on two consecutive occasions, approximately 24 hours apart. In the dose-sighting phase, three groups of two male mice were given SYN548764 as a suspension in 0.5 % w/v aqueous carboxymethylcellulose with 0.1 % v/v Tween 80 at 500, 1250 or 2000 mg/kg/day on two consecutive occasions, in order to determine the maximum tolerated dose (MTD). In the range-finding phase, a group of three male and three female mice were given SYN548764 at 2000 mg/kg/day, in order to confirm the MTD in both male and female mice. The MTD was confirmed to be greater than the limit dose of 2000 mg/kg/day in male and female mice, and as there was no inter-sex difference in toxicity, the main study was conducted in males only.
A proof of exposure phase was conducted to demonstrate that the bone marrow was exposed to the test item, via analysis of test item in the whole blood of treated animals. The presence of SYN548764 was confirmed by analysis of the study samples alongside samples of blank matrix and matrix spiked with the test item.
For the main study phase, three groups, each of six male mice were dosed with 500, 1000 or 2000 mg/kg/day SYN548764. A group of six male mice (negative Controls) was dosed with the vehicle alone and a positive Control group, also of six male mice, was given a single 4 mg/kg intraperitoneal dose of Mitomycin C (MMC).
Bone marrow was harvested from all range-finding and main study animals approximately 24 hours after the final dose administration and smears were prepared. The stained slides prepared for the main study were coded and 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei and the group frequencies were statistically analysed.

Test Material:	SYN548764
Description:	white to off white powder
Lot/Batch number:	MES 377/1
Purity:	95 % (w/w), concentration was adjusted for purity
Stability of test compound:	Retest date : 28 February 2017

Results

Preliminary toxicity assay: There were no clinical signs observed and no effect on body weight following administration of SYN548764 at dose levels up to 2000 mg/kg/day.
Based on the results of this phase, the MTD was considered to exceed the limit dose of 2000 mg/kg/day in males and females. As there was no difference between the MTD in males and females, the main study was conducted in male mice only.

Exposure to SYN548764 was confirmed in all range-finder blood samples. Bone marrow smears were not analysed in the range-finding phase since the presence of SYN548764 was confirmed in the blood samples.

Micronucleus test: There were no adverse clinical observations following administration of SYN548764 to male mice. There were no adverse clinical observations in Group 1 (negative Control) or in Group 5 (positive Control).

There were no statistically significant increases in micronucleus frequency in male mice treated at any dose level of SYN548764, compared with the concurrent negative Control group.

There was no evidence of a statistically significant reduction in the PCE/NCE ratio in male mice treated with SYN548764, and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of SYN548764 to the bone marrow.

The animals dosed with MMC, the positive Control item, had statistically significant increases in the number of micronucleated cells compared to the concurrent Control group, which demonstrated that the test system was capable of detecting a known clastogen and that the scorers were capable of detecting micronuclei.

There was no statistically significant decrease in the PCE/NCE ratio in the positive Control group, indicating a lack of toxicity to the bone marrow.

Conclusion

There was no evidence of clastogenicity or aneugenicity in male mice following oral (gavage) administration of SYN548764 up to the OECD 474 limit dose of 2000 mg/kg/day. SYN548764 is considered to be neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

B.6.8.1 – 6.4.54b Proof of exposure - In vivo MN test in mice with SYN548764[7] - study 54

Data requirement 2.30

Applicant to provide evidence of tissue exposure in the in vivo MN study with SYN548764 – study 54.

See also 2(92)
See reporting table 2(76)

NL (August 2017): The applicant provided proof of exposure for the in vivo mouse micronucleus test (Dunton, 2015a; study 54) by providing raw data on the presence of SYN548764 in blood samples. The RMS agrees with the conclusion that SYN548764 is systemically available and thus may reach the bone marrow.

[7] Comment by RMS: After the Ames, in vitro chromosome aberration, in vitro mouse lymphoma and in vivo micronucleus assays had been performed for metabolite SYN548764 (M11, SYN548581), the notifier discovered that the tested substance was not correctly synthesized and was actually the alternative isomer R417888 (M12). Therefore the studies presented below should be regarded as additional data generated on R417888.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

During the Expert meeting, the proof of bone marrow exposure in the mouse micronucleus assay is discussed. In this assay, no bone marrow toxicity was evident. Bone marrow exposure was demonstrated by detectable blood and plasma concentrations of R417888 in male Crl:CD-1 mice, though at 24 hours very low levels were detectable. Based on the presence of the metabolite in blood and plasma at 4 hours post dosing, no concern for clastogenicity exists. However, no conclusion can be drawn on aneugenicity and therefore a data gap was recognized.

The notifier provided the following evidence of tissue exposure in the in vivo MN study:
" The in vivo bone marrow micronucleus assay is a widely used in vivo genotoxicity test. In the case that a negative result is obtained evidence must be presented that the compound of interest was systemically available to the bone marrow. As the bone marrow is a well-perfused tissue, concentrations of compound-related material(s) in blood or plasma are generally similar to those observed in bone marrow. Therefore, proof of exposure can be obtained by measurement of the compound of interest either in blood or plasma.

Concern that the compound is not available for distribution into bone marrow because of irreversible binding to red blood cells can be addressed two ways. Either analyse for the compound in plasma or demonstrate that any binding to a matrix in whole blood (plasma or red blood cells) was reversible. The preparation of whole blood for analysis will yield reversibly bound compounds, but not those irreversibly bound and compound quantified will be that distributed in blood and plasma.
Blood samples were taken 1 and 4 hours following oral administration and again after the terminal blood sample approximately 24 hours after the second test item administration.
After sampling each blood sample was diluted with1 % (v/v) formic acid in acetonitrile [1:3 (v/v)]. Prior to analysis each sample was extracted using organic solvent. Following centrifugation, to pellet the protein, the resulting supernatant was analysed via mass spectrometry.

As shown in the table below, SYN548764 was freely available at concentrations much greater than the 40 ng/mL low standard and quantifiable over several time points demonstrating sustained exposure. It can therefore be concluded that SYN548764 was systemically available to allow bone marrow exposure.

SYN548764 Analytical Response and Retention Time Data

SYN548764		
Sample I.D.	Peak area counts	Retention time (min)
Blank	0.0	N/A
STD Low	10568.4	1.65
STD High	555450.9	1.64
Blank	0.0	N/A
RF1 71 M DAY 1 1h	1657871.3*	1.65
RF1 72 M DAY 1 1h	1253778.6*	1.64
RF1 73 M DAY 1 1h	1249976.5*	1.63
RF1 71 M DAY 1 4h	608962.8*	1.63
RF1 72 M DAY 1 4h	750327.8*	1.63
RF1 73 M DAY 1 4h	348532.5*	1.64
RF1 71 M DAY 1 TER	5962.2	1.64
RF1 72 M DAY 1 TER	4086.9	1.64
RF1 73 M DAY 1 TER	3729.9	1.64
RF1 74 F DAY 1 1h	2987609.3*	1.64
RF1 75 F DAY 1 1h	2358316.9*	1.64
RF1 76 F DAY 1 1h	2493394.9*	1.63
RF1 74 F DAY 1 4h	1031999.3*	1.64
RF1 75 F DAY 1 4h	1276989.3*	1.64
RF1 76 F DAY 1 4h	1390574.7*	1.63
RF1 74 F DAY 1 TER	1445.7	1.64
RF1 75 F DAY 1 TER	16041.0*	1.64
RF1 76 F DAY 1 TER	5751.4	1.63
Blank	0.0	N/A
STD Low	12490.0	1.64
STD High	593930.4	1.63
Blank	0.0	N/A

*Study samples exhibit analytical response for SYN548764 greater than the mean response observed in the low spiked standard (40 ng/mL blood concentration)
TER = terminal sample

A re-examination of the analytical verification data for newly synthesized standard M11 revealed that this proposed structure had not been correctly synthesized. The synthesized standard thought to be M11 was actually the alternative isomer M12 (R417888), therefore the results originally presented in the MCA S5 for SYN548581 should be regarded as additional data generated on R417888. The MCA S5 has been updated accordingly to reflect this. "

Genotoxicity R611968 – in vitro

B.6.8.1 – 6.4.55 Reverse mutation assay in bacteria with R611968 - study 55

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/47 Sokolowski A (2015m) R611968 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1677801, issue date: 09 March 2015. Unpublished Syngenta File No.SYN548651_10000.

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.

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

Study design.

The test substance R611968 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:

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

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

Test Material:	R611968
Description:	Off-White, solid
Lot/Batch number:	DAH-XXIX-85-2
Purity:	99.5 % (HPLC Area Distribution)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	Recertification Date: 31 January 2017
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)

Results

No precipitation of the test item occurred up to the highest investigated concentration.

The plates incubated with the test substance 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 any of the bacterial strains tested neither with or without metabolic activation.

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with R611968 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 and all mutation rates were within the range of normal biological variability. Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.
The results of the study are given in Tables 6.8.1 – 6.4.55-1 and -2.

Table 6.8.1 – 6.4.55-1: Summary of the Amest test, experiment I

Study Name: 1677801
Experiment: 1677801 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1677801
Date Plated: 02/02/2015
Date Counted: 05/02/2015

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 ± 2	9 ± 3	22 ± 7	152 ± 5	201 ± 8	334 ± 14
	Untreated		9 ± 3	8 ± 3	24 ± 2	166 ± 17	229 ± 19	391 ± 14
	R611968	3 µg	12 ± 4	9 ± 1	21 ± 5	137 ± 17	174 ± 14	384 ± 33
		10 µg	13 ± 2	8 ± 1	24 ± 7	139 ± 9	157 ± 3	358 ± 39
		33 µg	10 ± 2	7 ± 1	21 ± 1	155 ± 13	188 ± 14	341 ± 23
		100 µg	11 ± 2	8 ± 1	22 ± 5	138 ± 9	177 ± 35	369 ± 15
		333 µg	11 ± 2	7 ± 2	22 ± 4	149 ± 15	200 ± 17	350 ± 40
		1000 µg	8 ± 2	9 ± 4	18 ± 1	165 ± 15	162 ± 20	305 ± 31
		2500 µg	11 ± 2	8 ± 1	22 ± 6	145 ± 23	124 ± 41	239 ± 10
		5000 µg	9 ± 3	6 ± 2	21 ± 3	153 ± 17	106 ± 40	220 ± 60
	NaN3	10 µg	1159 ± 133			2104 ± 224		
	4-NOPD	10 µg			296 ± 13			
	4-NOPD	50 µg		65 ± 11				
	MMS	2.0 µL					3641 ± 449	3909 ± 611
With Activation	DMSO		13 ± 1	17 ± 3	28 ± 1	120 ± 6	217 ± 29	371 ± 18
	Untreated		18 ± 3	19 ± 3	32 ± 2	130 ± 12	255 ± 26	377 ± 45
	R611968	3 µg	12 ± 2	14 ± 4	31 ± 9	107 ± 23	238 ± 54	409 ± 27
		10 µg	11 ± 4	13 ± 1	32 ± 3	119 ± 10	223 ± 25	402 ± 23
		33 µg	12 ± 3	15 ± 6	23 ± 6	106 ± 5	223 ± 29	353 ± 35
		100 µg	12 ± 3	16 ± 4	30 ± 9	121 ± 7	231 ± 22	356 ± 34
		333 µg	11 ± 3	18 ± 4	32 ± 3	127 ± 14	244 ± 25	369 ± 44
		1000 µg	8 ± 2	13 ± 4	20 ± 2	144 ± 20	219 ± 30	370 ± 13
		2500 µg	13 ± 1	11 ± 3	25 ± 11	141 ± 12	137 ± 8	270 ± 25
		5000 µg	14 ± 4	10 ± 3	21 ± 6	140 ± 2	142 ± 30	233 ± 48
	2-AA	2.5 µg	430 ± 33	203 ± 42	3954 ± 540	3537 ± 650		
	2-AA	10.0 µg					1113 ± 30	1716 ± 45

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 – 6.4.55-2: Summary of the Amest test, experiment II

Study Name: 1677801
Experiment: 1677801 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1677801
Date Plated: 12/02/2015
Date Counted: 16/02/2015

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 ± 1	12 ± 4	26 ± 6	137 ± 18	206 ± 19	339 ± 14
	Untreated		8 ± 0	13 ± 3	20 ± 4	197 ± 10	233 ± 15	403 ± 7
	R611968	33 µg	9 ± 3	12 ± 2	23 ± 1	151 ± 17	208 ± 8	365 ± 11
		100 µg	10 ± 3	12 ± 3	28 ± 8	151 ± 6	212 ± 32	347 ± 2
		333 µg	10 ± 1	9 ± 1	23 ± 4	134 ± 4	202 ± 19	355 ± 25
		1000 µg	9 ± 2	10 ± 2	29 ± 9	105 ± 12	174 ± 11	346 ± 27
		2500 µg	10 ± 3	9 ± 1	23 ± 8	155 ± 13	141 ± 15	323 ± 11
		5000 µg	9 ± 1	11 ± 1	26 ± 0	137 ± 2	109 ± 32	323 ± 63
	NaN3	10 µg	1253 ± 72			2101 ± 48		
	4-NOPD	10 µg			322 ± 69			
	4-NOPD	50 µg		79 ± 8				
	MMS	2.0 µL					3389 ± 92	2501 ± 114
With Activation	DMSO		10 ± 3	15 ± 2	39 ± 5	125 ± 13	242 ± 8	368 ± 16
	Untreated		10 ± 2	19 ± 5	31 ± 10	186 ± 12	302 ± 16	402 ± 13
	R611968	33 µg	11 ± 3	16 ± 5	40 ± 5	115 ± 20	252 ± 9	381 ± 13
		100 µg	11 ± 2	17 ± 1	39 ± 2	108 ± 3	252 ± 13	395 ± 9
		333 µg	10 ± 2	17 ± 2	32 ± 4	128 ± 26	249 ± 5	385 ± 16
		1000 µg	10 ± 2	19 ± 4	36 ± 11	131 ± 9	214 ± 31	392 ± 19
		2500 µg	9 ± 1	16 ± 2	36 ± 12	119 ± 7	152 ± 20	319 ± 36
		5000 µg	8 ± 2	15 ± 2	37 ± 1	107 ± 8	133 ± 20	357 ± 31
	2-AA	2.5 µg	401 ± 17	157 ± 21	3346 ± 564	3568 ± 510		
	2-AA	10.0 µg					1122 ± 138	2299 ± 165

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusion

In the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay, R611968 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. R611968 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.56 In vitro chromosome aberration assay with R611968 - study 56

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/48 Sokolowski, A. (2015n) R611968 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1677802, issue date: 04 August 2015. Unpublished. Syngenta File No. SYN548651_10004

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

GLP: Signed and dated GLP and Quality Assurance statements were provided.
Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of R611968 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/β-naphthoflavone treated male rats).
Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods were 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance. In each treatment group two parallel cultures were analysed. 150 metaphases per culture were evaluated for structural chromosomal aberrations. 1000 cells per culture were counted for determination of mitotic index.
The highest treatment concentration in this study, 2000.0 µg/mL was chosen with respect to the current OECD Guideline 473 (2014). Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473 (2014).

Test Material:	R611968
Description:	White solid
Lot/Batch number:	DAH-XXIX-85-2
Purity:	99.5 %
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	DMSO 0.5%
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825.0 µg/mL (Exp. I), 660 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 7.5 µg/mL

Results

No precipitation of the test substance in the culture medium was observed at the end of treatment. The osmolarity and pH, measured with osmometer and pH meter, were determined in the solvent control and the maximum concentration without metabolic activation:
No relevant influence on the osmolarity or pH was observed. In Experiment I in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration. In Experiment II in the absence and presence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentrations.

In Experiment I with or without metabolic activation neither a statistically significant nor a biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item. The aberration rates of the cells after treatment with the test substance (0.0 – 3.0 % aberrant cells, excluding gaps) were close to the solvent control values (1.3 – 2.3 % aberrant cells, excluding gaps) and although some values exceeded the two-fold 95 % control limits of the laboratory historical solvent control data (2.6 %, excluding gaps), these observed chromosomal aberrations were not statistically significant in relation to the concurrent solvent control.
In Experiment II in the absence of S9 mix, statistically significant increases were observed after treatment with 213.2 and 373.2 µg/mL (14.3 and 11.0 % aberrant cells, excluding gaps).
However, the mean MI value at 373.2 µg/mL (38.1%) is considered just acceptable, although it should be noted this is above the recommended maximum cytotoxicity for the assay and so the aberration frequency noted may be of limited biological relevance. A consideration of the types of chromosome damage reported only 1 exchange aberration was observed and this was of a chromosome rather than chromatid nature so may be considered less likely to be treatment related. In addition only a single chromatid gap aberration was noted with the majority of aberrations being chromatid breaks. In Experiment II in the presence of S9 mix, one statistically significant increase was observed after treatment with 2000.0 µg/mL (5.0 % aberrant cells, excluding gaps). These values exceeded the two-fold standard deviation 95 % control limit (with and without S9 pulse treatment: 2.6 % aberrant cells, excluding gaps; without S9 continuous exposure: 2.3 % aberrant cells, excluding gaps), as well as the range of the laboratory historical solvent control data (without S9 mix: 0.0 – 3.0 % aberrant cells, excluding gaps; with S9 mix: 0.0 – 3.5% aberrant cells, excluding gaps). Although for OECD 473 (2014) a repeat exposure was not strictly required, this was conducted to satisfy the EPA and EU test guidelines. It may be noted there is a lack of reproducibility between Experiment I and Experiment II (cytotoxicity and aberration frequencies) and therefore the observed response in Experiment II may be of questionable biological relevance.
A trend test was not performed because the number of evaluated concentrations was too small to give a significant result. No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (660.0 or 825.0 µg/mL) or CPA (7.5 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

The restuls of the study are given in Table 6.8.1 – 6.4.56.

Table 6.8.1 – 6.4.56: Summary of the chromosome aberration test

Exp.	Preparation interval	Test item concentration in µg/mL	Mitotic indices in % of control	Aberrant cells in %		
				incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	2.0	1.7	0.0
		Positive control[2]	82.1	11.7	**11.7**[S]	2.7
		13.2	109.7	1.7	1.3	0.0
		23.1	106.1	1.0	1.0	0.0
		40.3	97.8	1.3	1.0	0.0
Exposure period 22 h without S9 mix						
II	22 h	Solvent control[1]	100.0	0.7	0.7	0.0
		Positive control[3]	62.3	17.0	**16.7**[S]	7.0
		13.2	99.4	2.3	2.0	0.3
		23.1	91.6	2.3	2.3	0.3
		40.3	90.7	14.3	**13.7**[S]	0.0
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	3.0	2.3	0.0
		Positive control[4]	64.2	12.3	**10.0**[S]	0.3
		13.2	81.9	2.7	2.3	0.0
		23.1	72.7	3.7	3.0	0.3
		40.3	74.1	2.7	2.7	0.0
II	22 h	Solvent control[1]	100.0	1.7	1.7	0.0
		Positive control[4]	64.2	10.0	**10.0**[S]	0.3
		14.9	86.9	2.0	2.0	0.0
		29.8	78.4	4.0	3.3	0.3
		59.6	102.5	8.0	**7.3**[S]	1.0

* 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 550.0 µg/mL
4 CPA 7.5 µg/mL

Conclusion

In the *in vitro* chromosome aberration assay equivocal results were seen: a putative positive result was seen in the absence of metabolic activation at concentrations showing higher levels of cytotoxicity. In addition in the presence of metabolic activation, whilst a putative response was observed in the second experiment, this was not seen under the same conditions in the first experiment and hence the lack of reproducibility again questions the relevance of these findings. Therefore, no final conclusion can be drawn with regard to the clastogenic properties of R611968.

B.6.8.1 – 6.4.57 In vitro cell mutation assay in mouse lymphoma cells with R611968 - study 57

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/49 Wollny H (2015e) R611968 - 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. 1677803 issue date: 04 May 2015. Unpublished. Syngenta File No.SYN548651_10002.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 476 of 1997. There were no deviations from the guideline of 1997 considered to compromise the scientific validity of the study.

Study design The study was performed to investigate the potential of R611968 to induce mutations at the mouse lymphoma thymidine kinase locus of the cell line L5178Y. The assay was performed in three independent experiments, using two parallel cultures each. Experiments I, II, and III were performed with and without metabolic activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations:

Experiment I:

without metabolic activation: 83.1; 166.3; 332.5; 665.0; and 997.5 µg/mL

with metabolic activation: 83.1; 166.3; 332.5; 665.0; and 1330.0 µg/mL

Experiment II:

without metabolic activation: 100.0; 200.0; 400.0; and 600.0 µg/mL

with metabolic activation: 200.0; 400.0; 600.0; and 800.0 µg/mL

Experiment III:

without metabolic activation: 400.0; 500.0; 600.0; 700.0; and 800.0* µg/mL

with metabolic activation: 400.0; 500.0; 600.0; 700.0; and 800.0 µg/mL

* evaluated in culture I only

Test Material:	R611968
Description:	White, solid
Lot/Batch number:	DAH-XXIX-85-2
Molecular weight:	265.5 g/mol
Purity	99.5 % w/w, concentration calculation adjusted to purity
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: Methylmethanesulphonate, 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

Relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in experiment I at 332.5 µg/mL and above without metabolic activation. In experiment II cytotoxic effects occurred at 600.0 µg/mL without metabolic activation and at 800.0 µg/mL with metabolic activation. In experiment III cytotoxic effects were noted

at 700.0 µg/mL without metabolic activation and at 600.0 µg/mL and above with metabolic activation. The recommended cytotoxic range of approximately 10-20% relative total growth was covered with and without metabolic activation. The data generated at 997.5 µg/mL in the first experiment without metabolic activation, and at 1330.0 µg/mL with metabolic activation were not considered valid as the Relative Total Growth (RTG) was markedly below the lower limit of 10% in both parallel cultures. In experiment III, the data generated at 800.0 µg/mL with metabolic activation were considered valid as the mean RTG of both parallel cultures was 10%. The recommended cytotoxic range of 10-20% relative total growth was covered 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 at acceptable levels of cytotoxicity with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible concentration-dependent increase of mutant frequencies. No statistically significant trend occurred in any of the experiments.

In this study the range of the solvent controls was from 48 up to 134 mutant colonies per 10^6 cells; the range of the groups treated with the test item was from 27 up to 234 mutant colonies per 10^6 cells.

The lowest solvent control value fell just short of the lower limit of the spontaneous mutation frequency of the acceptance criteria (48 versus 50 colonies per 10^6 cells). This deviation was judged as biologically irrelevant as it was very minor and the mean value of both parallel cultures (48 and 63 cultures, equal to a mean of 55.5 cultures) met the acceptance criteria.

The viability slightly exceeded the acceptable range with the solvent control of the first culture of experiment II without metabolic activation. Again, the data are acceptable as the deviation was very minor (121% versus 120%) and the mean value of both parallel cultures was fully acceptable (121% and 80% equal to a mean of 100.5%). The total suspension growth of the solvent control in experiment I in the presence of metabolic activation was just very marginally below the acceptance criteria (7.8% vs 8%, but this difference was considered trivial and the parallel culture values (12.2%) was fully satisfactory, hence the data were considered acceptable.

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 mutant colonies at acceptable levels of toxicity with at least one of the concentrations of the controls.

The results of the study are given in Table 6.8.1 – 6.4.57.

Table 6.8.1 – 6.4.57: Summary of the TK assay

			relative	relative	mutant		relative	relative	mutant	
	conc. µg	S9	cloning	total	colonies/		cloning	total	colonies/	
	per mL	mix	efficiency 1	growth	10^6 cells	threshold	efficiency 1	growth	10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	95	221	100.0	100.0	126	252
Pos. control with MMS	19.5	-	40.5	26.0	317	221	44.0	36.2	326	252
Test item	41.6	-	93.4	culture was not continued#			70.6	culture was not continued#		
Test item	83.1	-	118.4	90.0	87	221	69.6	103.8	164	252
Test item	166.3	-	90.4	66.7	112	221	80.8	91.0	110	252
Test item	332.5	-	79.7	48.3	137	221	5.4	66.5	135	252
Test item	665.0	-	37.0	23.2	109	221	18.1	20.1	121	252
Test item	997.5	-	**10.9**	**0.7**	**386**	221	**0.6**	**3.6**	**61**	252
Test item	1330.0	-	3.6	culture was not continued##			0.0	culture was not continued##		
Solv. control with DMSO		+	100.0	100.0	118	244	100.0	100.0	134	260
Pos. control with CPA	3.0	+	72.7	135.8	400	244	55.3	37.2	634	260
Pos. control with CPA	4.5	+	53.0	116.9	536	244	48.6	64.1	512	260
Test item	83.1	+	86.0	105.0	158	244	81.3	110.2	140	260
Test item	166.3	+	88.8	248.2	102	244	74.8	127.8	126	260
Test item	332.5	+	83.3	178.9	71	244	72.4	77.4	138	260
Test item	665.0	+	66.6	59.8	216	244	23.7	36.5	90	260
Test item	1330.0	+	**3.5**	**2.0**	**1299**	244	**23.3**	**6.7**	**364**	260
Test item	1995.0	+	0.0	culture was not continued##			8.4	culture was not continued##		
Test item	2660.0	+	0.0	culture was not continued##			8.7	culture was not continued##		
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	48	174	100.0	100.0	63	189
Pos. control with MMS	19.5	-	28.0	17.0	340	174	14.8	13.3	333	189
Test item	100.0	-	76.3	61.3	59	174	107.3	77.1	112	189
Test item	200.0	-	112.5	63.8	64	174	86.3	82.3	84	189
Test item	400.0	-	53.8	29.4	74	174	72.0	64.9	68	189
Test item	600.0	-	18.4	9.0	66	174	24.8	26.9	60	189
Test item	800.0	-	1.3	culture was not continued#			4.0	culture was not continued#		
Test item	1000.0	-	0.4	culture was not continued#			0.0	culture was not continued#		
Test item	1200.0	-	1.7	culture was not continued#			0.0	culture was not continued#		
Solvent control with DMSO		+	100.0	100.0	128	254	100.0	100.0	109	235
Pos. control with CPA	3.0	+	111.2	43.0	265	254	90.4	52.4	243	235
Pos. control with CPA	4.5	+	85.2	60.9	294	254	73.1	65.8	289	235
Test item	200.0	+	111.2	197.1	44	254	114.3	139.6	76	235
Test item	400.0	+	120.4	119.1	76	254	36.9	93.4	88	235
Test item	600.0	+	58.8	90.1	88	254	58.0	55.9	55	235
Test item	800.0	+	29.0	22.3	92	254	18.7	19.1	72	235
Test item	1000.0	+	7.3	culture was not continued#			3.8	culture was not continued#		
Test item	1200.0	+	6.9	culture was not continued#			0.0	culture was not continued#		
Test item	1400.0	+	0.0	culture was not continued#			0.0	culture was not continued#		
Experiment III / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	56	182	100.0	100.0	60	186
Pos. control with MMS	19.5	-	56.5	42.2	274	182	53.0	26.1	290	186
Test item	400	-	125.6	96.8	52	182	12.4	70.3	40	186
Test item	500	-	45.9	34.1	27	182	5.1	20.6	45	186
Test item	600	-	66.3	72.5	30	182	17.7	53.0	49	186
Test item	700	-	30.9	24.3	41	182	10.3	28.2	42	186
Test item	800	-	**5.6**	**0.4**	**507**	**182**	0.0	5.8	culture was not continued##	
Test item	900	-	3.3	culture was not continued#			3.3	culture was not continued#		
Test item	1000	-	0.0	culture was not continued##			0.0	culture was not continued##		
Solvent control with DMSO		+	100.0	100.0	119	245	100.0	100.0	84	210
Pos. control with CPA	3	+	62.6	29.7	432	245	82.9	54.3	158	210
Pos. control with CPA	4.5	+	42.7	16.1	593	245	77.2	33.1	324	210
Test item	400	+	98.4	111.2	93	245	41.3	114.4	102	210
Test item	500	+	110.9	76.5	103	245	54.7	96.7	86	210
Test item	600	+	37.2	37.1	176	245	26.4	47.5	112	210
Test item	700	+	25.6	20.1	163	245	4.5	17.1	100	210
Test item	800	+	5.5	10.6	234	245	2.8	9.3	84	210
Test item	900	+	0.6	culture was not continued#			1.6	culture was not continued#		
Test item	1000	+	0.0	culture was not continued#			0.4	culture was not continued#		

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126
\# culture was not continued as a minimum of only four analysable concentrations is required
\## culture was not continued due to exceedingly severe cytotoxic effects
The values printed in bold are judged as invalid, since the acceptance criteria (page 23) are not met (RTG < 10%).

Conclusion

In the mouse lymphoma assay test substance R611968 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, R611968 is considered to be non-mutagenic in this mouse lymphoma assay.

Genotoxicity R611968 – in vivo

B.6.8.1 – 6.4.58a In vivo micronucleus test in mice with R611968 - study 58

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/52 Dunton, J. (2015b) R611968 – Oral (Gavage) Mouse Micronucleus Test. Sequani Ltd. Sequani Report No. BFI0406, issue date: 17 November 2015. Unpublished. Syngenta File No.SYN548651_10006.

GUIDELINES: Mouse 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.

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

Study design

R611968 was tested to evaluate its potential to cause damage to chromosomes or cell division apparatus, or to cause cell cycle interference, leading to micronucleus formation in polychromatic erythrocytes in the bone marrow of young adult mice. In all phases, the dosing of the vehicle and test item was by oral (gavage) administration either once or twice, depending on the severity of the clinical signs, separated by approximately 24 hours.

In the dose-sighting phase, groups of two male mice were given R611968 as a suspension in 0.5 % (w/v) aqueous carboxymethylcellulose with 0.1 % (v/v) Tween 80 at 500, 1250 or 800 mg/kg/day, in order to determine the maximum tolerated dose (MTD). In the range-finding phase, groups of three male and three female mice were given R611968 at 800 mg/kg/day or 500 mg/kg/day, in order to confirm the MTD in both male and female mice. The MTD was confirmed as 500 mg/kg/day in both male mice and female mice. As there was no inter-sex differences in toxicity (a difference in MTD of three-fold or greater), the main study was conducted in males only, with the high dose selected as 500 mg/kg/day.

Since bone marrow is well perfused, exposure of the bone marrow to the test item was indirectly assessed by collection of blood. The presence of R611968 was confirmed by LC-MS/MS analysis.

For the main study phase, three groups, each of six male mice were dosed with 125, 250 or 500 mg/kg/day R611968 on two successive days, separated by approximately 24 hours.

A group of six male mice (negative Controls) was dosed with the vehicle alone and a positive Control group, also of six male mice, was given a single 4 mg/kg intraperitoneal dose of Mitomycin C (MMC).

Bone marrow was harvested from all animals approximately 24 hours after the final dose administration and smears were prepared. The stained slides prepared for the main study were coded and 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei and the group frequencies were statistically analysed.

Test Material:	R611968
Description:	Beige powder
Lot/Batch number:	MES 411/1
Purity:	99 % w/w
Stability of test compound:	Retest date : 31 July 2017

Results

Preliminary toxicity assay: Dose-sighting phase: Clinical signs observed following administration at 500 mg/kg/day included increased activity after the first dose and decreased activity and piloerection after the second dose. At 1250 mg/kg/day, clinical signs included decreased activity, piloerection, slow breathing, partially closed eyes, hunched posture, abnormal gait, distended abdomen and coldness to the touch. Animals were killed due to poor clinical condition four hours after the second dose. Clinical signs observed in one male (Animal 66) given 800 mg/kg/day, included decreased activity, laboured breathing and closed eyes. Animal 66 was euthanised one hour after the first dose. At necropsy, Animal 66 showed early onset of rigor mortis but no abnormalities were detected. Body weight loss was observed in Animal 61 given 500 mg/kg/day and in Animal 65 given 800 mg/kg/day.

Range-finding phase: Clinical signs observed in males and females given 800 mg/kg/day included decreased activity, slow breathing, eyes closed or partially closed, distended abdomen, abnormal gait, hunched and low posture and piloerection. Males 71 and 73 and Female 76 were euthanised due to poor clinical condition two hours after the first dose. The remaining animals were killed two hours after the first dose as this dose level exceeded the MTD.
There were no adverse clinical signs following administration at 500 mg/kg/day.
Minor body weight loss was observed in Animals 78 to 81 given 500 mg/kg/day.
Based on the results of this phase, the MTD was considered to be 500 mg/kg/day in both males and females. As there was no inter-sex difference, the main study was conducted in male mice only.
Exposure to R611968 was confirmed in all range-finder blood samples. Bone marrow smears were not analysed in the range-finding phase since the presence of R611968 was confirmed in the blood samples.

Micronucleus test: There were no adverse clinical observations following administration of R611968 to male mice at 125 mg/kg/day (Group 2) or 250 mg/kg/day (Group 3). Nor were there any adverse clinical observations in Group 1 (negative Control) or Group 5 (positive Control).
Clinical signs observed in one male (Animal 21) following administration at 500 mg/kg/day (Group 4) included decreased activity, eyes closed or partially closed, abnormal and unsteady gait, hunched posture, laboured breathing and piloerection. Animal 21 was euthanised due to poor clinical condition 4 hours after the second dose. At necropsy, Animal 21 showed early onset of rigor mortis but no abnormalities were detected.
There were no statistically significant increases in micronucleus frequency in male mice given any dose level of R611968, compared with the negative Control group.

There was no evidence of a statistically significant reduction in the PCE/NCE ratio in male mice given R611968, and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of R611968 to the bone marrow.
The animals dosed with MMC, the positive Control item, had statistically significant increases in the number of micronucleated cells compared with the concurrent Control group, which demonstrated that the test system was capable of detecting a known clastogen and that the scorers were capable of detecting micronuclei. There was no statistically significant decrease in the PCE/NCE ratio in the positive Control group, indicating a lack of toxicity to the bone marrow.

Conclusion

There was no evidence of clastogenicity or aneugenicity following oral (gavage) administration of R611968 up to the MTD of 500 mg/kg/day in male mice. R611968 is considered to be neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

B.6.8.1 – 6.4.58b Proof of exposure - In vivo micronucleus test with R611968

Data requirement 2.31

Applicant to provide evidence of tissue exposure in the in vivo MN study with R611968 – study 58.

See also 2(89, 92)
See reporting table 2(77)

NL (August 2017): The applicant provided proof of exposure for the in vivo mouse micronucleus test (Dunton, 2015b; study 58) by providing raw data on the presence of R611968 in blood samples.
The RMS agrees with the conclusion that R611968 is systemically available and thus may reach the bone marrow.

The notifier provided the following evidence of tissue exposure in the in vivo MN study:
"The in vivo bone marrow micronucleus assay is a widely used in vivo genotoxicity test. In the case that a negative result is obtained evidence must be presented that the compound of interest was systemically available to the bone marrow. As the bone marrow is a well-perfused tissue, concentrations of compound-related material(s) in blood or plasma are generally similar to those observed in bone marrow. Therefore, proof of exposure can be obtained by measurement of the compound of interest either in blood or plasma.

Concern that the compound is not available for distribution into bone marrow because of irreversible binding to red blood cells can be addressed two ways. Either analyse for the compound in plasma or demonstrate that any binding to a matrix in whole blood (plasma or red blood cells) was reversible. The preparation of whole blood for analysis will yield reversibly bound compounds, but not those irreversibly bound and compound quantified will be that distributed in blood and plasma.

Blood samples were taken 1 and 4 hours following oral administration and again after the terminal blood sample approximately 24 hours after the second test item administration.
After sampling each blood sample was diluted with1 % (v/v) formic acid in acetonitrile [1:3 (v/v)]. Prior to analysis each sample was extracted using organic solvent. Following centrifugation, to pellet the protein, the resulting supernatant was analysed via mass spectrometry.

As shown in the table below, SYN548651 (R611968) was freely available at concentrations much higher than the 40 ng/mL low standard and quantifiable over several time points demonstrating sustained exposure. It can therefore be concluded that SYN548651 (R611968) was systemically available to allow bone marrow exposure.

R548651 (R611968) Analytical Response and Retention Time Data

SYN548651 (R611968)		
Sample I.D.	Peak area counts	Retention time (min)
Blank	0.0	0.00
STD Low	36203.9	1.99
STD High	3775123.7	1.99
Blank	0.0	0.00
RF2 77 M DAY 2 1h	37875837.5	1.99
RF2 78 M DAY 2 1h	36197848.7	2.00
RF2 79 M DAY 2 1h	32604369.4	2.00
RF2 77 M DAY 2 4h	31551712.1	1.99
RF2 78 M DAY 2 4h	37435413.2	1.99
RF2 79 M DAY 2 4h	37475229.7	1.99
RF2 77 M DAY 2 TER	5382953.7	1.99
RF2 78 M DAY 2 TER	18944360.1	1.99
RF2 79 M DAY 2 TER	2985434.6	2.00
RF2 80 F DAY 2 1h	43099504.0	1.99
RF2 81 F DAY 2 1h	41003414.0	1.99
RF2 82 F DAY 2 1h	37429060.1	1.99
RF2 80 F DAY 2 4h	32855148.9	2.00
RF2 81 F DAY 2 4h	36195497.9	2.00
RF2 82 F DAY 2 4h	23155304.6	2.00
RF2 80 F DAY 2 TER	13470205.2	2.00
RF2 81 F DAY 2 TER	12250788.2	1.99
RF2 82 F DAY 2 TER	11936120.7	1.99
Blank	0.0	0.00
STD Low	45380.0	2.00
STD High	3962248.9	1.99
Blank	0.0	0.00

All Study samples exhibit analytical response for SYN548651 (R611968) greater than the mean response observed in the low spiked standard (10 ng/mL blood concentration)
TER = terminal sample

Genotoxicity R613636 – in vitro

B.6.8.1 – 6.4.59 Reverse mutation assay in bacteria with R613636 - study 59

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/50 Sokolowski A (2015o) R613636 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1677701, issue date: 10 March 2015. Unpublished Syngenta File No. R613636_10006.

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.

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

Study design

This study was performed to investigate the potential of R613636 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 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 both experiments: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate

Test Material:	R613636
Description:	White, solid
Lot/Batch number:	DAH-XXIX-96
Purity:	98.6 % (HPLC Area Distribution)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	Recertification Date: 31 January 2017
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)

Results

The test item precipitated in the overlay agar in the test tubes from 2500 to 5000 µg/plate. Precipitation of the test item in the overlay agar on the incubated agar plates was observed in experiment I and II in all strains without S9 mix at 5000 µg/plate and in experiment I and II in all strains with S9 mix from 2500 to 5000 µg/plate. The undissolved particles had no influence on the data recording.

The plates incubated with the test substance showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

Toxic effects, evident as a reduction in the number of revertants (below the induction factor of 0.5), were observed at the following concentrations (µg/plate):

Strain	Experiment I		Experiment II	
	without S9 mix	with S9 mix	without S9 mix	with S9 mix
TA 1535	2500 – 5000	5000	5000	5000
TA 1537	2500 – 5000	/	5000	/
TA 98	5000	2500 – 5000	2500 – 5000	2500 – 5000
TA 100	5000	2500 – 5000	5000	1000 – 5000
WP2 pKM101	2500 – 5000	5000	1000 – 5000	1000 – 5000
WP2 *uvrA* pKM101	5000	5000	5000	5000

/ = no toxic effects

No increase in revertant colony numbers of any of the six tester strains was observed following treatment with R613636 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 and all mutation rates were within the range of normal biological variability. Appropriate reference mutagens were used as positive controls. They showed a distinct increase of induced revertant colonies.

The results of this study are presented in Tables 6.8.1 – 6.4.59-1 and -2

Table 6.8.1 – 6.4.59-1: Summary of the Ames test

Study Name: 1677701
Experiment: 1677701 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1677701
Date Plated: 28/01/2015
Date Counted: 02/02/2015

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		8 ± 2	13 ± 3	23 ± 2	167 ± 9	208 ± 27	350 ± 52
	Untreated		8 ± 1	9 ± 1	29 ± 9	178 ± 25	225 ± 15	334 ± 17
	R613636	3 µg	9 ± 1	13 ± 3	24 ± 3	145 ± 12	199 ± 10	351 ± 23
		10 µg	9 ± 3	12 ± 4	23 ± 1	155 ± 7	226 ± 19	268 ± 21
		33 µg	9 ± 3	10 ± 2	21 ± 2	151 ± 9	194 ± 12	295 ± 30
		100 µg	9 ± 3	11 ± 1	21 ± 1	165 ± 29	183 ± 1	322 ± 20
		333 µg	8 ± 3	10 ± 2	18 ± 7	146 ± 12	156 ± 18	346 ± 37
		1000 µg	5 ± 1	9 ± 2	16 ± 4	152 ± 22	132 ± 20	268 ± 5
		2500 µg	3 ± 1	6 ± 1	22 ± 1	136 ± 21	49 ± 6	176 ± 31
		5000 µg	2 ± 1 P	1 ± 1 PM	5 ± 2 PM	12 ± 4 PM	3 ± 2 PM	52 ± 3 P
	NaN3	10 µg	1121 ± 64			2502 ± 79		
	4-NOPD	10 µg			285 ± 25			
	4-NOPD	50 µg		61 ± 9				
	MMS	2.0 µL					4677 ± 83	4334 ± 135
With Activation	DMSO		12 ± 3	20 ± 5	35 ± 4	149 ± 14	223 ± 30	363 ± 16
	Untreated		13 ± 4	24 ± 8	37 ± 4	176 ± 24	278 ± 22	389 ± 28
	R613636	3 µg	12 ± 4	19 ± 2	37 ± 3	162 ± 14	265 ± 8	380 ± 29
		10 µg	12 ± 4	23 ± 2	38 ± 13	162 ± 17	251 ± 30	373 ± 39
		33 µg	12 ± 4	16 ± 6	41 ± 8	163 ± 7	269 ± 53	325 ± 14
		100 µg	9 ± 2	15 ± 1	34 ± 4	146 ± 11	226 ± 28	354 ± 15
		333 µg	10 ± 1	16 ± 5	40 ± 3	133 ± 11	205 ± 13	382 ± 12
		1000 µg	9 ± 3	18 ± 3	23 ± 6	93 ± 16	230 ± 37	310 ± 26
		2500 µg	7 ± 1 P	12 ± 4 P	15 ± 1 P	27 ± 6 PM	118 ± 5 P	187 ± 15 P
		5000 µg	3 ± 1 PM	9 ± 3 PM	7 ± 3 PM	5 ± 2 PM	64 ± 10 P	40 ± 9 PM
	2-AA	2.5 µg	440 ± 12	238 ± 11	4195 ± 228	4017 ± 283		
	2-AA	10.0 µg					1117 ± 112	2559 ± 170

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Key to Plate Postfix Codes

P	Precipitate
M	Manual count

Table 6.8.1 – 6.4.59-2: Summary of the Ames test

Study Name: 1677701
Experiment: 1677701 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1677701
Date Plated: 12/02/2015
Date Counted: 16/02/2015

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		11 ± 3	12 ± 4	26 ± 7	167 ± 10	249 ± 29	368 ± 36
	Untreated		10 ± 1	15 ± 2	29 ± 7	195 ± 29	245 ± 11	411 ± 27
	R613636	3 µg	9 ± 3	10 ± 2	28 ± 6	144 ± 28	218 ± 17	442 ± 38
		10 µg	10 ± 3	10 ± 2	26 ± 6	153 ± 16	226 ± 4	424 ± 29
		33 µg	13 ± 1	14 ± 4	22 ± 5	150 ± 10	240 ± 44	405 ± 24
		100 µg	11 ± 4	11 ± 1	20 ± 1	166 ± 31	197 ± 8	377 ± 30
		333 µg	10 ± 3	9 ± 0	27 ± 4	155 ± 8	224 ± 10	388 ± 41
		1000 µg	13 ± 3	7 ± 2	21 ± 6	137 ± 21	89 ± 3	306 ± 57
		2500 µg	8 ± 1	6 ± 1	10 ± 3	128 ± 29	84 ± 1	306 ± 49
		5000 µg	2 ± 1 P	3 ± 1 PM	2 ± 1 PM	17 ± 1 PM	8 ± 2 PM	21 ± 3 PM
	NaN3	10 µg	1206 ± 65			2356 ± 155		
	4-NOPD	10 µg			294 ± 12			
	4-NOPD	50 µg		80 ± 9				
	MMS	2.0 µL					3838 ± 201	2903 ± 76
With Activation	DMSO		12 ± 3	18 ± 1	44 ± 15	142 ± 11	258 ± 5	438 ± 17
	Untreated		13 ± 3	23 ± 8	33 ± 5	193 ± 28	328 ± 33	454 ± 7
	R613636	3 µg	12 ± 3	20 ± 4	44 ± 7	167 ± 35	315 ± 46	458 ± 40
		10 µg	11 ± 2	20 ± 6	43 ± 3	137 ± 13	258 ± 2	437 ± 10
		33 µg	12 ± 4	14 ± 5	41 ± 7	169 ± 20	252 ± 19	427 ± 20
		100 µg	12 ± 2	16 ± 4	39 ± 4	139 ± 10	258 ± 22	468 ± 21
		333 µg	12 ± 2	20 ± 3	32 ± 10	138 ± 8	234 ± 35	441 ± 26
		1000 µg	8 ± 2	23 ± 3	20 ± 7	33 ± 6	108 ± 33	341 ± 32
		2500 µg	7 ± 0 P	17 ± 3 P	10 ± 4 P	14 ± 4 PM	79 ± 14 P	308 ± 30 P
		5000 µg	3 ± 1 PM	16 ± 5 P	4 ± 1 PM	3 ± 1 PM	24 ± 7 PM	71 ± 11 PM
	2-AA	2.5 µg	395 ± 32	165 ± 13	4207 ± 192	3830 ± 250		
	2-AA	10.0 µg					1169 ± 67	2443 ± 68

Key to Positive Controls		Key to Plate Postfix Codes	
NaN3	sodium azide	P	Precipitate
2-AA	2-aminoanthracene	M	Manual count
4-NOPD	4-nitro-o-phenylene-diamine		
MMS	methyl methane sulfonate		

Conclusion

In the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay R613636 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. R613636 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.60 In vitro chromosome aberration study with R613636 - study 60

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/51 Sokolowski, A. (2015p) R613636 - *In Vitro* Chromosome Aberration Test in Human Lymphocytes. Harlan Cytotest Cell Research GmbH (Harlan CCR), In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1677702, issue date: 04 August 2015. Unpublished. Syngenta File No. R613636_10008

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473

(2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

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

Acceptability: The study was performed in accordance with OECD guideline 473. There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

Study design

This *in vitro* assay was performed to assess the potential of R613636 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/β-naphthoflavone treated male rats).

In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure periods were 4 hours with S9 mix and 22 hours without S9 mix. The chromosomes were prepared 22 hours after start of treatment with the test substance.

In each treatment group two parallel cultures were analysed. 150 metaphases per culture were evaluated for structural chromosomal aberrations. 1000 cells per culture were counted for determination of mitotic index.

Test Material:	R613636
Description:	White solid
Lot/Batch number:	DAH-XXIX-96
Purity:	98.6 % (concentrations corrected for purity)
CAS#:	-
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO 0.5%
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825.0 µg/mL (Exp. I), 550 µg/plate (Exp. II) Presence of S9 mix: Cyclophosphamide 7.5 µg/mL

Results

Cytogenetic assay:

Precipitation of the test substance in the culture medium was observed in Experiment I in the absence and presence of S9 mix at 216.2 µg/mL and above at the end of treatment. The osmolarity and pH, measured with osmometer and pH meter were determined in the solvent control and the maximum concentration without metabolic activation: No relevant influence on the osmolarity or pH was observed. In both experiments, concentrations showing clear cytotoxicity were not evaluable because of non-specific chromosome morphological changes that prevented the evaluation of higher concentrations. In Experiment I at 70.6 µg/mL chromosomes could not be evaluated because single chromatids were not distinguishable. Additionally in Experiment II at 70.6 µg/mL without S9 mix as well

as at 71.5 µg/mL with S9 mix the chromosomal sets were incomplete. At the next highest concentration of 123.6 µg/mL in both experiments no evaluable metaphases were present.
In Experiment I with and without S9 the onset of non-specific chromosomal changes correlated with the onset of cytotoxicity. Even though the MI at 70.6 µg/mL would have been still acceptable (48 % and 83.3%, respectively) at the next highest concentration of 123 µg/mL there were no surviving cells left. Similar observations were made for Experiment II without S9 mix. Here the MI at 70.6 µg/mL was 30.2% and at the next highest concentration no cells survived. Only in Experiment II with S9 mix was this correlation with the onset of cytotoxicity not observed.
In Experiment I in the absence and presence of S9 mix no statistically significant and biologically relevant increase in structural chromosomal aberrations was observed at the concentrations evaluated. The increase in chromosomal aberrations above the two-fold standard deviation 95% control limits (2.6 %, excluding gaps) in Experiment I with S9 at 23.1 µg/mL (3.0%) and above (2.7%) was judged as biologically irrelevant since it was not statistically significant in relation to the concurrent solvent control.
In Experiment II a statistically significant increase in structural chromosomal aberrations was observed in the absence of S9 mix at a concentration of 40.3 µg/mL (13.7% aberrant cells, excluding gaps) and in the presence of S9 mix at a concentration of 59.6 µg/mL (7.3% aberrant cells, excluding gaps), both clearly exceeding the two-fold standard deviation 95 % control limits (without S9: 2.3 %, excluding gaps; with S9: 2.6 %, excluding gaps) as well as the historical solvent control (without S9 mix: 0.0 – 3.0 % aberrant cells, excluding gaps; with S9 mix: 0.0 – 3.5 % aberrant cells, excluding gaps).
A trend test was not performed because the number of evaluated concentrations was too small to give a significant result.
In both experiments, in the absence and presence of S9 at the next highest concentration above those read, the presence of non-specific chromosome morphological changes may indicate a contribution of these non-specific effects to the observed aberration frequency.
Furthermore, the lack of reproducibility between Experiment I and Experiment II in the presence of S9 mix should be noted. Hence, the aberration frequencies noted at the highest reported concentration may not be of true biological significance.
No evidence of an increase in polyploid metaphases was noticed after treatment with the test substance as compared to the control cultures.
Either EMS (825.0 or 550.0 µg/mL) or CPA (7.5 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations.

The results of the chromosome aberration test are given in Table 6.8.1 – 6.4.60.

Table 6.8.1 – 6.4.60: Summary of the chromosome aberration test

Exp.	Preparation interval	Test item concentration in µg/mL	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 h	Solvent control[1]	100.0	2.0	1.7	0.0
		Positive control[2]	82.1	11.7	**11.7**[S]	2.7
		13.2	109.7	1.7	1.3	0.0
		23.1	106.1	1.0	1.0	0.0
		40.3	97.8	1.3	1.0	0.0
Exposure period 22 h without S9 mix						
II	22 h	Solvent control[1]	100.0	0.7	0.7	0.0
		Positive control[3]	62.3	17.0	**16.7**[S]	7.0
		13.2	99.4	2.3	2.0	0.3
		23.1	91.6	2.3	2.3	0.3
		40.3	90.7	14.3	**13.7**[S]	0.0
Exposure period 4 h with S9 mix						
I	22 h	Solvent control[1]	100.0	3.0	2.3	0.0
		Positive control[4]	64.2	12.3	**10.0**[S]	0.3
		13.2	81.9	2.7	2.3	0.0
		23.1	72.7	3.7	3.0	0.3
		40.3	74.1	2.7	2.7	0.0
II	22 h	Solvent control[1]	100.0	1.7	1.7	0.0
		Positive control[4]	64.2	10.0	**10.0**[S]	0.3
		14.9	86.9	2.0	2.0	0.0
		29.8	78.4	4.0	3.3	0.3
		59.6	102.5	8.0	**7.3**[S]	1.0

* 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 550.0 µg/mL
4 CPA 7.5 µg/mL

Conclusion

In this chromosome aberration test the test substance R613636 induced structural chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of a metabolic activation system. Therefore, R613636 is considered to be clastogenic in this chromosome aberration test, when tested up to highest evaluable concentrations.

B.6.8.1 – 6.4.61 In vitro cell mutation assay in mouse lymphoma cells with R613636 - study 61

Data requirement 2.32

Applicant to provide the results table of the in vitro cell mutation assay in mouse lymphoma cells with R613636 – study 61.

See reporting table 2(78)

NL (August 2017): The results table provided by the applicant is presented below. The conclusion by the RMS does not change.

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. After evaluation of additional information provided by the applicant, the RMS agreed with the applicant's conclusion that the test substance is non-mutagenic in this study.

Report:	K-CA 5.8.1/55 Wollny H (2015f), R613636- 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. 1677703, issue date: 15 October 2015. Unpublished. Syngenta File No. R613636_10012.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

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

Study design

The study was performed to investigate the potential of R613636 to induce mutations at the mouse lymphoma thymidine kinase locus using the cell line L5178Y. The maximum concentration of the pre-experiment was 2880 µg/mL, equal to approximately 10 mM, based on the molecular weight (283.926 g/mol) of the test substance and the purity (98.6%) of the test item. The maximum concentration of the main experiments was limited by cytotoxicity of the test item.

The main experiments were evaluated at the following concentrations:

Experiment I:

without metabolic activation: 2.5: 5.0; 10.0; 20.0; and 30.0 µg/mL

with metabolic activation: 5.0; 10.0; 20.0; 30.0; and 40.0 µg/mL

Experiment II:

without metabolic activation: 2.5; 5.0; 10.0; and 20.0 µg/mL

with metabolic activation: 5.0; 10.0; 20.0; 25.0; and 30.0 µg/mL

Experiment III:

without metabolic activation: 2.5; 5.0; 10.0; 15.0; and 20.0 µg/mL

with metabolic activation: 10.0; 15.0; 20.0; 25.0; and 30.0 µg/mL

Test Material:	R613636
Description:	White solid
Lot/Batch number:	DAH-XXIX-96
Molecular weight:	283.926 g/mol
Purity	98.6 % w/w (concentration calculation adjusted to purity)
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 % in the pre-experiment and 1.0% in the main experiments)
Positive control:	Absence of S9 mix: Methylmethanesulphonate (MMS), 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

Mutation assay:

Relevant cytotoxic effects indicated by a relative total growth (RTG) of less than 50% in both cultures occurred in experiment I at 20.0 µg/mL and above with and without metabolic activation. In experiment II cytotoxic effects, as determined by RTG, occurred at 10.0 µg/mL and above without metabolic activation and at 20.0 µg/mL and above with metabolic activation. In experiment III relevant cytotoxic effects, as determined by RTG, were noted at 10.0 µg/mL and above without metabolic activation and at 15.0 µg/mL and above with metabolic activation.

A substantial but not reproducible dose dependent increase of the mutation frequency was observed in the main experiments with and without metabolic activation at RTG levels around 10% or even below. At RTG levels down to 10%, the threshold of 126 colonies per 10^6 cells was exceeded in both cultures of the first experiment without metabolic activation at 20.0 µg/mL. In culture II of the first experiment with metabolic activation, the threshold was exceeded at 30.0 µg/mL. In the second experiment without metabolic activation the threshold was not exceeded at RTG levels down to 10%. In the second experiment with metabolic activation the mutation frequency exceeded the threshold at 10.0 µg/mL in culture I and at 5.0, 20, and 25 µg/mL in culture II. In the third experiment the threshold was not exceeded with and without metabolic activation at RTG levels down to 10%.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of the mutation frequency. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in both cultures of the first experiment with and without metabolic activation and in the second culture of experiment III without metabolic activation.

In this study the range of the solvent control values was from 59 up to 175 mutant colonies per $10P^{6P}$ cells; the range of the group values treated with the test substance was from 63 up to 383 mutant colonies per $10P^{6P}$ cells at RTG levels of at least 10%. The highest solvent control value of 175 slightly exceeded the upper limit of the acceptance criteria of 170. The data are acceptable however, as the spontaneous rate of the parallel culture was 131 resulting in a mean of 153 colonies per 10^6 cells.

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 toxicity with at least one of the

concentrations of the controls. Although the MMS control of the second culture of the third experiment induced a substantial increase of total colonies (325 colonies compared to 92 of the corresponding control), the acceptance criterion of at least 150 induced small colonies was just not met (172 compared to 33 small colonies) as many of the induced colonies were large ones. The MMS control of the parallel culture met the criteria with 194 induced small colonies compared to 32 small colonies of the corresponding solvent control. Results of the study are given in the table below.

Table 6.8.1 – 6.4.61: Results of the mouse lymphoma assay (experiment I, II, and III)

	conc. µg per mL	S9 mix	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	80	206	100.0	100.0	88	214
Pos. control with MMS	19.5	-	41.0	9.6	427	206	48.1	21.1	383	214
Test item	1.3	-	74.0	culture was not continued*			98.2	culture was not continued*		
Test item	2.5	-	49.3	95.5	79	206	93.1	103.3	127	214
Test item	5.0	-	45.9	54.0	68	206	80.2	86.6	125	214
Test item	10.0	-	40.4	56.6	112	206	60.4	68.8	104	214
Test item	20.0	-	24.2	16.7	267	206	25.3	16.1	313	214
Test item	30.0	-	5.4	2.0	398	206	2.5	2.8	253	214
Test item	40.0	-	0.5	culture was not continued**			0.0	culture was not continued**		
Solv. control with DMSO		+	100.0	100.0	96	222	100.0	100.0	86	212
Pos. control with CPA	3.0	+	72.2	57.0	191	222	51.4	65.5	191	212
Pos. control with CPA	4.5	+	45.1	40.2	402	222	42.1	43.3	333	212
Test item	1.3	+	82.5	culture was not continued*			83.2	culture was not continued*		
Test item	2.5	+	77.7	culture was not continued*			73.1	culture was not continued*		
Test item	5.0	+	53.5	84.0	133	222	58.4	95.9	64	212
Test item	10.0	+	49.1	62.2	144	222	37.0	65.2	70	212
Test item	20.0	+	24.3	44.6	124	222	24.6	46.1	110	212
Test item	30.0	+	7.7	8.2	304	222	12.8	10.6	243	212
Test item	40.0	+	2.1	0.7	871	222	4.5	2.3	442	212
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	175	301	100.0	100.0	131	257
Pos. control with MMS	19.5	-	16.8	10.5	592	301	40.0	10.6	433	257
Test item	2.5	-	56.3	80.1	128	301	85.1	67.9	121	257
Test item	5.0	-	103.6	88.6	139	301	75.5	67.3	63	257
Test item	10.0	-	66.8	26.9	177	301	48.4	17.9	161	257
Test item	20.0	-	7.5	3.3	289	301	3.4	1.6	342	257
Test item	25.0	-	1.8	culture was not continued**			0.0	culture was not continued**		
Test item	30.0	-	1.5	culture was not continued**			0.0	culture was not continued**		
Test item	35.0	-	1.2	culture was not continued**			0.0	culture was not continued**		
Solv. control with DMSO		+	100.0	100.0	59	185	100.0	100.0	125	251
Pos. control with CPA	3.0	+	95.7	52.4	213	185	57.3	34.9	540	251
Pos. control with CPA	4.5	+	64.2	22.0	430	185	34.5	17.7	1007	251
Test item	2.5	+	85.4	culture was not continued*			87.1	culture was not continued*		
Test item	5.0	+	116.1	62.8	134	185	70.1	53.8	272	251
Test item	10.0	+	79.5	60.0	383	185	63.3	59.2	222	251
Test item	20.0	+	22.1	18.2	121	185	30.9	23.0	330	251
Test item	25.0	+	8.7	9.3	161	185	11.3	10.9	281	251
Test item	30.0	+	6.4	4.4	116	185	5.5	3.7	634	251
Test item	35.0	+	4.2	culture was not continued**			1.4	culture was not continued**		
Experiment III / 4 h treatment			culture I				culture II			
Solv. control with DMSO		-	100.0	100.0	80	206	100.0	100.0	92	218
Pos. control with MMS	19.5	-	60.0	36.0	306	206	91.1	35.9	325	218
Test item	2.5	-	100.0	58.9	90	206	88.4	56.6	129	218
Test item	5.0	-	81.0	49.8	73	206	91.1	47.0	93	218
Test item	10.0	-	31.5	21.0	83	206	48.3	19.3	175	218
Test item	15.0	-	10.6	6.9	90	206	10.8	4.7	280	218
Test item	20.0	-	3.8	1.0	160	206	0.3	0.4	697	218
Test item	25.0	-	1.0	culture was not continued**			0.0	culture was not continued**		
Solv. control with DMSO		+	100.0	100.0	111	237	100.0	100.0	138	264
Pos. control with CPA	3.0	+	74.0	64.7	227	237	81.8	63.9	262	264
Pos. control with CPA	4.5	+	49.3	32.0	377	237	50.6	32.9	352	264
Test item	5.0	+	76.5	culture was not continued*			40.4	culture was not continued*		
Test item	10.0	+	76.5	55.9	160	237	51.4	66.9	115	264
Test item	15.0	+	40.4	49.1	115	237	70.0	31.1	150	264
Test item	20.0	+	31.6	27.6	125	237	47.6	24.9	155	264
Test item	25.0	+	17.9	6.5	301	237	21.1	12.0	169	264
Test item	30.0	+	8.1	3.6	225	237	5.5	3.8	335	264

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126, # culture was not continued since a minimum of only four analysable concentrations is required, ## culture was not continued due to exceedingly severe cytotoxic effects

Conclusions

In conclusion it can be stated that during the mutagenicity test described and under the experimental conditions reported, a substantial but not reproducible dose dependent increase of the mutation frequency was observed in the main experiments with and without metabolic activation.
However, the applicant indicated that interpretation of the study followed the methodology laid out by the IWGT (Moore et al 2003) by use of the global evaluation factor (GEF) to assess increases in mutant frequency for significance. In this interpretation a test substance is considered positive if the GEF is exceeded and a positive trend test is also observed. However, an important caveat to this as described by the IWGT where effects are seen only at higher cytotoxicity levels, i.e. lower RTG: “It is generally advisable to conduct one or more additional experiments to better define the assay response (particularly in the (30 – 10%) RTG cytotoxicity range”.

In the *absence of metabolic activation*.
Experiment 1. The mutant frequency was raised above the GEF value threshold only at a concentration approaching the cytotoxicity limit (20 µg/mL) with RTG values of 16.7 and 16.1% in cultures I and II. Concentrations above this were too cytotoxic (RTG less than 10%) and so should not considered as part of the mutagenicity assessment. In line with the IWGT recommendations these conditions were further investigated in experiments 2 and 3. In experiment 2 concentrations of 20 µg/mL and above were too cytotoxic and in experiment 3 concentrations of 15 µg/mL and above were too cytotoxic. In experiments 2 and 3 in the absence of metabolic activation no relevant increases in GEF above the threshold were observed. Hence, the relevance of the response in experiment 1 must be considered questionable and overall the lack of reproducibility and cytotoxic mediated response would indicate these effects not be of biological significance.

In the *presence of metabolic activation*, no increase in mutant frequency above the GEF threshold was seen under any condition and in any experiment that was reproducible in both cultures and with an acceptable RTG (>10%). Here again increases in mutant frequency was associated with principally cytotoxic (RTG <10%) concentrations. Hence on this basis, the lack of reproducibility and influence of cytotoxicity, the sporadic increases in mutant frequency are considered to be not biologically relevant.

The RMS agrees that the nonreproducible increase in mutation frequency mainly at (near) toxic doses, are not biologically relevant. Therefore, R613636 is considered non-mutagenic in this mouse lymphoma assay.

Moore, M.M., Honma, M., Clements, J., Bolcsfoldi, G., Burlinson, B., Cifone, M., Clarke, J., Delongchamp, R., Durward, R., Fellows, M., Gollapudi, B., Hou, S., Jenkinson, P., Lloyd, M., Majeska, J., Myhr, B., O'Donovan, M., Omori, T., Riach, C., San, R., Stankowski Jr., L.F., Thakur, A.K., Van Goethem, F., Wakuri, S., Yoshimura, I., Assay: Follow-up Meeting of the International Workshop on Genotoxicity Testing Aberdeen, Scotland, 2003 Assay Acceptance Criteria, Positive Controls, and Data Evaluation. Environmental and Molecular Mutagenesis 47: 1-5 (2006) Mouse Lymphoma Thymidine Kinase Gene Mutation

Data requirement 2.42f

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse lymphoma assay. The results will be discussed in an experts' meeting.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the Expert meeting, it was concluded that the response should be considered equivocal with and without metabolic activitation. Additional information is added below.

Response by notifier to the above data requirement:
"R613636 showed extreme cytotoxicity towards the L5178Y cell line. Increases in mutant frequency were in the main associated with high cytotoxicity. Experiments conducted in the absence of metabolic activation: In Experiments I, II and III with the exception of 20.0 µg/mL in Experiment I, all increases in MF are at unacceptably high cytotoxicity (RTG < 10%) and so considered to be not biologically relevant. The increase in MF observed at 20.0 µg/mL was itself associated with high cytotoxicity (RTG <20%). Hence, in the absence of metabolic activation it is apparent MF increases are in response to cytotoxicity.

Experiments conducted in the presence of metabolic activation: In experiments I (30 and 40 µg/mL) and III (25 and 30 µg/mL) increases in MF were only observed at unacceptably high cytotoxicity levels (RTG < 10%). In experiment II, increases in MF were observed at concentrations showing no response in experiments I or III. Hence, these increases in MF are not considered reproducible or reliable.
Overall, the lack of reproducibility of increases MF and the observation of increases in MF most often associated with high cytotoxicity leads to the conclusion that R613636 is displaying a cytotoxic rather than mutagenic response in this assay and the assay is concluded to be negative. R613636 is considered to be negative in the in vitro mammalian gene mutation assay conducted in L5178y TK+/- cells."

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the Expert meeting, it was concluded that the response should be considered equivocal considering the positivity observed at non-cytotoxic concentrations in Experiment 1 and 2 with and without metabolic activation.

B.6.8.1 – 6.4.62 In vitro cell gene mutation assay in Hamster V79 cells with R613636 - study 62

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/56 Wollny H (2015g) R613636 - Cell Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1711600 issue date: 20 November 2015. Unpublished. Syngenta File No. R613636_10014.

GUIDELINES: Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT)
OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)
GLP: Signed and dated GLP and Quality Assurance statements were provided.
Acceptability: There were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study

Study design
This *in vitro* study was performed to investigate the potential of R613636 to induce mutations at the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus in the Chinese hamster cell line V79. 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 maximum concentration of the pre-experiments (80.0 µg/mL) was based on the toxicity data generated in the Mouse Lymphoma Assay 1677703 (Syngenta Task Number: TK0256017). The test substance was dissolved in DMSO. The pre-experiment was repeated in the absence of metabolic activation using a lower concentration range of 0.04 to 5.0 µg/mL).
The main experiments were evaluated at the following concentrations:

Experiment I	
without metabolic activation:	0.31; 0.63; 1.25; 1.9; and 2.5 µg/mL
with metabolic activation:	5.0; 10.0, 20.0; 30.0; and 40.0 µg/mL
Experiment II	
without metabolic activation:	1.25; 1.5; 1.75; 2.0; and 2.5 µg/mL
with metabolic activation:	15.0; 20.0; 25.0; 30.0; and 40.0 µg/mL

Test Material:	R613636
Description:	White, solid
Lot/Batch number:	DAH-XXIX-96
Molecular weight:	283.926 g/mol
Purity	98.6 % w/w
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: Ethylmethane sulfonate (EMS), 0.15 mg/mL 1.1 µg/mL = 4.3 µM (experiment I) 2.2 µg/mL = 8.6 µM (experiment II)

Results

Mutation assay:

Relevant cytotoxic effects indicated by a relative cloning efficiency I or cell density below 50% occurred in the first experiment at 20.0 µg/mL and above with and at 1.25 µg/mL and above without metabolic activation. In the second experiment toxic effects as described above were noted at 30.0 µg/mL and above with and at 1.75 µg/mL and above without metabolic activation. The cytotoxic gradient induced by the test item was exceptionally steep. For example in experiment II without metabolic activation the cloning efficiency I levels were 77.0% and 63.7% at 1.5 µg/mL versus 8.8% and 1.9% at the next higher concentration of 1.75 µg/mL. The recommended cytotoxic range of approximately 10-20% cloning efficiency I was covered with and without metabolic activation.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. The mutant frequency did not exceed the historical range of solvent controls.

The threshold of three times the corresponding solvent control was reached but not exceeded in the second culture of the second experiment without metabolic activation at an intermediate concentration of 1.25 µg/mL. The effect however, was judged as biologically irrelevant as it was clearly based upon a rather low solvent control of 5.6 mutant colonies/10^6 cells. Furthermore, the threshold was not reached at any other, even higher concentration or in the parallel culture under identical conditions.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

In both experiments of this study (with and without S9 mix) the range of the solvent controls was from 5.6 up to 16.5 mutants per 10^6 cells; the range of the groups treated with the test item was from 7.0 up to 23.9 mutants per 10^6 cells. The cloning efficiency II (absolute value) Experiment II Culture I minus S9-mix was 50% but was considered to be acceptable.

EMS (150 µg/mL) and DMBA (1.1 µg/mL in experiment I and 2.2 µg/mL in experiment II) were used as positive controls and showed a distinct increase in induced mutant colonies.

Results of the study are given in the table below.

Table 6.8.1 – 6.4.62: Summary of the gene mutation assay

	conc.	S9	cloning	cell	cloning	colonies/	induction	cloning	cell	cloning	colonies/	induction
	µg/mL	mix	efficiency I	density	efficiency II	10^8 cells	factor	efficiency I	density	efficiency II	10^8 cells	factor
			%	%	%			%	%	%		
Column	1	2	3	4	5	6	7	8	9	10	11	12
Experiment I / 4 h treatment			culture I					culture II				
Solvent control with DMSO		-	100.0	100.0	100.0	12.8	1.0	100.0	100.0	100.0	12.0	1.0
Positive control (EMS)	150.0	-	110.8	72.1	90.0	165.5	12.9	105.0	103.3	95.8	198.8	16.5
Test item	0.078	-	74.4	culture was not continued#				89.4	culture was not continued#			
Test item	0.16	-	96.9	culture was not continued#				99.4	culture was not continued#			
Test item	0.31	-	75.3	95.2	102.7	9.8	0.8	79.4	99.0	110.7	10.8	0.9
Test item	0.63	-	85.5	94.3	104.0	12.3	1.0	85.0	104.7	105.4	9.9	0.8
Test item	1.25	-	9.3	82.4	93.6	10.9	0.9	10.6	97.7	104.2	8.8	0.7
Test item	1.9	-	6.2	91.0	91.9	10.0	0.8	9.4	97.1	103.4	16.6	1.4
Test item	2.5	-	0.0	86.2	87.7	14.5	1.1	0.0	91.0	91.6	23.9	2.0
Solvent control with DMSO		+	100.0	100.0	100.0	12.2	1.0	100.0	100.0	100.0	9.1	1.0
Positive control (DMBA)	1.1	+	95.7	74.1	87.3	119.8	9.8	92.5	92.2	92.0	102.9	11.3
Test item	1.25	+	88.6	culture was not continued#				104.7	culture was not continued#			
Test item	2.5	+	100.5	culture was not continued#				104.4	culture was not continued#			
Test item	5.0	+	91.1	110.0	102.9	18.3	1.5	91.4	108.5	89.0	15.6	1.7
Test item	10.0	+	85.0	101.7	91.3	14.3	1.2	84.2	86.4	99.0	10.7	1.2
Test item	20.0	+	28.3	62.0	106.5	14.1	1.1	23.6	72.8	99.0	12.6	1.4
Test item	30.0	+	7.0	42.7	102.8	15.8	1.3	10.6	67.4	87.6	10.3	1.1
Test item	40.0	+	0.0	43.5	83.2	12.4	1.0	0.6	70.1	79.8	23.2	2.6
Experiment II / 4 h treatment			culture I					culture II				
Solvent control with DMSO		-	100.0	100.0	100.0	16.2	1.0	100.0	100.0	100.0	5.6	1.0
Positive control (EMS)	150.0	-	94.3	83.1	91.8	289.0	17.8	97.0	93.7	81.4	209.3	37.2
Test item	0.25	-	98.4	culture was not continued#				93.7	culture was not continued#			
Test item	0.50	-	96.2	culture was not continued#				96.0	culture was not continued#			
Test item	1.00	-	91.7	culture was not continued#				96.7	culture was not continued#			
Test item	1.25	-	95.9	85.0	95.8	12.7	0.8	92.8	118.9	81.8	17.1	3.0
Test item	1.50	-	77.0	83.4	98.0	20.6	1.3	63.7	118.1	82.9	13.0	2.3
Test item	1.75	-	8.8	86.9	100.4	12.5	0.8	5.4	110.2	83.0	15.2	2.7
Test item	2.0	-	3.6	89.6	105.2	17.0	1.0	3.5	118.0	83.0	7.7	1.4
Test item	2.5	-	0.0	94.2	99.8	9.0	0.6	0.0	124.8	84.6	13.9	2.5
Experiment II / 4 h treatment			culture I					culture II				
Solvent control with DMSO		+	100.0	100.0	100.0	13.3	1.0	100.0	100.0	100.0	16.5	1.0
Positive control (DMBA)	2.2	+	76.3	97.9	85.3	150.3	11.3	61.9	95.3	61.2	317.1	19.2
Test item	2.5	+	97.3	culture was not continued#				102.7	culture was not continued#			
Test item	5.0	+	93.8	culture was not continued#				96.8	culture was not continued#			
Test item	10.0	+	91.6	culture was not continued#				84.1	culture was not continued#			
Test item	15.0	+	88.4	98.0	99.1	13.0	1.0	83.2	120.3	93.7	19.4	1.2
Test item	20.0	+	87.0	65.1	95.7	20.5	1.5	69.7	94.6	102.9	18.5	1.1
Test item	25.0	+	33.1	70.9	100.0	13.5	1.0	56.7	101.9	96.4	9.5	0.6
Test item	30.0	+	7.6	56.1	92.5	20.1	1.5	9.2	69.4	101.0	14.8	0.9
Test item	40.0	+	0.7	36.5	91.2	16.0	1.2	0.7	57.6	95.6	22.0	1.3

culture was not continued as a minimum of only four analysable concentrations is required by the guideline

Conclusion

In conclusion it can be stated that in the mutagenicity test described and under the experimental conditions reported, R613636 did not induce mutations in the HPRT locus in the Chinese hamster cell line V79 in absence or presence of metabolic activation. Therefore, R613636 is considered to be non-mutagenic in this HPRT assay.

Data requirement 2.42g

Applicant to provide a technical position on the results of in vitro studies with metabolites reported in column 2.

See reporting table 2(91)

NL (August 2017): The notifier provided a further explanantion on the results of the in vitro mouse lymphoma assay. The results will be discussed in an experts' meeting.

Response by notifier to the above data requirement:

"This assay was conducted to the OECD 476 (1997) test guideline. The response in the assay was assessed using the statistical significance of increase in MF, and a trend test for concentration response relationship. R613636 was tested up to cytotoxic concentrations and no relevant increase in MF was observed. No statistically significant increases in MF were observed with respect to concurrent controls and no concentration related trend was observed for any of the cultures. Additionally, all observed MF values were within the solvent historical control range and within the 95% control limits for the HCD (Mean + 2StDev). For these reasons, lack of statistical significance, and no concentration related trend, R613636 is concluded to be inactive, i.e. non-mutagenic in this assay."

Genotoxicity R613636 – in vivo

B.6.8.1 – 6.4.63a In vivo oral mouse micronucleus test with R613636 - study 63

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/57 Dunton, J. (2015c) R613636 – Oral (Gavage) Mouse Micronucleus Test. Sequani Ltd. Sequani Report No. BFI0402, issue date: 05 October 2015. Unpublished. Syngenta File No. R613636_10010.

GUIDELINES: Mouse 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.

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

Study design

R613636 was tested to evaluate its potential to cause damage to chromosomes or cell division apparatus, or to cause cell cycle interference, leading to micronucleus formation in polychromatic erythrocytes in the bone marrow of young adult mice. In all phases, the dosing of the vehicle and test item was by oral (gavage) administration, on to consecutive occasions, approximately 24 hours apart. In the dose-sighting phase, three groups of two male mice were given R613636 as a suspension in 0.5 % w/v aqueous carboxymethylcellulose with 0.1 % v/v Tween 80 at 500, 1250 or 2000 mg/kg/day on two consecutive occasions, in order to determine the maximum tolerated dose (MTD). In the range-finding phase, groups of three male and/or three female mice were given R613636 at 1250 or 2000 mg/kg/day, in order to confirm the MTD in both male and female mice. The MTD was confirmed to be 1250 mg/kg/day in male and female mice, and as there was no inter-sex difference in toxicity, the main study was conducted in males only.

A proof of exposure phase was conducted to demonstrate that the bone marrow was exposed to the test item, via analysis of test item in the whole blood of treated animals. The presence of R613636 was confirmed by analysis of the study samples alongside samples of blank matrix and matrix spiked with the test item.

For the main study phase, three groups, each of six male mice were dosed with 312.5, 625 or 1250 mg/kg/day R613636. A group of six male mice (negative Controls) was dosed with the vehicle alone and a positive Control group, also of six male mice, was given a single 4 mg/kg intraperitoneal dose of Mitomycin C (MMC).

Bone marrow was harvested from surviving range-finding and main study animals approximately 24 hours after the final dose administration and smears were prepared. The stained slides prepared for the main study were coded and 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei and the group frequencies were statistically analysed.

Test Material:	R613636
Description:	white to off white powder
Lot/Batch number:	DAH-XXIX-96
Purity:	98.6 %
Stability of test compound:	Retest date : 31 January 2017

Results

Preliminary toxicity assay: Dose-sighting phase: At 2000 mg/kg/day, clinical observations included closed eyes, intermittent twitching, slow breathing, loose faeces, decreased activity and unsteady gait. Both animals were also prostrate and cold to the touch. Loose faeces was observed in both animals two and four hours after the second dose of 1250 mg/kg/day. There were no clinical signs observed following administration of R613636 at 500 mg/kg/day.

There was no effect on body weight at any dose level.

Range-finding phase: At 2000 mg/kg/day in females, following their second dose, signs included decreased activity, abnormal gait, distended abdomen, prostration, slow breathing and closed eyes. Females were killed due to clinical condition one hour post second-dose. Loose faeces was observed in males following administration at 1250 mg/kg/day.

Based on the results of this phase, the MTD was considered to be 1250 mg/kg/day in males and females. As there was no difference between the MTD in males or females, the main study was conducted in male mice only.

Exposure to R613636 was confirmed in all range-finder blood samples.

Bone marrow smears were not analysed in the range-finding phase since the presence of R613636 was confirmed in the blood samples.

Micronucleus test: There were no clinical observations following administration of R613636 to male mice, nor were there any clinical observations in Group 1 (negative Control) or Group 5 (positive Control).

There were no statistically significant increases in micronucleus frequency in male mice treated at any dose level of R613636, compared with the negative Control group.

There was no evidence of a statistically significant reduction in the PCE/NCE ratio in male mice treated with R613636, and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of R613636 to the bone marrow. The animals dosed with MMC, the positive Control item, had statistically significant increases in the number of micronucleated cells compared to the concurrent Control group, which demonstrated that the test system was capable of detecting a known clastogen and that the scorers were capable of detecting micronuclei. There was no statistically significant decrease in the PCE/NCE ratio in the positive Control group, indicating a lack of toxicity to the bone marrow. Results of the study are given in the table below.

Table 6.8.1 – 6.4.63: Summary of the micronucleus test

	Negative Control 0 mg/kg/day	R613636 312.5 mg/kg/day	R613636 625 mg/kg/day	R613636 1250 mg/kg/day	MMC 4 mg/kg
N	6	6	6	6	6
Mean MN-PCE per 2000 cells	0.50	1.00	1.50	0.33	56.83[WW]
SD	0.84	1.26	1.05	0.52	14.47
Mean MN-PCE +SD	1.34	2.26	2.55	0.85	71.30
Mean MN-PCE -SD	-0.34	-0.26	0.45	-0.18	42.36
Mean PCE/NCE ratio	0.46	0.63	0.56	0.47	0.45
SD	0.08	0.20	0.10	0.09	0.13
Mean PCE/NCE +SD	0.54	0.83	0.66	0.56	0.58
Mean PCE/NCE -SD	0.39	0.43	0.46	0.38	0.32

MMC: Mitomycin C
N: number of animals
WW: statistically significant (Wilcoxon's test) p<0.01
Note: any discrepancy in this table is due to rounding differences

Conclusions

There was no evidence of clastogenicity or aneugenicity following oral (gavage) administration of R613636 up to the MTD of 1250 mg/kg/day in male mice. Therefore, R613636 is considered to be neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

B.6.8.1 – 6.4.63b Proof of exposure in mouse micronucleus test with R613636

Data requirement 2.33

Applicant to provide evidence of tissue exposure in the in vivo MN study with R613636 – study 63.
See also 2(92)
See reporting table 2(79)

NL (August 2017): The applicant provided proof of exposure for the in vivo mouse micronucleus test (Dunton, 2015c; study 63) by providing raw data on the presence of R613636 in blood samples. The RMS agrees with the conclusion that R613636 is systemically available and thus may reach the bone marrow.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):
During the Expert meeting, the proof of bone marrow exposure in the mouse micronucleus assay was discussed. In this assay, no bone marrow toxicity was evident. Bone marrow exposure was demonstrated by detectable blood and plasma concentrations of R613636 in male Crl:CD-1 mice, though at 24 hours very low levels were detectable. Based on the presence of the metabolite in blood and plasma at 4 hours post dosing, no concern for clastogenicity exists. However, no conclusion can be drawn on aneugenicity and therefore a data gap was recognized.

The notifier provided the following evidence of tissue exposure in the in vivo MN study:
"The in vivo bone marrow micronucleus assay is a widely used in vivo genotoxicity test. In the case that a negative result is obtained evidence must be presented that the compound of interest was systemically available to the bone marrow. As the bone marrow is a well-perfused tissue, concentrations of compound-related material(s) in blood or plasma are generally similar to those observed in bone marrow. Therefore, proof of exposure can be obtained by measurement of the compound of interest either in blood or plasma.

Concern that the compound is not available for distribution into bone marrow because of irreversible binding to red blood cells can be addressed two ways. Either analyse for the compound in plasma or demonstrate that any binding to a matrix in whole blood (plasma or red blood cells) was reversible. The preparation of whole blood for analysis will yield reversibly bound compounds, but not those irreversibly bound and compound quantified will be that distributed in blood and plasma.
Blood samples were taken 1 and 4 hours following oral administration and again after the terminal blood sample approximately 24 hours after the second test item administration.

After sampling each blood sample was diluted with1 % (v/v) formic acid in acetonitrile [1:3 (v/v)]. Prior to analysis each sample was extracted using organic solvent. Following centrifugation, to pellet the protein, the resulting supernatant was analysed via mass spectrometry.

As shown in the table below, R613636 was freely available at concentrations equivalent or higher than the 40 ng/mL low standard and quantifiable over several time points demonstrating sustained exposure. It can therefore be concluded that R613636 was systemically available to allow bone marrow exposure.

R613636 Analytical Response and Retention Time Data

R613636		
Sample I.D.	Peak area counts	Retention time (min)
Blank	0.0	0.00
STD Low	5482.6	1.73
STD High	260969.6	1.72
Blank	0.0	0.00
RF1 71 M DAY 1 1h	42811.8*	1.73
RF1 72 M DAY 1 1h	304250.8*	1.72
RF1 73 M DAY 1 1h	78827.5*	1.72
RF1 71 M DAY 1 4h	337.6	1.74
RF1 72 M DAY 1 4h	26956.4*	1.72
RF1 73 M DAY 1 4h	3515.5	1.72
RF1 71 M DAY 1 TER	47.0	1.80
RF1 72 M DAY 1 TER	172.1	1.72
RF1 73 M DAY 1 TER	175.1	1.72
RF1 74 F DAY 1 1h	4170.9	1.73
RF1 75 F DAY 1 1h	5193.5	1.73
RF1 76 F DAY 1 1h	36217.9*	1.72
RF1 74 F DAY 1 4h	5120.9	1.73
RF1 75 F DAY 1 4h	70856.7*	1.73
RF1 76 F DAY 1 4h	7252.9*	1.72
RF1 74 F DAY 1 TER	124.7	1.74
RF1 75 F DAY 1 TER	37.4	1.75
RF1 76 F DAY 1 TER	167.8	1.75
Blank	32.0	1.49
STD Low	6006.3	1.72
STD High	278007.0	1.73
Blank	30.9	1.84

*Study samples exhibit analytical response for R613636 greater than the mean response observed in the low spiked standard (40 ng/mL blood concentration)
TER = terminal sample

"

Genotoxicity SYN507900 – in vitro

B.6.8.1 – 6.4.64 Reverse mutation assay in bacteria with SYN507900 - study 64

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/58 Sokolowski, A. (2015q) SYN507900 - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Envigo CRS GmbH GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf Germany. Laboratory Report No. 1699301, issue date: 05 November 2015. Unpublished. Syngenta File No.SYN507900_10004.

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.

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

Study design

This study was performed to investigate the potential of SYN507900 dissolved in DMSO 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 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 both experiments:

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

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

Test Material:	SYN507900
Description:	White solid
Lot/Batch number:	MES 393/2
Purity:	99.0 % (estimated error: ± 2%)
Stability of test compound:	Not indicated by the sponsor
Expiry date:	31 July 2017

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)

Results

No precipitation of the test substance was observed in the overlay agar either in the test tubes or on the incubated plates. The plates incubated with the test substance 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 increase in revertant colony numbers of any of the six tester strains was observed following treatment with SYN507900 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. They showed a distinct increase of induced revertant colonies. Results of this study are given in the tables below.

Table 6.8.1 – 6.4.64-1: Summary of the Ames test, results of pre-experiment / experiment I

Study Name: 1699301
Experiment: 1699301 VV Plate
Assay Conditions:

Study Code: Harlan CCR 1699301
Date Plated: 15/09/2015
Date Counted: 18/09/2015

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 ± 1	9 ± 3	24 ± 2	164 ± 6	190 ± 4	331 ± 35
	Untreated		8 ± 5	11 ± 2	35 ± 9	163 ± 16	216 ± 15	336 ± 25
	SYN507900	3 µg	9 ± 5	10 ± 4	21 ± 1	151 ± 15	174 ± 15	323 ± 26
		10 µg	11 ± 3	11 ± 2	27 ± 8	156 ± 12	153 ± 4	320 ± 25
		33 µg	9 ± 4	12 ± 5	27 ± 8	166 ± 3	193 ± 20	317 ± 29
		100 µg	11 ± 1	10 ± 5	29 ± 1	148 ± 6	185 ± 33	287 ± 34
		333 µg	11 ± 1	8 ± 2	26 ± 4	164 ± 8	169 ± 10	332 ± 29
		1000 µg	10 ± 1	11 ± 4	24 ± 5	164 ± 12	151 ± 5	318 ± 21
		2500 µg	7 ± 2	10 ± 5	22 ± 4	172 ± 26	144 ± 24	284 ± 15
		5000 µg	13 ± 5	8 ± 3	30 ± 4	186 ± 16	142 ± 2	278 ± 16
	NaN3	10 µg	1180 ± 53			2183 ± 196		
	4-NOPD	10 µg			337 ± 15			
	4-NOPD	50 µg		66 ± 5				
	MMS	2.0 µL					4412 ± 68	4189 ± 109
With Activation	DMSO		9 ± 2	12 ± 1	28 ± 3	141 ± 9	210 ± 3	361 ± 20
	Untreated		11 ± 3	14 ± 3	42 ± 13	145 ± 3	248 ± 16	402 ± 6
	SYN507900	3 µg	11 ± 3	13 ± 2	27 ± 7	148 ± 17	199 ± 8	333 ± 54
		10 µg	12 ± 2	13 ± 1	33 ± 4	134 ± 7	194 ± 5	356 ± 10
		33 µg	11 ± 2	13 ± 4	24 ± 4	155 ± 23	201 ± 26	361 ± 53
		100 µg	11 ± 3	12 ± 3	27 ± 3	155 ± 27	213 ± 22	367 ± 22
		333 µg	8 ± 5	10 ± 3	27 ± 5	142 ± 13	193 ± 20	364 ± 18
		1000 µg	9 ± 1	12 ± 4	33 ± 6	150 ± 2	184 ± 20	334 ± 17
		2500 µg	10 ± 3	11 ± 2	31 ± 10	145 ± 3	194 ± 19	302 ± 38
		5000 µg	12 ± 3	12 ± 2	22 ± 8	134 ± 8	181 ± 15	287 ± 19
	2-AA	2.5 µg	410 ± 39	200 ± 27	3375 ± 750	3612 ± 871		
	2-AA	10.0 µg					1140 ± 108	2098 ± 166

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 – 6.4.64-2: Summary of the Ame test, results of Experiment II

Study Name: 1699301
Experiment: 1699301 HV2 Pre
Assay Conditions:

Study Code: Harlan CCR 1699301
Date Plated: 25/09/2015
Date Counted: 28/09/2015

Metabolic Activation	Test Group	Concentration (per plate)	Revertant Colony Counts (Mean ±SD)					
			TA1535	TA1537	TA98	TA100	WP2 pKM101	WP2 uvrA pKM101
Without Activation	DMSO		12 ± 5	11 ± 4	26 ± 5	184 ± 8	194 ± 11	303 ± 28
	Untreated		12 ± 3	9 ± 5	35 ± 12	201 ± 12	227 ± 23	353 ± 45
	SYN507900	33 µg	12 ± 4	9 ± 1	31 ± 9	146 ± 23	202 ± 41	327 ± 24
		100 µg	10 ± 5	12 ± 2	26 ± 5	174 ± 13	173 ± 25	293 ± 26
		333 µg	12 ± 2	8 ± 2	19 ± 1	154 ± 17	160 ± 23	280 ± 37
		1000 µg	10 ± 6	9 ± 1	23 ± 7	148 ± 8	157 ± 14	290 ± 18
		2500 µg	7 ± 2	10 ± 2	27 ± 8	155 ± 16	155 ± 23	249 ± 15
		5000 µg	10 ± 2	10 ± 2	32 ± 1	129 ± 8	164 ± 18	272 ± 28
	NaN3	10 µg	1103 ± 71			1955 ± 171		
	4-NOPD	10 µg			282 ± 2			
	4-NOPD	50 µg		82 ± 15				
	MMS	2.0 µL					3300 ± 331	2527 ± 360
With Activation	DMSO		13 ± 3	8 ± 2	37 ± 6	139 ± 11	209 ± 15	348 ± 8
	Untreated		16 ± 3	9 ± 3	37 ± 9	197 ± 25	259 ± 17	376 ± 31
	SYN507900	33 µg	12 ± 6	9 ± 4	34 ± 6	137 ± 6	192 ± 18	346 ± 36
		100 µg	10 ± 3	9 ± 3	37 ± 6	138 ± 5	185 ± 21	357 ± 15
		333 µg	9 ± 4	9 ± 4	35 ± 2	145 ± 22	211 ± 35	327 ± 31
		1000 µg	13 ± 3	11 ± 3	34 ± 7	144 ± 36	173 ± 16	318 ± 52
		2500 µg	12 ± 3	10 ± 4	43 ± 13	143 ± 27	172 ± 28	293 ± 7
		5000 µg	10 ± 2	10 ± 3	44 ± 9	152 ± 12	162 ± 36	296 ± 3
	2-AA	2.5 µg	411 ± 56	106 ± 10	4448 ± 670	3928 ± 327		
	2-AA	10.0 µg					1111 ± 79	1978 ± 95

Key to Positive Controls

NaN3	sodium azide
2-AA	2-aminoanthracene
4-NOPD	4-nitro-o-phenylene-diamine
MMS	methyl methane sulfonate

Conclusions

During the described mutagenicity tests and under the experimental conditions reported, SYN507900 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. SYN507900 is considered to be non-mutagenic in the *Salmonella typhimurium* and *Escherichia coli* reverse mutation assay.

B.6.8.1 – 6.4.65 In vitro chromosome aberration study with SYN507900 - study 65

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.
Report:	K-CA 5.8.1/59 Sokolowski, A. (2015r) SYN507900 - Chromosome Aberration Test in Human Lymphocytes *In Vitro*. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1699302, issue date: 16 November 2015. Unpublished. Syngenta File No. SYN507900_10008

GUIDELINES: Chromosome Aberration Test in Human Lymphocytes *In Vitro*. OECD 473 (2014); EPA OPPTS 870.5375 (1998); EC 440/2008 B.10 (2008)

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

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

Study design

This *in vitro* assay was performed to assess the potential of SYN507900 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/β-naphthoflavone treated male rats). In each experimental group two parallel cultures were analysed. Per culture at least 150 metaphases were evaluated for structural chromosomal aberrations, except for the positive controls in Experiment I with and Experiment II without S9 mix, where only 75 metaphases were evaluated.

The highest applied concentration in this study (2655.0 µg/mL of the test substance, approx. 10 mM) was chosen with regard to the molecular weight of the test substance and with respect to the current EPA and EU test guideline meeting and exceeding the maximum concentrations required by the OECD Guideline 473 (2014). Concentration selection for the cytogenetic experiments was performed considering the toxicity data in accordance with OECD Guideline 473.

Test Material:	SYN507900
Description:	White solid
Lot/Batch number:	MES 393/2
Purity:	99 % (estimated error: ± 2%)
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	Culture medium
Positive control:	Absence of S9 mix: Ethylmethane sulfonate, 825.0 µg/mL (Experiment I), 550.0 µg/ml (Experiment II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL (Experiment I), 10.0 µg/mL (Experiment II)

Results

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 no clear cytotoxicity was observed up to the highest evaluated concentration. The highest applied concentration showed clear cytotoxic effects, but was not evaluable due to the steep cytotoxicity gradient and because no evaluable metaphases were present.

In Experiment I, in the absence of S9 mix, no statistically significant or biologically relevant increase in the number of cells carrying structural chromosomal aberrations was observed after treatment with the test item.

In Experiment II, in the absence of S9 mix, one statistically significant increase (5.2 % aberrant cells, excluding gaps), which exceeded the 95 % control limits of the laboratory historical solvent control

data (2.3 % aberrant cells, excluding gaps) was observed after treatment with 1517.1 µg/mL. The rates of chromosomal aberrations observed at 283.1 and 866.9 µg/mL were outside the 95 % control limits of the laboratory historical solvent control data (2.3 % aberrant cells, excluding gaps), but not statistically significant. At these concentrations heterogeneity in response was also observed.
In Experiment I, in the presence of S9 mix, one statistically significant increase (4.3 % aberrant cells, excluding gaps), which exceeded the 95% control limits of the laboratory historical solvent control data (2.6 % aberrant cells, excluding gaps) was observed after treatment with 2655.0 µg/mL.
In Experiment II, in the presence of S9 mix, two statistically significant increases (5.3 % and 5.5 % aberrant cells, excluding gaps, respectively) were observed after treatment with 1843.8 and 2655.0 µg/mL. These values exceeded the 95 % control limits of the laboratory historical solvent control data (2.6 % aberrant cells, excluding gaps). The value after treatment with 1536.5 µg/mL (3.8 % aberrant cells, excluding gaps) also exceeded the 95 % control limits of the laboratory historical solvent control data, but this increase was not statistically significant. The cultures at 1536.6 and 2212.5 µg/mL showed heterogeneity in response.
No evidence of an increase in polyploid metaphases was observed after treatment with the test substance as compared to the control cultures.
Either EMS (550.0 or 825.0 µg/mL) or CPA (10.0 or 15.0 µg/mL) were used as positive controls and showed distinct increases in cells with structural chromosome aberrations. Results of the study are given in the table below.

Table 6.8.1 – 6.4.65: Summary of the chromosome aberration assay

Exp.	Preparation interval	Test item concentration (µg/mL)	Mitotic indices (% of control)	Aberrant cells (%)		
				incl. gaps*	excl. gaps*	carrying exchanges
Exposure period 4 h without S9 mix						
I	22 hrs	Solvent control[1]	100.0	3.0	2.7	0.0
		Positive control[2]	96.8	11.3	**10.0**[5]	3.0
		495.4	91.5	1.0	0.7	0.0
		866.9	113.8	1.3	1.0	0.0
		1517.1	97.5	2.0	1.3	0.0
		2655.0	86.2	2.3	2.3	0.0
Exposure period 22 h without S9 mix						
II	22 hrs	Solvent control[1]	100.0	2.3	2.3	0.0
		Positive control[3#]	52.8	32.0	**32.0**[5]	8.0
		283.1[##]	96.9	3.3	3.3	0.2
		495.4[##]	99.8	2.8	2.3	0.0
		866.9[##]	82.6	3.0	2.5	0.0
		1517.1[##]	67.3	6.2	**5.2**[5]	0.3

* Including cells carrying exchanges
\# Evaluation of 75 metaphases per culture
\## Evaluation of 300 metaphases per culture
5 Aberration frequency statistically significant higher than corresponding control values
1 Culture medium
2 EMS 825.0 µg/mL
3 EMS 550.0 µg/mL
4 CPA 15.0 µg/mL
5 CPA 10.0 µg/mL

Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test substance induced structural chromosomal aberrations in human lymphocytes *in vitro* in the absence and presence of a metabolic activation system. Therefore, SYN507900 is considered to be clastogenic in this chromosome aberration test, when tested up to the highest evaluable or required concentrations.

B.6.8.1 – 6.4.66 Cell mutation assay in mouse lymphoma cells with SYN507900 - study 66

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/60 Wollny H (2015h), SYN507900 - 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. 1699303 issue date: 10 November 2015. Unpublished. Syngenta File No.SYN507900_10006.

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test OECD 476 (1997): OPPTS 870.5300 (1998): EC 440/2008 B17 (2008)

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

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

Study design

The study was performed to investigate the potential of SYN507900 to induce mutations at the mouse lymphoma thymidine kinase locus using 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 maximum concentration of the pre-experiment and the main experiments was 2655 µg/mL, equal to approximately 10 mM, based on the molecular weight (265.5 g/mol) and the purity (99%) of the test substance.

The main experiments I and II were evaluated at the following concentrations with and without metabolic activation: 165.9; 331.9; 663.8; 1327.5; and 2655.0 µg/mL

Test Material:	SYN507900
Description:	White, solid
Lot/Batch number:	MES 393/2
Molecular weight:	265.5 g/mol
Purity	99 % w/w (estimated error ± 2%), no correction for purity
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	culture medium
Positive control:	Absence of S9 mix: Methylmethanesulphonate, 19.5 µg/mL Presence of S9 mix: Cyclophosphamide (CPA), 3.0 / 4.5 µg/mL

Results

No relevant cytotoxic effects indicated by a relative cloning efficiency 1 (survival) or relative total growth of less than 50% in both cultures occurred in experiment I and II with and without metabolic activation.

No substantial or reproducible concentration-dependent increase of the mutation frequency was observed in the main experiments with and without metabolic activation. The threshold was exceeded at an intermediate concentration of 331.9 µg/mL in both cultures of the second experiment with metabolic activation. However, this isolated increase was judged as biologically irrelevant as it neither was dose dependent nor reproduced in the first main experiment and higher concentrations showed no similar effect.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies using RStudio (Version 0.98, RStudio Inc.) statistics software. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

In this study the range of the solvent control values was from 60 up to 123 mutant colonies per 10^6 cells; the range of the group values treated with the test substance was from 58 up to 259 mutant colonies per 10^6 cells.

The viability slightly exceeded the upper limit of 120% with the second culture of experiment II (127%). The data are acceptable however, as the mean of both parallel cultures (127% and 111%, equal to a mean of 119%) remained within the acceptable range.

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 toxicity with at least one of the concentrations of the controls. Results of the study are given in the table below.

Table 6.8.1 – 6.4.66: Summary of the TK assay

	conc. µg per mL	S9 mix	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold	relative cloning efficiency 1	relative total growth	mutant colonies/ 10^6 cells	threshold
Column	1	2	3	4	5	6	7	8	9	10
Experiment I / 4 h treatment			culture I				culture II			
Solv. control with medium		-	100.0	100.0	120	246	100.0	100.0	106	232
Pos. control with MMS	19.5	-	61.1	26.7	363	246	66.1	23.2	342	232
Test item	83.0	-	84.7	culture was not continued#			101.7	culture was not continued#		
Test item	165.9	-	88.8	94.1	138	246	105.4	118.8	98	232
Test item	331.9	-	96.6	91.9	152	246	101.7	109.6	91	232
Test item	663.8	-	94.9	78.1	161	246	81.1	91.8	100	232
Test item	1327.5	-	101.8	83.9	161	246	109.3	117.9	59	232
Test item	2655.0	-	70.6	53.6	134	246	109.3	139.2	74	232
Solv. control with medium		+	100.0	100.0	60	186	100.0	100.0	123	249
Pos. control with CPA	3.0	+	78.5	41.1	397	186	103.5	68.5	287	249
Pos. control with CPA	4.5	+	67.8	21.1	545	186	71.3	33.4	536	249
Test item	83.0	+	57.1	culture was not continued#			105.3	culture was not continued#		
Test item	165.9	+	93.4	125.8	62	186	101.7	106.4	156	249
Test item	331.9	+	111.6	75.9	102	186	103.5	71.2	195	249
Test item	663.8	+	75.1	76.1	88	186	135.9	100.9	140	249
Test item	1327.5	+	89.0	93.5	58	186	95.2	87.1	125	249
Test item	2655.0	+	101.8	78.2	103	186	142.4	101.0	109	249
Experiment II / 4 h treatment			culture I				culture II			
Solv. control with medium		-	100.0	100.0	116	242	100.0	100.0	97	223
Pos. control with MMS	19.5	-	35.4	17.6	346	242	37.4	29.4	293	223
Test item	83.0	-	130.7	culture was not continued#			89.6	culture was not continued#		
Test item	165.9	-	57.5	120.6	92	242	65.8	126.6	89	223
Test item	331.9	-	95.1	154.7	111	242	67.0	112.8	92	223
Test item	663.8	-	89.2	130.8	148	242	75.0	99.2	78	223
Test item	1327.5	-	85.1	106.1	83	242	64.6	94.8	100	223
Test item	2655.0	-	140.0	140.3	91	242	70.8	60.1	67	223
Solv. control with medium		+	100.0	100.0	99	225	100.0	100.0	111	237
Pos. control with CPA	3.0	+	79.3	56.5	286	225	51.3	66.4	116	237
Pos. control with CPA	4.5	+	66.0	34.1	358	225	66.1	57.3	279	237
Test item	83.0	+	120.6	culture was not continued#			107.6	culture was not continued#		
Test item	165.9	+	149.3	78.5	195	225	125.3	50.0	169	237
Test item	331.9	+	131.2	61.3	232	225	162.3	42.6	259	237
Test item	663.8	+	122.6	65.2	204	225	140.5	48.4	198	237
Test item	1327.5	+	113.1	55.0	208	225	135.9	48.8	183	237
Test item	2655.0	+	87.7	103.6	89	225	123.3	101.6	76	237

threshold = number of mutant colonies per 10^6 cells of each solvent control plus 126

\# culture was not continued since a minimum of only four analysable concentrations is required

Conclusion

In conclusion, it can be stated that under the experimental conditions reported the test substance SYN507900 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, SYN507900 is considered to be non-mutagenic in this mouse lymphoma assay.

Genotoxicity SYN507900 – in vivo

B.6.8.1 – 6.4.67a Mouse micronucleus test with SYN507900 - study 67

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/61 Dunton J (2016), SYN507900 – Oral (gavage) Mouse Micronucleus Test. Sequani Limited, Bromyard Road, Ledbury, United Kingdom. Laboratory Report No. BFI0444 issue date: 01 March 2016. Unpublished. Syngenta File unknown.

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

Study design
SYN507900 was tested to evaluate its potential to cause damage to chromosomes or cell division apparatus, or to cause cell cycle interference, leading to micronucleus formation in polychromatic erythrocytes in the bone marrow of young adult mice.
In all phases, the dosing of the vehicle and test item was by oral (gavage) administration, on two consecutive occasions, approximately 24 hours apart. In the range-finding phase, a group of three male and three female mice were given SYN507900 at 2000 mg/kg/day in order to confirm the MTD. The MTD was confirmed to be greater than the limit dose of 2000 mg/kg/day in male and female mice, and as there was no inter-sex difference in toxicity, the main study was conducted in males only.
A proof of exposure phase was conducted to demonstrate that the bone marrow was exposed to the test item, via LC-MS/MS analysis of test item in the whole blood of treated animals. The presence of SYN507900 was confirmed by analysis of the study samples alongside samples of blank matrix and matrix spiked with the test item.
For the main study phase, three groups, each of six male mice were dosed with 500, 1000 or 2000 mg/kg/day SYN507900 on two successive occasions, approximately 24 hours apart. A group of five male mice (negative Controls) were similarly dosed with the vehicle alone and a positive Control group, of six male mice, were given a single 4 mg/kg intraperitoneal dose of Mitomycin C (MMC). Bone marrow was harvested from all range-finding and main study animals approximately 24 hours after the final dose administration and smears were prepared. The stained slides prepared for the main study were coded and 2000 polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei and the group frequencies were statistically analysed.

Test Material:	SYN507900
Description:	white to off white powder
Lot/Batch number:	CSCC210323
Purity:	99 %
Stability of test compound:	Retest date : 31 January 2017

Control Materials:

Negative control	N/A	Final Volume: N/A	Route: N/A
Vehicle:	0.5 % w/v aqueous carboxymethylcellulose with 0.1 % v/v Tween 80	Final Volume: 20 mL/kg	Route: oral
Positive control :	Mitomycin C	Final Doses: 4 mg/kg	Route: i.p.

Results
There were no clinical signs observed following administration of SYN507900 to male mice at dose levels up to 2000 mg/kg/day, nor were there any adverse clinical observations in Group 1 (negative Control) or Group 5 (positive Control).

There were no statistically significant increases in micronucleus frequency in male mice given any dose level of SYN507900, compared with the negative Control group, when tested up to the limit dose. There was no evidence of a statistically significant reduction in the PCE/NCE ratio in male mice given SYN507900, and, since proof of exposure to the bone marrow was demonstrated in the range finding phase of the study, this indicated a lack of toxicity of SYN507900 to the bone marrow.
The animals dosed with MMC, the positive Control item, had statistically significant increases in the number of micronucleated cells compared with the concurrent Control group, which demonstrated that the test system was capable of detecting a known clastogen and that the scorers were capable of detecting micronuclei. There was no statistically significant decrease in the PCE/NCE ratio in the positive Control group, indicating a lack of toxicity to the bone marrow. Results are given in table 6.8.1 – 6.4.67.

Table 6.8.1 – 6.4.67: Summary of the mouse micronucleus test

	Negative Control 0 mg/kg/day	SYN507900 500 mg/kg/day	SYN507900 1000 mg/kg/day	SYN507900 2000 mg/kg/day	MMC 4 mg/kg
N	5	6	6	6	6
Mean MN-PCE	1.20	0.83	0.67	1.17	53.33[WW]
SD	1.30	0.98	0.52	0.98	17.08
Mean MN-PCE +SD	2.50	1.82	1.18	2.15	70.42
Mean MN-PCE -SD	-0.10	-0.15	0.15	0.18	36.25
Mean PCE/NCE ratio	0.54	0.70	0.51	0.57	0.49
SD	0.09	0.13	0.13	0.06	0.07
Mean PCE/NCE +SD	0.62	0.83	0.64	0.64	0.56
Mean PCE/NCE -SD	0.45	0.57	0.38	0.51	0.42

MMC: Mitomycin C
N: number of animals
WW: statistically significant (Wilcoxon's test) $p<0.01$
Note: any discrepancy in this table is due to rounding differences

Conclusion

There was no evidence of clastogenicity or aneugenicity in male mice following oral (gavage) administration of SYN507900 up to the OECD 474 limit dose of 2000 mg/kg/day. SYN507900 is considered to be neither clastogenic nor aneugenic in the mouse bone marrow micronucleus assay.

B.6.8.1 – 6.4.67b Proof of exposure in mouse micronucleus test with SYN507900

Data requirement 2.34

Applicant to provide evidence of tissue exposure in the in vivo MN study with SYN507900 – study 67.

See also 2(89, 92)
See reporting table 2(80)

NL (August 2017): The applicant provided proof of exposure for the in vivo mouse micronucleus test

(Dunton, 2016; study 67) by providing raw data on the presence of SYN507900 in blood samples. The RMS agrees with the conclusion that SYN507900 is systemically available and thus may reach the bone marrow.

The notifier provided the following evidence of tissue exposure in the in vivo MN study:
"The in vivo bone marrow micronucleus assay is a widely used in vivo genotoxicity test. In the case that a negative result is obtained evidence must be presented that the compound of interest was systemically available to the bone marrow. As the bone marrow is a well-perfused tissue, concentrations of compound-related material(s) in blood or plasma are generally similar to those observed in bone marrow. Therefore, proof of exposure can be obtained by measurement of the compound of interest either in blood or plasma.

Concern that the compound is not available for distribution into bone marrow because of irreversible binding to red blood cells can be addressed two ways. Either analyse for the compound in plasma or demonstrate that any binding to a matrix in whole blood (plasma or red blood cells) was reversible. The preparation of whole blood for analysis will yield reversibly bound compounds, but not those irreversibly bound and compound quantified will be that distributed in blood and plasma.
Blood samples were taken 1 and 4 hours following oral administration and again after the terminal blood sample approximately 24 hours after the second test item administration.

After sampling each blood sample was diluted with1 % (v/v) formic acid in acetonitrile [1:3 (v/v)]. Prior to analysis each sample was extracted using organic solvent. Following centrifugation, to pellet the protein, the resulting supernatant was analysed via mass spectrometry.

As shown in the table below, SYN507900 was freely available at concentrations much higher than the 40 ng/mL low standard and quantifiable over several time points demonstrating sustained exposure. It can therefore be concluded that SYN507900 was systemically available to allow bone marrow exposure.

SYN507900 Analytical Response and Retention Time Data

SYN507900		
Sample I.D.	Peak area counts	Retention time (min)
Blank	0.0	0.00
STD Low	62903.9	2.15
STD High	2200546.9	2.13
Blank	0.0	0.00
RF1 71 M DAY 2 1h	3430354.1	2.13
RF1 72 M DAY 2 1h	2680065.0	2.13
RF1 73 M DAY 2 1h	4179504.3	2.12
RF1 71 M DAY 2 4h	4810023.7	2.13
RF1 72 M DAY 2 4h	4060622.5	2.10
RF1 73 M DAY 2 4h	4157262.0	2.12
RF1 71 M DAY 2 TER	486729.4	2.10
RF1 72 M DAY 2 TER	917112.7	2.12
RF1 73 M DAY 2 TER	138014.8	2.11
RF1 74 F DAY 2 1h	5476332.2	2.11
RF1 75 F DAY 2 1h	5742941.8	2.13
RF1 76 F DAY 2 1h	5588349.3	2.12
RF1 74 F DAY 2 4h	6229557.8	2.11
RF1 75 F DAY 2 4h	7975540.1	2.13
RF1 76 F DAY 2 4h	4889971.6	2.10
RF1 74 F DAY 2 TER	465645.1	2.11
RF1 75 F DAY 2 TER	401482.2	2.10
RF1 76 F DAY 2 TER	328079.1	2.11
Blank	0.0	0.00
STD Low	78897.0	2.11
STD High	2291301.7	2.09
Blank	931.5	2.09

All study samples exhibit analytical response for SYN507900 greater than the mean response observed in the low spiked standard (40 ng/mL blood concentration)
TER = terminal sample

"

B.6.8.1 - 6.5 Long-term toxicity and carcinogenicity

B.6.8.1 – 6.5.1 Long-term toxicity and carcinogenicity with SDS-3701 - study 1 rat

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. In addendum 14 of the DAR, the NOAEL of the study was set at 3.0 mg/kg bw/d, after a re-evaluation of the haemosiderin observed in females at 3.0 mg/kg bw/d.

Characteristics

reference	: Ford et al, 1983	exposure	: 104 weeks [2]
type of study	: chronic toxicity and carcinogenicity	doses	: 0, 0.5, 3, 10/15 and 20/30 mg/kg bw/day [2]
years of execution	: 1980-1982		
test substance	: SDS-3701 (purity 99%)	vehicle	: food
route	: oral	GLP statement	: yes
species	: rat, Charles River CD	guideline	: not according to OECD 453
group size	: 75/sex/dose[1]	**NOAEL**	: 3.0 mg/kg bw/day [3]

[1] an interim sacrifice was conducted on 10/sex/dose at 12 months
[2] animals of the 20/30 mg/kg bw/day were killed after 12 months, see also study design
[3] the authors of the report considered 3.0 mg/kg/day the NOEL for males and females

Study design

Due to poor survival, decreased body weight and anaemia, the dosage of group 4 (15 mg/kg/day) was reduced to 10 mg/kg/day for all animals beginning week 30 and remained at that level for the duration of the study. One-half of the males and all females in group 5 (30 mg/kg/day) were withdrawn from treatment during week 29 and fed control diet. The dosage for the other half of the males was reduced to 20 mg/kg/day was reduced to 20 mg/kg/day beginning week 30. All surviving group 5 animals were necropsied at one year.

Results

The results are summarized in Table **B.6.8.1 – 6.5.1.1**

Table B.6.8.1 – 6.5.1.1 Summary of results, Study 1

Dose (mg/kg bw/day)	0		0.5		3		10/15		30/20		dr
	M	F	M	F	M	F	M	F	M	F	
Mortality								i	i	i	
Clinical signs											
pale skin and eye							+	+	+	+	
Body weight							dc	dc	dc	dc	MF
Food consumption							d[1]	d[1]	d	i	
Ophthalmoscopy											
pale ocular structures									+	+	
haemorrhage									+	+	
bilateral cataract							i[2]				
Haematology											
erythrocyte counts									dc	dc	
haematocrit								dc	dc	dc	F
Hb								dc	dc	dc	F
MCV								dc	dc	dc	F
MCH								dc	dc	dc	F
MCHC							ic	ic	ic	ic	MF
reticulocyte counts									ic	ic	
relative neutrophil										ic	
counts								ic		ic	F
Clinical chemistry											
total protein								dc	dc	dc	F
albumin								dc	dc	dc	F
globulin								dc	dc	dc	F
cholesterol								dc	dc	dc	F
Urinalysis	No treatment-related findings										
Bone marrow smears											
haemosiderin						+		+	+	+	
12 month							+	+			
end kill								+			
increased cellularity								ic			
myeloid:erythroid ratio											
Organ weights											
kidneys								dc[a]	dc[a]	dc[a]	
heart								dc[a]	dc[a]	dc[a]	
spleen								ic[r]	dc[a]	dc[a]	
Pathology											
macroscopy											

Dose (mg/kg bw/day)	0		0.5		3		10/15		30/20		dr
	M	F	M	F	M	F	M	F	M	F	
hemorrhagic areas and pale tissues							+[1]	+[1]	+	+	
microscopy											
non-neoplastic lesions											
bone marrow											
- hypocellularity (1)								i	i	i	
liver											
- iron deposits								i	i	i	

dr dose related
dc/ic statistically significantly decreased/increased
d/i decreased/increased, but not statistically significantly
a/r absolute/relative organ weight
\+ present in one/a few animals
++ present in most/all animals
1 first 28 weeks before reduction of dosages
2 at 24 months

Conclusions

- 104 weeks of oral exposure of rats to the metabolite DS-3701 (purity 99%) via the food resulted in treatment-related changes in mortality, clinical signs, food consumption, haematology, clinical chemistry, bone marrow smear , organ weights, and pathology. Main target organ was the bone marrow.
- The 20-30 mg/kg/day group was included in the evaluation because the animals were sacrificed after 52 weeks. The treatment-related changes were observed at 10 - 15 mg/kg/day.
- Haemosiderin in bone marrow smears was observed in females of the 3 mg/kg/day group. The toxicological significance of the last finding in the absence of haematological findings is unclear. Based on the re-evaluation it can be concluded that the slight effect on haemosiderin seen in females at 3 mg/kg bw/day by Wright's stain at the termination of the study was not confirmed by the Prussian Blue stain. Furthermore, the slight change in haemosiderin at 3 mg/kg bw/day was not accompanied by other changes in bone smears as overall cellularity, maturity of the erythroid and myeloid cells, the presence of other cells and the estimated myeloid:erythroid ratios. In addition at 3 mg/kg bw/day no changes in haematology were observed. Based on this re-evaluation of bone marrow smear parameters, the effect on haemosiderin is not considered adverse.

Therefore, the NOAEL of the study can be established at 3 mg/kg bw/day, based on treatment related changes in body weight, food consumption, haematology, clinical chemistry, bone marrow smears, organ weights, hypocellularity in bone marrow and iron deposits in liver.

- There were no indications for carcinogenicity.

Guidelines and limitations

Deviations from OECD 453:

- The highest dose group (20-30 mg/kg/day) was killed before termination of the study due to severe toxicity. The dose of the 10 - 15 mg/kg/day group was decreased from 15 to 10 mg at week 30. The highest dose group was excluded from the evaluation of carcinogenicity. Still, three test groups are left for evaluation.
- Swollen necks were temporarily observed in most animals including controls during the study. The authors suggested that this clinical observation was related to a transient infection.
- Haematology was performed in ten instead of twenty animals/sex/group.

Nevertheless this toxicity study is considered appropriate for the overall evaluation.

B.6.8.1 – 6.5.2 Long-term toxicity and carcinogenicity with SDS-3701 - study 2 mouse

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Data requirement 2.35

Applicant to provide updated result tables of the long term toxicity study with SDS-3701 with the actual values/% of changes.

See reporting table 2(82)

NL (August 2017): The updated result tables provided by the notifier are added to the summary below. The conclusion by the RMS does not change.

Characteristics

reference	: Ford and Killeen, 1982b	exposure	: 24 months [1]
type of study	: chronic carcinogenicity study	doses	: 0, 375, 750 and 1500 mg/kg food (equivalent to 54, 107 and 214 mg/kg bw/day)
years of execution	: 1978-1980		
test substance	: SDS-3701		
route	: oral	vehicle	: food
species	: mouse CD-1	GLP	: yes
group size	: 60/sex/groups	statement	: not according to OECD 451 [2]
		guideline	: see guidelines and limitations
		NOAEL	: -

[1] females of groups 375 and 750 mg/kg food were sacrificed in month 20, females of controls and the 1500 mg/kg food groups were sacrificed in month 22.
[2] In the report stated as 'not applicable'

Study design

Due to poor survival in both female intermediate dose groups, 10 control females and all surviving females in the intermediate groups were sacrificed at month 20. All surviving females in the control and highest dose group and all surviving males were sacrificed at month 22. The increased mortality rates occurred from month 10 on in the mid dose group and in month 12 in the low dose group. After month 17, an accelerated mortality rate was seen in the high dose group females.

Results

The results are summarized in Table 6.8.1 – 6.5.2-1. Actual values and % of changes are given in result Tables 6.8.1 – 6.5.2-2/5).

Table B.6.8.1 – 6.5.2-1 Summary of results, Study 2

Dose (mg/kg food)	0.00		375		750		1500		dr
	M	F	M	F	M	F	M	F	
Mortality				i		i		i	
Clinical signs	No treatment-related findings								
Body weight						d	dc	dc	F
Food consumption					i	i	ic	ic	MF
Ophthalmoscopy	Not determined								
Haematology RBC[1]				dc	dc	dc	dc	dc	
Clinical chemistry	Not determined								
Urinalysis	Not determined								
Organ weights liver[2]			ic^{ar}		ic^{ar}	i^{r}	ic^{ar}	ic^{ar}	
kidneys							ic^{ar}	ic^{ar}	

Dose (mg/kg food)	0.00		375		750		1500		dr
	M	F	M	F	M	F	M	F	
Pathology macroscopy	No treatment-related findings								
microscopy *non-neoplastic lesions* spleen - haemosiderin								ic	
kidneys - chronic interstitial nephritis				ic		ic		ic	F

dr dose related
dc/ic statistically significantly decreased/increased
d/i decreased/increased, but not statistically significantly
a/r absolute/relative organ weight
\+ present in one/a few animals
++ present in most/all animals
1 At 12 months only (males), and at 12 and 18 months (females)
2 increases in liver weight of males were observed only when livers with grossly visible masses and/or cysts were excluded

Table B.6.8.1 – 6.5.2-2 Inter-group Comparison of Bodyweights at Selected Time Points (g)

	Achieved Intake of Chlorothalonil (mg/kg food)							
	Males				Females			
Week	0	375	750	1500	0	375	750	1500
1	26.2 (100.0)	25.5 (97.3)	25.1* (95.8)	23.6** (90.1)	22.3 (100.0)	22.2 (99.6)	21.9 (98.2)	21.1** (94.6)
4	30.7 (100.0)	30.5 (99.3)	28.3** (92.2)	26.2** (85.3)	24.7 (100.0)	24.1 (97.6)	23.1** (93.5)	22.4** (90.7)
12	34.7 (100.0)	34.6 (99.7)	34.2 (98.6)	32.0** (92.2)	29.1 (100.0)	28.0* (96.2)	27.3** (93.8)	25.6** (88.0)
24	37.3 (100.0)	36.6 (98.1)	36.2 (97.1)	34.8** (93.3)	32.4 (100.0)	32.3 (99.7)	31.1* (96.0)	28.5** (88.0)
47	40.7 (100.0)	39.9 (98.0)	39.9 (98.0)	38.3* (94.1)	34.0 (100.0)	33.0 (97.1)	31.9** (93.8)	29.4** (86.5)
83	41.0 (100.0)	41.0 (100.0)	39.9 (97.3)	38.1** (92.9)	35.1 (100.0)	33.4 (95.2)	33.5 (95.4)	29.9** (85.2)
103	40.0 (100.0)	39.5 (98.8)	39.5 (98.8)	38.1 (95.3)	-	-	-	-

** Statistically significant difference from control group mean, 1% level
* Statistically significant difference from control group mean, 5% level
-Groups terminated when mortality reached 75%

Table B.6.8.1 – 6.5.2-3: Inter-group Comparison of Food Consumption (g/kg/day) at Selected Time Points

	Achieved Intake of Chlorothalonil (mg/kg food)							
	Males				Females			
Week	0	375	750	1500	0	375	750	1500
1	230.2 (100.0)	235.0 (102.1)	240.2 (104.3)	227.3 (98.7)	276.8 (100.0)	264.8 (95.7)	296.2 (107.0)	297.7 (107.6)
4	196.8 (100.0)	205.8 (104.6)	218.8 (111.2)	283.7 (144.2)	273.5 (100.0)	270.0 (98.7)	332.5 (121.6)	334.2 (122.2)
12	174.7 (100.0)	174.1 (99.7)	181.7 (104.0)	215.5 (123.4)	222.1 (100.0)	235.9 (106.2)	234.5 (105.6)	276.8 (124.6)
24	141.7 (100.0)	158.9 (112.1)	163.1 (115.1)	178.9 (126.3)	207.7 (100.0)	197.5 (95.1)	216.3 (104.1)	289.2** (139.2)
47	120.8 (100.0)	114.3 (94.6)	117.2 (97.0)	124.3 (102.9)	175.5 (100.0)	149.8 (85.4)	162.0 (92.3)	239.0** (136.2)
83	133.5 (100.0)	132.5 (99.3)	134.8 (101.0)	139.3 (104.3)	168.9 (100.0)	145.8* (86.3)	142.3** (84.3)	179.7 (106.4)

103	127.4 (100.0)	140.4 (110.2)	137.5 (107.9)	131.1 (102.9)	-	-	-	-

** Statistically significant difference from control group mean, 1% level
* Statistically significant difference from control group mean, 5% level
-Groups terminated when mortality reached 75%

Table B.6.8.1 – 6.5.2-4: Inter-group Comparison of Selected Biochemistry Values

	Achieved Intake of Chlorothalonil (mg/kg bw/day)							
	Males				Females			
RBC (10^6/mm^3)	0	375	750	1500	0	375	750	1500
12 Month	8.45 (100.0)	7.64* (90.4)	7.93 (93.8)	7.81* (92.4)	9.34 (100.0)	8.36** (89.5)	8.31** (89.0)	8.47** (90.7)
18 Month	8.62 (100.0)	8.19 (95.0)	7.07 (82.0)	8.04 (93.3)	8.90 (100.0)	7.44 (83.6)	6.85* (77.0)	7.01* (78.8)

** Statistically significant difference from control group mean, 1% level
* Statistically significant difference from control group mean, 5% level

Table B.6.8.1 – 6.5.2-5: Inter-group Comparison of Organ Weights (g) (Month 24 for Males and Month 20 for Females)

	Achieved Dose of Chlorothalonil (mg/kg bw/day)							
	Males				Females			
Observation	0	375	750	1500	0	375	750	1500
Absolute Liver weight	1.550 (100.0)	1.785* (115.2)	1.765* (113.9)	1.718* (110.8)	1.482 (100.0)	1.659 (111.9)	1.714 (115.7)	-
Liver /Bodyweight	4.51 (100.0)	5.32** (118.0)	5.29** (117.3)	5.49** (121.7)	5.05 (100.0)	5.71 (113.1)	6.10** (120.8)	-
Absolute Kidney weight	0.739 (100.0)	0.694 (93.9)	0.688 (93.1)	0.641** (86.7)	0.548 (100.0)	0.495 (90.3)	0.503 (91.8)	-
Kidney/ Bodyweight	2.17 (100.0)	2.06 (94.9)	2.07 (95.4)	2.04 (94.0)	1.88 (100.0)	1.70 (90.4)	1.80 (95.7)	-

** Statistically significant difference from control group mean, 1% level
* Statistically significant difference from control group mean, 5% level

Conclusions

- 24 months of oral exposure of mice to DS-3701 (purity 99.6%) resulted in changes in body weight, food consumption, haematology, organ weights, and non-neoplastic histopathology of the spleen and kidneys. Although mortality was increased in all female test groups, the relationship with the treatment is not clear because a dose-related pattern was absent. A further evaluation of the increased mortality was hampered because the report did not commented on the causes of death. - Kidney histopathology and haematology changes were observed still in females at the lowest dose level tested, while liver weight changes were observed still in males of the lowest dose level.
- There were no indications for carcinogenicity.

Guidelines and limitations

Deviations from OECD 451:

- The mortality in both female intermediate dose groups exceeded 50% by month 18, a situation that is not allowed in a negative carcinogenicity study. The relationship with the treatment is not clear. Because no comments were made with respect to causes of death, no further analysis of the increased mortality was possible. The study is considered inappropriate for evaluation.

B.6.8.1 – 6.5.3 Long-term toxicity and carcinogenicity with SDS-46851 - study 3 rat

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Data requirement 2.36

Applicant to provide updated result tables of the long term toxicity study with SDS-46851, in particular with regards to progressive nephropathy, with the actual values/% of changes.

See reporting table 2(83)

NL (August 2017): The updated result tables provided by the notifier and a technical position regarding chronic progressive nephrophathy are added to the summary below. The conclusion by the RMS does not change.

Characteristics

reference	: ZENECA, Serrone et al. 1993	exposure	: 104 weeks
type of study	: chronic toxicity and carcinogenicity	doses	: 0, 80, 200, 500 and 1000 mg/kg bw/day
years of execution	: 1990-1992		
test substance	: SDS-46851 (purity 99.3%)	vehicle	: food
route	: oral	GLP statement	: yes
species	: rat, CD VAF/Plus Sprague-Dawley	guideline	: not according to OECD 453
group size	: 60/sex/dose	**NOAEL**	: 200 mg/kg bw/day (1)

1 According to the authors, 500 mg/kg bw/day was the NOAEL for males, based on retinal atrophy data.

Study design

The study was performed in accordance with OECD guideline 453, with the exception of the deviations described in 'guideline and limitations'.

Results

The results are summarized in Table 6.8.1 – 6.5.3-1. Actual values and % of changes are given in result Tables 6.8.1 – 6.5.3-2/4).

Table 6.8.1 – 6.5.3-1 Summary of results, Study 3

Dose (mg/kg bw/day)	0		80		200		500		1000		dr
	M	F	M	F	M	F	M	F	M	F	
Mortality	No treatment-related findings										
Clinical signs	No treatment-related findings										
Body weight	No treatment-related findings										
Food consumption							ic		ic	ic	MF
Ophthalmoscopy	No treatment-related findings										
Haematology	No treatment-related findings										
Clinical chemistry	No treatment-related findings										
Urinalysis	No treatment-related findings										

Dose (mg/kg bw/day)	0		80		200		500		1000		dr
	M	F	M	F	M	F	M	F	M	F	
Organ weights	No treatment-related findings										
Pathology											
macroscopy	No treatment-related findings										
microscopy											
non-neoplastic lesions											
eyes:											
- retinal atrophy [1]								i	i	ic	MF
kidneys:											
- severity of chronic progressive nephropathy			i		i		i		i		
neoplastic lesions											
kidneys											
- adenoma									1/60		
- cystadenoma									1/60		

dr dose related
dc/ic statistically significantly decreased/increased
d/i decreased/increased, but not statistically significantly
1 historical control data indicated the rare occurrence of the lesion

Table 6.8.1 – 6.5.3-2 Relative Food consumption g/kg/day

Week	Dose of SDS-46851 (mg/kg bw/day)									
	Males					Females				
	0	80	200	500	1000	0	80	200	500	1000
0	107.5 (100.0)	108.2 (100.7)	109.4 (101.8)	107.7 (100.2)	109.3 (101.7)	110.7 (100.0)	109.5 (98.9)	111.4 (100.6)	111.7 (100.9)	112.1 (101.3)
1	87.8 (100.0)	89.5 (101.9)	91.7 ** (104.4)	92.9 ** (105.8)	94.3 ** (107.4)	95.4 (100.0)	94.6 (99.2)	99.2** (104.0)	100.1** (104.9)	99.6** (104.4)
2 ++m / f	82.1 (100.0)	82.0 (99.9)	83.7 (101.9)	83.7 (101.9)	86.2 ** (105.0)	92.1 (100.0)	91.0 (98.8)	93.6 (101.6)	95.1* (103.3)	94.6* (102.7)
4 ++m[j] / f	67.4 (100.0)	68.2 (101.2)	69.9 ** (103.7)	70.3** (104.3)	70.5 ** (104.6)	80.6 (100.0)	81.4 (101.0)	83.2* (103.2)	83.5** (103.6)	83.8** (104.0)
5 ++ f	64.9 (100.0)	65.4 (100.8)	67.8 ** (104.5)	67.4 ** (103.9)	62.4 ** (96.1)	80.1 (100.0)	78.8 (98.4)	79.8 (99.6)	80.2 (100.1)	82.5* (103.0)
6 ++m[j] / f	60.5 (100.0)	61.9 * (102.3)	63.8 ** (105.5)	62.7 ** (103.6)	62.4 ** (103.1)	74.4 (100.0)	75.4 (101.3)	75.6 (101.6)	75.8 (101.9)	78.8** (105.9)
7 ++ m[j]	58.2 (100.0)	59.2 (101.7)	60.7 ** (104.3)	60.4 ** (103.8)	60.6 ** (104.1)	72.1 (100.0)	71.9 (99.7)	74.8** (103.7)	73.5 (101.9)	76.6** (106.2)
10 ++m	54.4 (100.0)	55.0 (101.1)	55.6 (102.2)	54.6 (100.4)	56.8** (104.4)	62.8 (100.0)	67.9 (108.1)	70.7** (112.6)	67.7 (107.8)	70.7** (112.6)
11 ++ m[j]	51.8 (100.0)	52.6 (101.5)	52.6 (101.5)	52.4 (101.2)	52.4 * (101.2)	65.5 (100.0)	65.4 (99.8)	68.2** (104.1)	65.5 (100.0)	67.8* (103.5)
41# ++ m[j]	39.3 (100.0)	38.7 (98.5)	39.4 (100.3)	40.2 (102.3)	40.8 * (103.8)	52.6 (100.0)	55.0 (104.6)	54.6 (103.8)	54.4 (103.4)	55.3* (105.1)
45 ++ m	39.1 (100.0)	38.9 (99.5)	39.7 (101.5)	40.3 (103.1)	41.7** (106.6)	52.5 (100.0)	52.1 (99.2)	53.0 (101.0)	53.9 (102.7)	54.3 (103.4)
49 ++ m[j]	38.0 (100.0)	38.6 (101.6)	41.3** (108.7)	40.0** (105.3)	40.3** (106.1)	52.0 (100.0)	53.8 (103.5)	53.2 (102.3)	54.7* (105.2)	53.9 (103.7)
55 ++ f	37.7 (100.0)	39.6 (105.0)	39.2* (104.0)	38.6 (102.4)	38.8 (102.9)	51.3 (100.0)	52.9 (103.1)	52.9 (103.1)	53.9 (105.1)	54.6* (106.4)
61 ++ m[j]	38.8 (100.0)	38.5 (99.2)	39.1 (100.8)	39.5 (101.8)	41.2** (106.2)	51.3 (100.0)	50.9 (99.2)	51.6 (100.6)	52.8 (102.9)	52.9 (103.1)
65 ++ m	38.4 (100.0)	38.0 (99.0)	39.3 (102.3)	41.1** (107.0)	41.6** (108.3)	50.0 (100.0)	50.2 (100.4)	50.6 (101.2)	52.0 (104.0)	52.0 (104.0)
69 ++m[j]	37.2 (100.0)	37.9 (101.9)	39.5* (106.2)	40.3** (108.3)	40.1** (107.8)	52.5 (100.0)	51.6 (98.3)	49.8 (94.9)	50.2 (95.6)	53.5 (101.9)

73 ++ m[j]	35.7 (100.0)	36.2 (101.4)	37.3 (104.5)	38.0** (106.4)	37.8** (105.9)	48.8 (100.0)	46.6 (95.5)	48.7 (99.8)	46.6 (95.5)	47.5 (97.3)
77 ++ m[j]	35.6 (100.0)	36.1 (101.4)	35.3 (99.2)	38.0 (106.7)	37.9 (106.5)	48.3 (100.0)	49.6 (102.7)	49.7 (102.9)	48.1 (99.6)	47.9 (99.2)
81 ++ m[j]	36.0 (100.0)	38.0 (105.6)	36.8 (102.2)	38.4 (106.7)	39.3* (109.2)	44.7 (100.0)	46.0 (102.9)	46.2 (103.4)	47.8 (106.9)	47.9 (107.2)
93 ++ f[j]	35.9 (100.0)	37.0 (103.1)	37.7 (105.0)	38.3 (106.7)	38.0 (105.8)	41.5 (100.0)	41.1 (99.0)	41.7 (100.5)	45.9* (110.6)	44.5 (107.2)

Numbers in brackets represent percent of control
Food consumption based on 5 rather than 6 days
* Statistically significant difference from control group at the 0.05 level (Bonferroni t test; unless otherwise indicated)
** Statistically significant difference from control group at the 0.01 level (Bonferroni t test; unless otherwise indicated)
++ Statistically significant trend at the 0.01 level; regression analysis, unless otherwise indicated (m / f denotes significance in males/ females respectively). m[j] / f[j] indicate statistical tests conducted with Dunn's test for multiple comparisons and Jonckheere's trend test due to signifcance in Bartletts test at the 0.01 level.

Table 6.8.1 – 6.5.3-3 Retinal atrophy incidence and severity

	Dose of SDS-46851 (mg/kg bw/day)									
	Males++					Females++				
	0	80	200	500	1000	0	80	200	500	1000*
Retinal atrophy, bilateral (Number examined)	60	60	60	60	60	60	60	60	60	60
Minimal	-	1	2	3	2	1	2	2	4	3
Slight/mild	1	-	-	-	2	-	-	-	1	4
Moderate	-	-	-	-	2	-	-	-	1	1
Moderately severe	-	-	-	-	2	-	-	-	-	1
Total	1	1	2	3	6	1	2	2	6	9

++ Statistically significant at the 0.01 level; (trend test, two tailed)
* Statistically significant at the 0.05 level; (Mantel-Haenszel permutation test)

Table 6.8.1 – 6.5.3-4 Chronic Progressive Nephropathy (includes data from sceduled sacrifice and unscheduled sacrifice and death)

	Dose of SDS-46851 (mg/kg bw/day)									
	Males+					Females				
	0	80	200	500**	1000	0	80	200	500	1000
Chronic Progressive Nephropathy (Number examined)	60	60	60	60	60	60	60	60	60	60
Minimal	25	14	18	18	13	40	34	38	39	35
Slight/mild	15	14	16	9	13	5	4	7	2	10
Moderate	7	7	9	14	10	-	2	2	2	3
Moderately severe	7	10	4	2	8	-	2	1	1	1
Severe / high	3	14	12	17	15	3	3	1	1	1
Total	57	59	59	60	59	48	45	49	45	50

+ Statistically significant at the 0.05 level; (trend test, two tailed)
** Statistically significant at the 0.01 level; (Peto mortality-prevalance test)

Conclusions

- 104 Weeks of oral exposure of CD rats to sds-46851 (purity 99.3%) via the food resulted in a slight increase in the incidence of males with severe chronic progressive nephropathy (CCPN) at all levels tested. The total incidence of CCPN did not differ between test groups and controls: almost all males were affected. A dose-response relationship was absent. Historical control data indicated that the incidence of severe CCPN in the control group of the present study was low. Although the historical control incidences of severe CCPN were lower than the incidences of severe CCPN found in the test groups of the present study, the absence of a dose-response relationship in the present study indicated that the relationship with treatment was rather unlikely.
- The NOAEL for males was 200 mg/kg bw/day, based on the food consumption data. The NOAEL for females was 200 mg/kg/day, based on the retinal atrophy data.
- No distinct incidences for carcinogenicity were obtained.

Co-RMS

A link between retinal degeneration and fungicide use has been suggested previously (see notably "Retinal degeneration in licensed pesticide applicators", by Kamel F et al. Am J Ind Med. 2000 Jun;37(6):618-28 and "Retinal degeneration and other eye disorders in wives of farmer pesticide applicators enrolled in the agricultural health study", by Kirrane EF et al. Am J Epidemiol. 2005 Jun 1;161(11):1020-9.). Although this effect has not been described for chlorothalonil, its occurrence in rat treated with SDS-46851 raises questions about the mechanism of action of this metabolite. Has this mechanism been examined to date?

coRMS-BE agrees on the NOAEL but is of the opinion that the adversity of an increase in food consumption could be questioned.

After the commenting round, the **notifier** provided addition tables addressing the requested endpoints (shown above) for this study. The notifier also provided the following statement regarding progressive nephropathy: "The incidence of progressive nephropathy can be seen to be similar across control and all dose groups. In the absence of any clear dose response relationship and no clear evidence of any

increase in severity with dose considering the >10 fold range, this findings is considered to incidental to treatment. The task force agree with the position stated by the RMS that although the historical control incidences of severe CCPN were lower than the incidences of severe CCPN found in the test groups of the present study, the absence of a dose-response relationship in the present study indicated that the relationship with treatment was rather unlikely. "

The **RMS** agrees with the statement provided by the notifier. In addition to the arguments above, as the findings are only observed in males and chronic progressive nephropathy is considered a rat-specific condition, the observed effects are not consided relevant for humans.

Guidelines and limitations
Deviations from OECD 453 guidelines:
10 instead of 20 animals/sex/dose were used for haematology. Weights of adrenals and ovaries were not determined. Nevertheless this study is considered appropriate for the evaluation.

B.6.8.1 – 6.5.4 Long-term toxicity and carcinogenicity with SDS-46851 - study 4 mouse

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: ZENECA, Lucas and Laveglia, 1994	exposure	: 18 months
type of study	: carcinogenicity study	doses	: 0, 1000, 3500 and 7000 mg/kg food[1]
years of execution	: 1991-1992	vehicle	: food
test substance	: SDS-46851 (purity 99.3%)	GLP statement	: yes
route	: oral	guideline	: not according to OECD 451
species	: mouse Crl:CD-1 (ICR); BR VAF/Plus	**NOAEL**	: carcinogenicity study
group size	: 60/sex/dose		

[1] equal to 0, 157, 548 and 1132 mg/kg bw/day for males and 0, 185, 642 and 1295 mg/kg bw/day for females

Study design
The study was performed in accordance with OECD guideline 451, with the exception of the deviations described in 'guideline and limitations'. Dose levels were obtained from a range-finding study (ZENECA, Mizens, 1991), in which mice received 0, 250, 750, 2200 and 7500 mg/kg food for 90 days (dose levels equal to 0, 41, 122, 368 and 1270 mg/kg bw/day for males and 0, 47, 145, 456 and 1532 mg/kg bw/day for females). The only observed effect was renal tubular epithelial hyperplasia in 1/10, 2/10 and 4/10 animals in the 250, 750 and 7500 mg/kg food groups, but not in the control nor in the 2200 mg/kg food group.

Results
The results are summarized in Table 6.8.1 – 6.5.1.4

Table 6.8.1 – 6.5.4-1 Summary of results, Study 4

Dose (mg/kg food)	**0**		**1000**		**3500**		**7000**		**dr**
	M	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
Mortality	No treatment-related findings								
Clinical signs - increased activity and aggression							i		

Dose (mg/kg food)	0		1000		3500		7000		dr
	M	F	M	F	M	F	M	F	
- anogenital staining							i	i	
Body weight							i[1]		
Food consumption				ic		ic	ic	ic	
Ophthalmoscopy	Not determined								
Haematology	No treatment-related findings								
Clinical chemistry	Not determined								
Urinalysis	Not determined								
Organ weights	No treatment-related findings								
Pathology macroscopy	No treatment-related findings								
microscopy *non-neoplastic lesions*	No treatment-related findings								

dr dose related
1 increased only during the first 4 weeks of exposure

Conclusions

- 18 Months of oral exposure of mice to SDS-46851 via the food resulted in slightly increased incidences of some clinical signs in the 7000 mg/kg food group, increased food consumption in 7000 mg/kg food males and in all test group females. The increases in females did not show a dose-response relationship and, therefore, the relationship with the treatment is doubtful.
- Based on the clinical signs and food consumption results in males the authors established a NOAEL of 3500 mg/kg food (equal to 548 mg/kg/day).
- No indications for carcinogenicity were obtained.

Guidelines and limitations

Deviations from OECD 451:
Haematology was performed on 10 animals/sex/dose instead of all control and top-dose animals. Nevertheless this study is considered appropriate for the oncogenicity evaluation.

B.6.8.1 - 6.6 Reproductive toxicity

B.6.8.1 - 6.6.1 Generational studies

B.6.8.1 – 6.6.1.1 Generational studies with SDS-3701 - study 1 rat

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: ZENECA, Ford and Killeen., 1982c	exposure	: during a pre-pairing period and throughout pairing, gestation, lactation, and rest periods
type of study	: reproductive toxicity (1 generation)		
years of execution	: 1981-1982		
test substance	: SDS-3701, 99%	doses[1]	: 0, 10, 20, 30, 60, 120 mg/kg food[2]
route	: oral	vehicle	: diet
species	: rats, CD Sprague-Dawley	GLP statement	: yes
group size	: 12 male/dose; 24 females/dose (F0 parents)	guideline	: in accordance with OECD 415
		NOAEL	: parental 3 mg/kg bw/day developmental 1.5 mg/kg bw/day reproductive >6 mg/kg bw/day

[1] The present one-generation study was conducted following up a 3-generation reproduction study (ZENECA, Ford et al. 1981a,b)**.** Dose levels of 0, 10, 60, 125 mg/kg food (equivalent to 0, 0.5, 3, 6.3 mg/kg bw/day) were administered in diet through three successive generations of CD Sprague-Dawley rats. The rats were mated during two-weeks period, followed by rest period, and a second mating period. Histopathology was not performed on the F0 parental animals. The NOAEL for parental and developmental effects was established at 10 mg/kg food based on lower mean pup weights observed in all three generations, and a reduction in body weight in the parental animals at the mid and high dose level. The NOAEL for reproductive effects is >125 mg/kg food (equivalent to >6.3 mg/kg bw/day).
The one-generation study was performed to define the NOAEL using intermediate dosage levels between 10 and 60 mg/kg food.

[2] equivalent to 0, 0.5, 1, 1.5, 3, 6 mg/kg bw/day

Study design

The study is in accordance with OECD 415 except for a two-week mating period that was carried out twice with a rest period within of three weeks, resulting in two litters (F1a, F1b). Given the poor mating index in the control and high dose group, the first mating period was extended with 5 additional days. In the first mating period, the animals were mated on a 1:2 basis. The animals were mated 1:1 during the second mating period. No tests were carried out to investigate non-fertile animals. Physical examination was not performed during the mating, gestation, and lactation period. The number of males included in this study was too low (n=12).

Results

The results are summarized in Table 6.8.1 – 6.6.1.1-1.

Table 6.8.1 – 6.6.1.1-1 Summary of results, Study 1

Dose / effect (mg/kg food)	**0**		**10**		**20**		**30**		**60**		**120**		**dr**
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
F0 Animals													
Mortality				1[1]									
Clinical signs	no treatment-related findings												
Body weight	no treatment-related findings												
Food consumption week 6-28											ic[r]		
Mating/fertility/ gestation[2]	no treatment-related findings												

Dose / effect (mg/kg food)	**0**		**10**		**20**		**30**		**60**		**120**		**dr**
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
Pathology macroscopic	no treatment-related findings												
F1 pups													
Litter size	no treatment-related findings												
Survival index viability index on day 4											dc		
Sex ratio	no treatment-related findings												
Body weight									dc[3]		dc[4]		
Pathology macroscopic	no treatment-related findings												

dr dose-related
r relative, in g/kg bw
1 one female died during gestation (week 24 of study); enlarged spleen and liver were found at necropsy
2 a relatively poor mating index was observed in the control group
3 day 10-21, F1b litters
4 day 7-21

Table 6.8.1 – 6.6.1.1-1: Pup body weights (g)

	Dietary concentration (ppm) DS-3701					
	F1A					
	0	**10**	**20**	**30**	**60**	**120**
Mean pup weight at birth	6.6	6.6	6.6	6.7	6.7	6.6
Mean pup weight day 7	16.8	16.7	16.1	16.7	15.6	**13.1****
Mean pup weight day 14	32.3	31.5	30.2	32.2	29.5	**25.1****
Mean pup weight day 21	52.6	51.1	49.7	51.5	47.1	**42.0****
	F1B					
	0	**10**	**20**	**30**	**60**	**120**
Mean pup weight at birth	6.7	6.7	6.8	6.5	6.5	6.6
Mean pup weight day 7	17.9	17.1	17.2	16.6	15.7	13.7**
Mean pup weight day 14	34.1	32.2	33.1	31.9	**29.3****	**26.6****
Mean pup weight day 21	55.3	52.0	54.6	52.2	**48.1***	**44.3****

* Significantly different from control P < 0.05
** Significantly different from control P < 0.01

Conclusions

- The NOAEL for parental toxicity is 60 mg/kg food (equivalent to 3 mg/kg bw/day) based on the increased relative food consumption in the F0-males.
- Given the decrease in body weight of the F1 pups, the NOAEL for developmental effects is established at 30 mg/kg food (equivalent to 1.5 mg/kg bw/day). It is noted that pup weight at birth was not decreased at birth and day 7, statistical significance was only reached from day 14 onwards when pups start eating solids.
- The NOAEL for reproductive effects is >120 mg/kg food (equivalent to >6 mg/kg bw/day).

Guidelines and limitations

The deviations from the OECD 415 guideline (see study design) are considered to be of no influence on the results of this study. The results of the study are considered acceptable for the overall evaluation.

Data requirement 2.37

Applicant to provide a technical review of the generational study in rat with SDS-3701.

See reporting table 2(84)

NL (August 2017): The notifier provided a statement regarding the NOAEL in the generational study in rats. This statement and the response by the RMS is provided below.

Comment by EFSA:

" It is questioned whether a (parental) NOAEL should be based on increased food consumption. Anyway, offspring's seem to be more sensitive than parents since effects on bw were observed in the absence of parental toxicity. Reproductive toxicity investigations were limited compared with current data requirements for this type of study."

Response by Notifier:

"The Task Force considers that as the pattern of changes in absolute and relative food consumption parameters is diverse, these effects are not considered to be treatment-related, and therefore should not be used to determine a NOAEL."

Response by RMS:

With the setting of the NOAEL, also the preceding three-generation study (Ford et al. 1981a,b, refer to B.6.8.1 – 6.6.1.1) was considered. In this study, the effect level for parental and developmental effects was established at 60 mg/kg food (equivalent to 3 mg/kg bw/day) based on lower mean pup weights observed in all three generations, and a reduction in body weight in the parental animals. Therefore, the NOAEL is set at 60 mg/kg food (equivalent to 3 mg/kg bw/day) for parental toxicity.

Data requirement 2.39

Applicant to submit a classification proposal for SDS-3701.

See experts' consultation in 2(97)
See reporting table 2(87)

NL (August 2017): The RMS agrees with the Weight of Evidence Assessment provided by the notifier and that based on the available studies sufficient information is available to conclude that no classification for developmental and reproductive effects is warrented.

The Weight of Evidence Assessment provided by the applicant focusses on the Wazeter (1976b) and

Schroeder (1998) studies.

In the reproductive study (Ford and Killeen 1982c), a decreased pup weight was observed at a dose level of 3 mg/kg bw/day while no maternal toxicity was observed at this dose. However, it is noted that the effect on pup weight only occurred from day 14 of lactation when pups start eating the diet on their own. This effect should therefore not be consided for classification and labelling for developmental toxicity.

The Task Force provided a Weight of Evidence Assessment for developmental toxicity for SDS-3701 based on the findings of the available studies, all of which have been evaluated in the EU previously and the most informative are presented in this RAR. This Weight of Evidence Assessment is summarized below.

Table 6.8.1 - 6.6.1.1-3: Developmental and Reproductive Toxicity Studies - SDS-3701

Reference	Species and strain	GLP	Guideline	Reference in RAR
Schroeder, 1998a	Crl:CD® (SD) rat	Yes	Non-Guideline	A range-finding developmental toxicity study, not included in the RAR.
Schroeder, 1998b	Crl:CD® (SD) rat	Yes	OECD 414 (1981)	B.6.8.1 – 6.6.2.2/3
Wazater & Goldenthal, 1976b	Dutch Belted rabbit	No	Non-Guideline	B.6.8.1 – 6.6.2.1
Ford & Killeen, 1981	Crl:CD® (SD) rat	Yes	OECD 416 (1983) equivalent	No study summary is provided in the RAR; study mentioned in summary of Ford and Killeen, 1982c; B.6.8.1 – 6.6.1.1
Ford and Killeen, 1982c	Crl:CD® (SD) rat	Yes	OECD 415 (1983) equivalent	B.6.8.1 – 6.6.1.1

In these studies, observations support the conclusion that SDS-3701 induced embryolethality in the rat following dosing via the oral route at doses above 15 mg/kg bw/day (Schroder, 1998a; Schroeder, 1998b) and the rabbit at doses above 5 mg/kg/day (Wazater & Goldenthal, 1976b), by inducing significant maternal toxicity, specifically maternal anaemia, and not from a direct and specific effect of SDS-3701 on the foetus. In addition, there is also further signs of developmental toxicity reported (foetal oedema, growth retardation and foetal death) which are also considered related to the hypoxia produced by the anaemic condition in the foetus. This embryo-foetal anaemia leads to hypoxia in tissues, and it has been shown that hypoxia causes abnormal embryo-foetal development (Grabowski, 1970).

In the table below, the outcome of the Weight of Evidence assessment is summarized.

Table 6.8.1 - 6.6.1.1-3: Process decisions and classification outcome for SDS-3701 using the classification framework for developmental and reproductive toxicity as shown in Moore et al. (2013).

	SDS-	*Rationale*

	3701	
1. Compile all available relevant data	✓	The studies were adequately conducted and reported, and the prinicipal studies had sufficient group sizes for evaluation
2. Is there a substance-related response	✓	Yes, observed in three studies (Schroeder, 1998a; Schroeder, 1998b; Wazater & Goldenthal, 1976b). There was reductions in foetal weight (≥30 mg/kg/day), an increase incidence of total resorptions (60 mg/kg/day), increased post-implantation losses (≥30 mg/kg/day) and an increased incidence of foetuses with oedema (≥30 mg/kg/day) (Schroeder, 1998a). Increased post-implanation losses (≥15 mg/kg/day), an increase incidence of total resorptions (25 mg/kg/day), and reductions in foetal weight (≥15 mg/kg/day) were also observed in Schroeder, 1998b. Increased post-implanation losses (5 mg/kg/day), an increase incidence of abortions (5 mg/kg/day) were also observed in the rabbit (Wazater & Goldenthal, 1976b). However, the above findings were observed in the presence of significant maternal toxicity (reductions in both food consumption, body weight and body weight gains, and anaemia).
3. What is the level of concern	**H**	The level of concern is HIGH.
4. Consider response in context of all available data (weight of evidence)	✓	The reproductive and developmental effect (i.e. post-implantation loss) is considered to be secondary to significant maternal toxicity (anaemia). There was no effect on fertility or litter size in a dietary three-generation reproduction toxicity study in the rat (Ford & Killeen, 1981a,b) and a one-generation study reproduction toxicity study in the rat (Ford and Killeen, 1982c).
5. Can a MoA or causal relationship be established?	✓	There is data to support a MoA or causal relationship for reductions in foetal weight, an increase incidence of total resorptions, increased post-implantation losses and an increased incidence of foetuses with oedema, these effects are considered due to the evident maternal toxicity (anaemia).
6. Is the MoA or causal relationship relevant to humans	✓	It is well acknowledged from observations in humans that anaemia during pregnancy can be associated with a number of developmental problems in the newborn and developing child (Mihaila *et al*., 2011).
7. What is the final level of concern?	**L**	The final level of concern is considered to be low, no classification.
8. Is there data to indicate effects on or via lactation?	✘	There were no observations that might indicate an effect mediated on via the milk.
Classification	**None**	

H, high concern; M, moderate concern; L, low concern;

To summarize, several observations support the conclusion that SD-3701 induced embryolethality in the rat following dosing via the oral route at doses above 15 mg/kg bw/day and the rabbit at doses above 5 mg/kg/day, by inducing significant maternal toxicity, specifically maternal anaemia, and not

from a direct and specific effect of SD-3701 on the foetus. In addition, there is also further signs of developmental toxicity reported (foetal oedema, growth retardation and foetal death) which are also considered related to the hypoxia produced by the anaemic condition in the foetus. There was no effect on fertility or litter size in a dietary three-generation reproduction toxicity study in the rat and a one-generation study reproduction toxicity study in the rat.

When comparing the findings of the aforementioned studies with the classification and labelling criteria in accordance with Regulation (EC) No. 1272/2008), can be concluded that classification of R182281 for developmental and reproductive effects is not warranted on based on the following:
- *Category 1A* (known human reproductive toxicant) is not appropriate as there is no human evidence establishing a causal relationship between exposure to R182281 and an adverse effect on development.
- *Category 1B* (presumed human reproductive toxicant) is also not appropriate as there is no clear evidence of an adverse effect on development in experimental animals that is considered not to be the secondary, non-specific consequence of other toxic effects.
- *Category 2* (suspected human reproductive toxicant) is also not appropriate because there is no evidence of an adverse effect on development in experimental animals that is considered not to be the secondary, non-specific consequence of other toxic effects.

Response by RMS:
The RMS agrees with the Weight of Evidence Assessment provided by the notifier. The RMS adds to this that the induction of anaemia by SDS-3701 is clearly demonstated in the 60-day oral toxicity study (B6.8.1 – 6.3.1) and the carcinogenicity study (B.6.8.1 – 6.5.1), with the latter at similar dose levels as in the reproduction toxicity studies.
The RMS agrees that no classification for developmental and reproductive effects is warrented. Also refer to commenting box for data requirement 2.39 above.

B.6.8.1 – 6.6.1.2 Generational studies with SDS-46851- study 2 rat

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: ZENECA, Lucas., 1993	exposure	: during a pre-pairing period and throughout pairing, gestation, lactation and rest periods
type of study	: reproductive toxicity, two-generation		
years of execution	: 1991-1992		
test substance	: SDS-46851, 99.3%	doses[1]	: 0, 2000, 6000, 20000 mg/kg food[2]
route	: oral	vehicle	: diet
species	: rats, Sprague-Dawley (CD-VAF/PLUS)	GLP statement	: yes
		guideline	: in accordance with OECD 416
group size	: 35/sex/dose; (F0 parents)	**NOAEL**	: parental 269 mg/kg bw/day developmental >911 mg/kg bw/day reproductive >911 mg/kg bw/day

[1] The dose levels were chosen on the basis of a combined 90-day feeding and one-generation reproduction study (ZENECA, Serrone et al., 1988). Male rats were administered dose-levels of 0, 5200, 15500, and 40000 mg/kg food in diet (equivalent to 0, 250, 750, and 2000 mg/kg bw/day) and female rats 0, 4000, 12000, and 32000 mg/kg food in diet (equivalent to 0, 200, 600, and 1600 mg/kg bw/day). The NOAEL for parental animals was 250 mg/kg bw/day based on an increase of relative and absolute kidney and liver weights in the mid and high dose group. The reproduction part of the study was not conform any specific guideline. No effects on reproduction were observed. Lower body weights in pups in

the highest dose group were found during the latter part of the lactation period. However, these were not considered an effect of the test material on reproduction.

[2] equal to 0, 88, 269, 911 mg/kg bw/day and 121, 370, 1246 mg/kg bw/day for males and females, respectively. The lowest mean compound consumption value for males was measured in week 22 in the F1 generation and for females in week 22 in the F0 generation.

Study design

The study is in accordance with OECD 416 except for a two-week mating period that was carried out twice with a rest period of three weeks, resulting in two litters per generation (F1a, F1b, F2a, F2b). All animals were mated during the second mating period irrespective if they had delivered a litter after the first mating period. No tests were carried out to investigate non-fertile animals. Individual food consumption was not measured during mating, gestation, and lactation periods for males and females.

Results

The results are summarized in Table 6.8.1 – 6.6.1.2

Table 6.8.1 – 6.6.1.2 Summary of results, Study 2

Dose / effect (mg/kg food)	**0**		**2000**		**6000**		**20000**		**dr**
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
F0 Animals									
Mortality		1		1	2	1		1	
Clinical signs	no treatment-related findings								
Body weight during gestation (day 7; F1b) during lactation (day 21; F1a)								ic ic	
Food consumption			ic[1]		ic[r,1] dc[r,2]		ic[a,r, 1]	ic[a,r, 3]	
Mating/fertility/ gestation	no treatment-related findings								
Pathology macroscopic microscopic kidneys: regenerative tubular epithelium								ic	
F1 pups									
Litter size	no treatment-related findings								
Survival index	no treatment-related findings								
Sex ratio	no treatment-related findings								
Body weight	no treatment-related findings								
Pathology macroscopic	no treatment-related findings								
F1 Parental animals									
Mortality	1	1			1				
Clinical signs	no treatment-related findings								
Body weight	no treatment-related findings								
Food consumption				ic[r, 4]		ic[r, 5]		ic[r, 6]	
Mating/fertility/ gestation	no treatment-related findings								
Pathology macroscopic microscopic	no treatment-related findings no treatment-related findings								
F2 pups									

Dose / effect (mg/kg food)	**0**		**2000**		**6000**		**20000**		**dr**
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	
Litter size	no treatment-related findings								
Survival index	no treatment-related findings								
Sex ratio	no treatment-related findings								
Body weight	no treatment-related findings								
Pathology macroscopic	no treatment-related findings								

dr dose-related
\+ present in one/a few animals
++ present in most/all animals
1 occasionally during week 6-8
2 decrease in week 20
3 occasionally during week 1-3
4 week 2-7
5 week 1, 3-7, 9-11
6 week 1-12

Conclusions

- As the pattern of changes in absolute and relative food consumption parameters is diverse, these effects are not considered to be treatment-related. The NOAEL for parental toxicity is 6000 mg/kg food (equal to 269 mg/kg bw/day) based on the microscopic effects on the kidneys observed in the F0 females and an increased body weight during gestation and lactation. The incidence of regenerative tubular epithelium was statistical significant increased. No treatment-related effects were found on the severity.
- No treatment-related effects were observed in the pups, therefore, the NOAEL for developmental effects is >20000 mg/kg food (equal to >911) mg/kg bw/day).
- The NOAEL for reproductive effects is >20000 mg/kg food (equal to >911 mg/kg bw/day).

Guidelines and limitations

The study is despite some deviations (see study design) in accordance with OECD 416 guideline. The results of the study are considered acceptable for the overall evaluation.

Data requirement 2.38

Applicant to provide a justification for the assessment of the reproductive toxicity of SDS-46851.

See reporting table 2(85)

NL (August 2017): The RMS agrees with the justification provided by the notifier (shown below) and remains with the original conclusion that despite the deviations, the results of the study are considered acceptable for the overall evaluation.

Comment by EFSA:

"Reproductive toxicity investigations were limited compared with current data requirements for this type of study."

Response by notifier:

"The two-generation study in rats was conducted in 1992 to 1993 (Lucas and Kileen, 1993). This study were therefore conducted according to the contemporaneous guideline OECD 416 (1983). Departures from the current OECD 416 (2001) are provided below, along with justification as to why the study provide sufficient assessment of reproductive toxicity to fill the 'data gaps'.

Endpoint specified for which no data are available 'data gaps'	**Data available which are considered to provide sufficient information to cover the 'data gaps'**
Adults	
Oestrus cyclicity Number of P and F1 females cycling normally and cycle length;	Oestrous cyclicity was not evaluated directly on the study, however, there were no treatment related effect on • The pre-coital interval in the F_0 or F_1 adults • Gestation length in the F_0 or F_1 adults • The number of successful pairings for P and F1 adults with two mating periods. • Litter size at day 0 at any dose level for F_{1a}, F_{1b}, F_{2a} or F_{2b} litters. • Histopathology of the ovary, uterus, cervix or vagina. If there had been an effect on oestrus cyclicity in the treated females on this study, there would have had a subsequent effect on one or more of the above parameters. Since there were no effects it can be concluded that there was no impact on oestrous cycling in treated animals.
Sperm parameters From a subset of 10 males from the P and F1 group one testis and epididymis should be used to evaluate sperm parameters. The report should include total cauda epididymal sperm number, percent progressively motile sperm, percent morphologically normal sperm, and percent of sperm with each identified abnormality;	Sperm parameters were not evaluated directly on the study, however, there was no treatment related effects on: • Histopathology of prostate, epididymis and seminal vesicles at all dose levels for P generation adults. • Histopathology of testes for high dose P generation adults and F1 generation adults at all dose levels. • The number of successful pairings for P and F1 adults with two mating periods. • Litter size at day 0 at any dose level for F_{1a}, F_{1b}, F_{2a} or F_{2b} litters.
Organ weights (sex organs specifically)	Organ weights (sex organs specifically) were not evaluated directly on the study, however, there were no treatment related effect on • Histopathology of prostate, epididymis and seminal vesicles at all dose levels for P generation adults. • Histopathology of testes for high dose P generation adults and F1 generation adults at all dose levels. • Histopathology of the vagina, uterus and ovaries for P and F1 generation adults at the high dose.
Histopathology A quantitative evaluation of primordial follicles should be conducted for F1 females.	There was no treatment related effect on: • The number of successful pairings for P and F1 adults with two mating periods. • Histopathology of the ovary for P and F1 generation adults at the high dose. • Litter size at day 0 at any dose level for F_{1a}, F_{1b}, F_{2a} or

Endpoint specified for which no data are available 'data gaps'	Data available which are considered to provide sufficient information to cover the 'data gaps'
	F_{2b} litters.
Offspring:	
Functional investigations (motor activity, sensory function, reflex ontogeny) particularly those related to sexual maturation recommended.	• Sexual maturation was not specifically investigated in the study, however, there were no treatment related effect on the histopathology of the accessory sex organs in males and females at any dose level. There is no effect of treatment on mating behaviour, or fertility and mating indices. • There was no evidence of a neurotoxic effect in acute and sub-chronic studies in adult rats hence it is concluded that SDS-46851 is not neurotoxic. • There was no evidence of abnormal clinical observations in weanlings suggestive of a treatment related effect on the nervous system.

In accordance with Article 8(d) of regulation (EC) 1107/2009 and Article 7(h) of regulation (EC) 844/2012, the notifier believes that sufficient information is available to fully assess the potential for SDS-46851 to impact reproduction and that the conduct of further vertebrate studies is not warranted."

Response by RMS:

The RMS agrees with the justification provided by the notifier above and remains with the original conclusion that despite the deviations, the results of the study are considered acceptable for the overall evaluation.

B.6.8.1 - 6.6.2 Developmental toxicity studies

B.6.8.1 – 6.6.2.1 Developmental toxicity study with SDS-3701 - study 1 rabbit

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. During the PPR Expert meeting (162 – session 2, September 2017) it was concluded that the maternal and developmental NOAELs are 1 mg/kg bw per day, based on 1 death and 1 abortion observed at 2.5 mg/kg bw per day. This has been added to the summary below.

Characteristics

reference	: Wazeter and Goldenthal, 1976b	exposure	: during day 6-18 of gestation
type of study	: oral embryotoxicity and teratogenicity study with rabbits	doses	: 0, 1, 2.5, 5 mg/kg/day[1]
		vehicle	: 0.5% Methocel
years of execution	: not reported	GLP statement	: not compulsory at the time of execution
test substance	: 4-hydroxy 2,5,6-trichloroiso-phthalonitrile, SDS-3701, white crystalline material, >94% pure	guideline	: not in accordance with OECD 414
		NOAEL	: maternal: ~~2.5~~ 1 mg/kg/day developmental: ~~2.5~~ 1 mg/kg/day no indications for teratogenicity
route	: oral		
species	: rabbit, Dutch Belted strain		
group size	: 10, 10, 12, 13/dose		

1 Dosages based on a dose range finding study (Wazeter and Goldenthal, 1976a). In this study maternal toxicity was established by dosage of 1,5,10, 25or 50 mg/kg/day 4-hydroxy 2,5,6-trichloroisophthalonitrile during day 6 to 18 of gestation. Hypothermia hypoactivity and soft stool were found in rabbits at the 10 mg/kg/day level. At the higher dose levels the compound was found to be extremely toxic.

Study design

The study design resembles the protocol described in the OECD guideline 414. However the four dose groups consisted of 10, 10, 12, and 13 animals respectively. The report is limited to data on maternal bodyweight. Only a summary on maternal and foetal parameters, and information on pathological effects in mothers and foetuses per litter is submitted.

The information on the purity of the test substance is restricted to a statement. No information on food intake is provided. All pathology data are limited reported, no information on individual foetuses is available.

Results

The results are summarized in Table 6.8.1 – 6.6.1.2.1

Table 6.8.1 – 6.6.2.1 Results developmental study in rabbit

Dose (mg/kg bw/day)	0	1	2.5	5	dr
Maternal effects					
Mortality			1	2	
Clinical signs and behaviour					
-hypothermia				+[1]	
-hypoactivity				+[1]	
Pregnant animals	10/10	10/10	10/10	9/10	
Abortions			1	4[2]	
Body-weight gain	no treatment related findings				
Pathology	no treatment related findings				
Litter response					
Live foetuses	no treatment related findings				
Fetal weight	no treatment related findings				
Post implantation loss				i	
Sex ratio	no treatment related findings				
Examination of foetuses					
External observations	not indicated				
skeletal findings	no treatment related findings				

Dose (mg/kg bw/day)	0	1	2.5	5	dr
Visceral findings	no treatment related findings				

dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls
dr dose-related
+/- increase/decrease without statistical analysis
1 Statement only
2 Two females aborting died during the study

Conclusions

- Clinical signs of toxicity were seen at 5 mg/kg/day. The NOAEL for maternal toxicity is established at 2.5 mg/kg/day.
- The increase in post implantation loss is considered to be a developmental effect and therefore the NOAEL for developmental toxicity is established at 2.5 mg/kg/day.
- No teratogenic effects were observed.

Co-RMS

Co-RMS remarks that one death and one abortion occurred at 2.5 mg/kg/day. Were these events discussed in the study? They seem to justify the setting of NOAEL (maternal) to 1 mg/kg/day.

Reply RMS

The death at 2.5 mg/kg bw/d is most probably incidental, although the deaths in the study were not discussed in the report. Lowering the NOAEL to 1.0 mg/kg bw/d will not affect the ADI (0.01 mg/kg bw/d) nor the ARfD (death is unlikely to be caused by a single dose) of SDS-3701.

Comment RMS after the PPR Expert meeting (162 – session 2, September 2017):

During the PPR Expert meeting it was concluded that the maternal NOAEL is 1 mg/kg bw per day, based on based on 1 death and 1 abortion observed at 2.5 mg/kg bw per day. Despite mortality occurred later in the study, in the absence of clear data on clinical signs, the mortality effect was considered an acute effect (single dose effect). For the NOAEL for developmental effects, the critical effect was abortion which was considered a developmental effect. Therefore, the NOAEL for developmental effects was set at 1 mg/kg bw per day.

Guidelines and limitations

This study has been performed prior to GLP regulations and OECD guideline 414 became effective. As a consequence there are several deviations from the guideline. The data are reported in a limited way. However, since this is the only study available with this metabolite, this study is considered suitable for evaluation.

B.6.8.1 – 6.6.2.2 Developmental toxicity study with SDS-3701 - study 2 rat

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment. Killeen (1998) is the same study as study 3 Schroeder (1998) which was evaluated in addendum14 DAR (April 2004)

Characteristics

reference	: Killeen, 1998	exposure	: during day 0-19 of gestation
type of study	: oral embryotoxicity and teratogenicity study with rats	doses	: 0, 5, 15 or 25 mg/kg/day[1]
		vehicle	: 1.0% Methocellulose
year of execution	: 1997	GLP statement	: yes
test substance	: 4-hydroxy 2,5,6-trichloroiso-phthalonitrile, SDS-3701, white crystalline material, 97.6% pure	guideline	: in accordance with OECD 414
		NOAEL	: maternal: 5 mg/kg/day developmental: 5 mg/kg/day no indications for teratogenicity
route	: oral (gavage)		
species	: rat, Albino rats VAF/Plus, CD		
group size	: 24 mated females/dose		

Study design

The study design resembles the protocol described in the OECD guideline 414. However, animals were treated during days 0-19 of gestation instead of days 6-15 of gestation.

Results

The results are summarized in Table 6.8.1 – 6.6.2.2

Table 6.8.1 – 6.6.2.2 Results developmental study in rat

Dose (mg/kg bw/day)	0	5	15	25	dr
Maternal effects					
Mortality	0	0	1	0	
Clinical signs and behaviour					
- red stains in ano-genital area				++	
- red exudate from vagina				+	
Pregnant animals	23/24	24/24	24/24	21/24	
Abortions	no treatment-related findings				
Food consumption			dc	dc	↘
Body-weight gain			dc	dc	↘
Haematology					
- haemoglobin				dc	
- haematocrit				dc	
- MCV			dc	dc	↘
- MCH			dc	dc	↘
- MCHC			ic	ic	↘
Pathology	no treatment related findings				
Litter response					
Corpora lutea				dc	
Live foetuses				dc[1]	
Fetal weight			dc	)[2]	
Early resorptions			i	ic	↗
Post implantation loss			i	ic	↗
Sex ratio	no treatment related findings				
Examination of foetuses					
External observations	no treatment related findings			)[2]	
skeletal findings	no treatment related findings			)[2]	
Visceral findings					
- 14th rudimentary rib			ic	)[2]	

dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls
dr dose-related
+/- increase/decrease without statistical analysis
1 In the 25 mg/kg bw/day group, significant *in utero* foetal death occurred. Only two foetuses (one live and the other dead) were seen in the 25 mg/kg bw/day group.
2 Since only one male foetus was seen in the 25 mg/kg bw/day group, no data were available.

Conclusions

- The NOAEL for maternal toxicity is established at 5 mg/kg bw/day, based on effects on body weight, food consumption and haematology at doses of 15 and 25 mg/kg bw/day.
- The NOAEL for developmental toxicity is established at 5 mg/kg bw/day, based on an increase in number of early resorptions at 15 and 25 mg/kg bw/day, a decrease in foetal weight, a decrease in corpora lutea and an increase of the 14th rudimentary rib at 15 mg/kg bw/day.
- No teratogenic effects were observed.

Guidelines and limitations

This study has been performed in accordance with OECD guideline 414 and is considered suitable for evaluation.

B.6.8.1 – 6.6.2.3 Developmental toxicity study with SDS-3701 - study 3 rat

Previous evaluation	In addendum 14 DAR (April 2004)
Evaluation RMS	No remarks on original assessment. Schroeder (1998) is the same study as study 2 Killeen (1998) which was evaluated in the DAR (2000)

Characteristics

reference	:	Schroeder, 1998	exposure	:	days 0-19 of gestation, gavage
type of study	:	teratogenicity study	doses	:	0, 5, 15, and 25 mg/kg bw/d
year of execution	:	1997	vehicle	:	methylcellulose (1%) in water
test substance	:	**SDS-3701**, lot no. 105H0489, purity 97.6%, solid gray powder	GLP statement	:	yes
route	:	oral	guideline	:	in accordance with OECD 414
species	:	rat, CD® (Sprague-Dawley) [Crl: CD® BR]	acceptability	:	acceptable
group size	:	24 females/dose	**NOAEL**mat	:	5 mg/kg bw/d
			NOAELdev	:	5 mg/kg bw/d
			teratogenic effects	:	yes

Study design

The study was performed in accordance with OECD 414.

Results

Table 6.8.1 – 6.6.2.3 Results developmental study in rat

Dose (mg/kg bw/day)	**0**	**5**	**15**	**25**	**dr**
Maternal effects					
Mortality (n=24)	0	0	1	0	
Clinical signs red stains anogenital area	0/24	0/24	0/23	10/24	
Pregnant animals at C-section (n=24)	23	24	23	21	
Early delivery	0/23	0/24	0/23	0/21	
Body weight/body weigth gain			dc	dc	dr

Dose (mg/kg bw/day)	**0**	**5**	**15**	**25**	**dr**
Food consumption			dc	dc	dr
Haematology					
HGB				dc	
HCT				dc	
MCV			dc	dc	
MCH			dc	dc	
MCHC			ic	ic	
Organ weight	not reported				
Pathology					
macroscopy	no treatment-related findings				
Litter response					
Live foetuses (average no. per animal)	13.3	13.3	12.4	0.0 (dc)	
Foetal weight (g)	3.4	3.4	2.7 (dc)	1.2	
Early resorption (% impl. per animal)	6.7	6.8	12.7	97.5 (ic)	
Post implantation loss (% impl. per animal)	6.7	6.8	15.1	99.5 (ic)	
Sex ratio (% males)	49.5	47	53.1	100	
Examination of the foetuses					
External observations	no treatment-related findings				
Skeletal findings rib(s) 14th rudimentary *litter incidence*	0/23	1/24	13/23 (ic)	0/1	
Visceral findings	no treatment-related findings				

dr dose related

dc/ic statistically significantly decreased/increased compared to the controls

Acceptability

The study is considered acceptable.

Conclusions

In this teratogenicity study with **SDS-3701** in rats, maternal effects were observed at 15 mg/kg bw/d and above. The effects consisted of a significant decreased bodyweight gain and food intake and in significant decreases in mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH) and increase in mean corpuscular haemoglobin concentration (MCHC). There was only one live foetus in the 25 mg/kg bw/d dose group. Effects on the foetuses were also observed at 15 mg/kg bw/d consisting of a significant decrease in foetal weight and significantly increased findings of a rudimentary 14th rib. The NOAEL for maternal and developmental effects is set at 5 mg/kg bw/d based on the above described effects. Teratogenic effects were found at 25 mg/kg bw/d consisting of significantly increased early resorptions. Findings of a rudimentary 14th rib were not considered to be teratogenic effects.

B.6.8.1 – 6.6.2.4 Developmental toxicity study with SDS-46851 - study 4 rat

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference : ZENECA, Schroeder et al., 1989
type of study : oral embryotoxicity and teratogenicity study with rats
year of execution : 1988
test substance : 3-carbamyl-2,4,5-trichlorobenzoic acid, beige powder, 99 % pure, SDS-46851
route : oral
species : rat, Sprague-Dawley
group size : 25/dose

exposure : on days 6-15 of gestation
doses : 0, 500, 1000, 2000 mg/kg/day[1]
vehicle : 1.0% aqueous methylcellulose
GLP statement : yes
guideline : in accordance to OECD 414
NOAEL : maternal: > 2000 mg/kg/day
developmental: > 2000 mg/kg/day
no indications for teratogenicity

1 Dosages based on a dose range finding study (ZENECA Chun, 1989). In this study maternal toxicity was established by dosage of 0, 250, 500, 1000, or 2000 mg/kg/day 3-carbamyl-2,4,5-trichlorobenzoic acid during day 6 to 15 of gestation. Marginal maternal toxicity was seen at the highest dose only.

Study design

Study performed according to OECD guideline 414.

Results

The results are summarized in Table 6.8.1 – 6.6.2.4.

Table 6.8.1 – 6.6.2.4

Dose (mg/kg bw/day)	0	500	1000	2000	dr
Maternal effects					
Mortality	none				
Clinical signs and behaviour	no treatment related findings				
Pregnant animals	22/25	25/25	23/25	25/25	
Abortions	none				
Females with viable foetuses at C-section	22	25	23	25	
Body-weight gain	no treatment related findings				
Food consumption	no treatment related findings				
Pathology	no treatment related findings				
Litter response					
Live foetuses	no treatment related findings				
Fetal weight	no treatment related findings				
Post implantation loss	no treatment related findings				
Sex ratio	no treatment related findings				
Examination of foetuses					
External observations	no treatment related findings				
skeletal findings	no treatment related findings				
Visceral findings	no treatment related findings				

dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls
dr dose-related
+/- increase/decrease without statistical analysis

Conclusions

- No maternal effects were seen in the doses tested. The NOAEL for maternal toxicity is > 2000 mg/kg/day.
- No developmental effects were seen at the doses tested. The NOAEL for developmental effects is determined > 2000 mg/kg/day.

Guidelines and limitations
The study meets the requirements of OECD guideline 414, and is considered suitable for evaluation.

B.6.8.1 – 6.6.2.5 Developmental toxicity study with SDS-46851 - study 5 rabbit

Previous evaluation	In DAR (2000)
Evaluation RMS	No remarks on original assessment.

Characteristics

reference	: ZENECA, Henwood et al., 1989	exposure	: on days 7-19 of gestation
type of study	: oral embryotoxicity and teratogenicity study with rabbits	doses	: 0, 250, 500, or 1000 mg/kg/day[1]
		vehicle	: 0.5% aqueous methylcellulose
year of execution	: 1988	GLP statement	: yes
test substance	: 3-carbamyl-2,4,5-trichlorobenzoic acid, light brown powder, >99 % pure, SDS-46851	guideline	: in accordance with OECD 414
		NOAEL	: maternal: < 250 mg/kg/day developmental: 500 mg/kg/day no indications for teratogenicity
route	: oral		
species	: rabbit, New Zealand White		
group size	: 20/dose		

1 Dosages based on a dose range finding study (ZENECA, Hoberman, 1989). In this study maternal toxicity was established by dosage of 0, 250, 500, 1000, or 2000 mg/kg/day 3-carbamyl-2,4,5-trichlorobenzoic acid during day 7 to 19 of gestation. Abnormal faeces was seen in the 1000 and 2000 mg/kg/day groups. Dose-related effects on body weight gain and food consumption were found at doses of 500, 1000 and 2000 mg/kg/day.

Study design
Study performed according to OECD guideline 414, the light dark regimen consisted of 16 hours light, 8 hours dark Dead foetuses were only examined externally. In case of apparent malformations these foetuses will be examined for skeletal abnormalities.

Results
The results are summarized in Table 6.8.1 – 6.6.2.5.

Table 6.8.1 – 6.6.2.5

Dose (mg/kg bw/day)	0	250	500	1000	dr
Maternal effects					
Mortality	3	1	0	3[1]	
Clinical signs and behaviour					
-faecal abnormalities[2]		+	+	++	dr
-anorexia		+	+	++	dr
-thin		+	+	++	dr
Pregnant animals	17/20	17/20	17/20	18/20	
Abortions/early deliveries	0	2	1	7[3]	
Females with viable foetuses at C-section	14	14	16	9	
Body weight gain					
-day 7-19			d	dc	dr
Food consumption					
-day 7-20			d	dc	dr
Pathology				+[4]	

Dose (mg/kg bw/day)	0	250	500	1000	dr
Litter response					
Live foetuses				-	dr
Fetal weight				-	
Post implantation loss	no treatment related findings				
Sex ratio	no treatment related findings				
Examination of foetuses					
External observations	no treatment related findings				
skeletal findings	no treatment related findings				
Visceral findings	no treatment related findings				

1 Two animals were found dead and one was sacrificed due to moribund condition.
2 Abnormalities are few or no faeces, soft faeces, and mucus-like faeces.
3 One of the rabbits with an abortion was sacrificed. This animal is included in the number of mortalities.
4 Gallbladder with hard, raised, black areas or enlarged gallbladder and foci/erosion of the stomach
dc/ic statistically significantly decreased/increased compared to the controls
d/i decreased/increased, but not statistically significantly compared to the controls
dr dose-related
+/- increase/decrease without statistical analysis
++/-- strong increase/decrease without statistical analysis

Conclusions

- Daily oral doses of 250 mg/kg/day resulted in maternal anorexia and concomitant thinness and effects on faeces. The NOAEL for maternal toxicity is established on < 250 mg/kg/day.
- Developmental effects were seen in the highest dose group only, therefore the NOAEL for developmental toxicity is determined at 500 mg/kg/day.
- No teratogenic effects were observed.

Guidelines and limitations

The study meets the requirements of OECD guideline 414 with minor deviations, and is considered suitable for evaluation.

B.6.8.1 – 6.8 Additional data with metabolites

B.6.8.1 – 6.8.1 Additional data with metabolites – study 1

Previous evaluation:	Submitted for the purpose of renewal, new data
RMS remarks	Acceptable. The RMS agrees with the conclusions drawn by the applicant.

Report:	K-CA 5.8.1/52. Parr-Dobrzanski B (2015) Chlorothalonil: Assessment of Relevance of Predicted Groundwater Metabolites, Derek Report Data Supplement to support Document N4 for AIR3, Syngenta Ltd. Jealott's Hill International Research, Bracknell, Berks RG42 6EY (Syngenta File Number : R044686_11288)

Methods

All soil metabolites (including those not exceeding 0.1 µg/L) and parent chlorothalonil were included in a Qualitative Structure Activity Relationship ((Q)SAR) analysis using the predictive toxicology program DEREK (**D**eductive **E**stimation of **R**isk from **E**xisting **K**nowledge), which is an expert system that searches a knowledgebase against a number of predictive endpoints. For the following metabolites the prediction of the toxicity was conducted with the DEREK NEXUS program:

- R44686 (SDS 2787)
- M2

- M3
- M11
- R182281 (SDS-3701) Compound 2
- R417888 (VIS01) Compund 10, M12
- R418503 (M13)
- R419492 Compound 12 (M8)
- R471811 Compound 13 (M4)
- R611553 Na
- R611965 (SDS 46851) Compound 4 (M5)
- R611966 (SDS 47523) Compound 5
- R611967 (SDS 47524) Compound 6
- R611968 (SDS 47525) (M9)
- R613636 (SDS 19221) Compound 3 (M14)
- SYN507900 (SDS 66882)
- VIS-02

Results
A summary of the results is given below:

Table 6.8.1 – 6.8: Summary of findings following DEREK analysis

Compound	Alert	Code and description	Likelihood
R44686 (SDS 2787) (Chlorothalonil)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Hepatotoxicity	557 Halobenzene	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	Skin sensitisation	415 Activated benzene	Plausible
	alpha-2-mu-Globulin nephropathy	264 Polyhalogenated benzene	Equivocal
R182281 (SDS-3701) Compound 2	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	Skin sensitisation	415 Activated benzene	Plausible
	Hepatotoxicity	557 Halobenzene	Plausible
R417888 (VIS01) Compound 10 (M12)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
R418503 (M13)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	Skin sensitisation	415 Activated benzene	Equivocal
R419492 Compound 12 (M8).	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
R471811 Compound 13 (M4)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
SYN507900 (SDS 66882)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
SYN548008 (M3)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
SYN548580 (M2)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Photoallergenicity	453 Halogenated aromatic compound	Plausible
SYN548581 (M11)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
R611553 Na[#]	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	Skin sensitisation	415 Activated benzene	Equivocal
R611965 (SDS 46851) Compound 4 (M5)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	alpha-2-mu-Globulin nephropathy	264 Polyhalogenated benzene	Equivocal
R611966 (SDS 47523) Compound 5[#]	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	alpha-2-mu-Globulin nephropathy	264 Polyhalogenated benzene	Equivocal
	Hepatotoxicity	557 Halobenziene	Plausible
R611967 (SDS 47524) Compound 6[#]	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	alpha-2-mu-Globulin nephropathy	264 Polyhalogenated benzene	Equivocal
	Hepatotoxicity	557 Halobenziene	Plausible
R611968 (SDS 47525) (M9)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Photoallergenicity	453 Halogenated aromatic compound	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
R613636 (SDS 19221) Compound 3 (M14)	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	Rapid prototypes: nephrotoxicity	RapidPrototype038 Aromatic nitrile	Equivocal
	alpha-2-mu-Globulin nephropathy	264 Polyhalogenated benzene	Equivocal
VIS – 02[#]	Carcinogenicity	116 Polyhalogenated aromatic	Plausible
	alpha-2-mu-Globulin	264 Polyhalogenated benzene	Equivocal

Compound	Alert	Code and description	Likelihood
	nephropathy		

metabolite does not exceed 0.1 µg/L in groundwater

(Q)SAR analysis revealed a number of alerts common to many of the metabolites and parent chlorothalonil, which are considered in detail below.

Structural alert for polyhalogenated aromatic – carcinogenicity (Chlorothalonil and 12 metabolites > 0.1 µg/L; R182281, R417888, R418503, R419492, R471811, SYN507900, SYN548008, SYN548580, SYN548581, R611965, R611968, R613636)

(Q)SAR analysis identified a common alert for carcinogenicity triggered by a polyhalogenated aromatic rule. This alert covers the carcinogenicity of polyhalogenated aromatic compounds. Examples include hexachlorobenzene [IARC 2001] and para-dichlorobenzene [IARC 1999], which have been classified as group 2B carcinogens by the IARC. The target organ for para-dichlorobenzene appears to be the liver in mice, whereas one study has indicated that it is not a liver tumour promoter in rats, instead carcinomas of the renal tubules and the kidney have been observed in rats [IARC 1999]. It has been proposed that the renal toxicity is caused through interaction with alpha-2-mu-globulin, in a mechanism thought not be relevant to humans

The kidney tumours observed with chlorothalonil are not consistent with the underlying mechanism reported for this alert. Furthermore, this alert was also triggered for R417888, R611965 and R182281 yet the reported toxicity data showed no evidence of kidney tumours/key precursor events likely to result in kidney tumour development, therefore this alert is not considered relevant to the identified soil metabolites.

Structures alert for rapid prototype aromatic nitrile - rapid prototypes – nephrotoxicity (Chlorothalonil and 8 metabolites > 0.1 µg/L; R182281, R417888, R418503, R419492, SYN507900, SYN548581, R611968, R613636)

(Q)SAR analysis also identified an alert for “rapid prototypes nephrotoxicity” which describes the potential nephrotoxicity of aromatic nitriles. This alert is derived using a proprietary data set of 731 chemicals, classified on the basis of the presence or absence of histopathologic lesions in the kidney in oral rat repeat dose studies mostly of 28-days duration. Eighteen chemicals in this data set activated this rapid prototype alert and thirteen of these were nephrotoxic.

Again, (Q)SAR analysis flagged this “rapid prototypes nephrotoxicity” alert for R417888 and R182281 yet, the reported toxicity data showed no evidence of kidney tumours/key precursor events likely to result in kidney tumour development therefore therefore this alert is not considered relevant to the identified soil metabolites.

Structural alert for activated benzene – skin sensitisation (chlorothalonil and 2 metabolites > 0.1 µg/L; R182281, R418503)

Electrophillic substituted benzenes may react with skin protein via a SnAr mechanism. The presence of a skin sensitisation structural alert within a molecule indicates the molecule has the potential to cause skin sensitisation. Whether or not the molecule will be a skin sensitiser will also depend on the relevant exposure concentration and its percutaneous absorption.

Chlorothalonil is classified as a skin sensitiser based on animal studies and reported findings following occupational exposure. In the absence of any significant exposure potential coming from groundwater metabolites, the sensitisation potential of groundwater metabolites is considered to be of limited relevance and has not been considered further.

Structural alert for polyhalogenated benzene – alpha-2-mu-Globulin nephropathy (chlorothalonil and 2 metabolites > 0.1 µg/L; R611965, R613636)
Alpha-2-mu-Globulin nephropathy is a male rat specific condition based on a mechanism thought not be relevant to humans and as such is not considered relevant.

Structural alert for halogenated aromatic – Photoallergenicity (1 metabolite > 0.1 µg/L; SYN548580)
Halogenated phenolic compounds of this type have been known to react covalently with proteins on irradiation with ultraviolet light. However whether or not the molecule will be photoallergenic will depend on the relevant exposure concentration, its percutaneous absorption and ability to absorb light. In the absence of any significant exposure potential coming from groundwater metabolites, the photoallergenicity potential of this single groundwater metabolite is considered to be of limited relevance and has not been considered further.

Conclusion
(Q)SAR analysis of the soil metabolites of chlorothalonil when considered alongside the available sub-chronic/chronic/carcinogenicity data of chlorothalonil did not provide any relevant toxicological alerts based on the known kidney mode of action for parent chlorothalonil.

B.6.8.2 Supplementary studies on the active substance

Refer to Volume 3, B.6a (AS)

B.6.8.3 Studies on endocrine disruption

Refer to Volume 3, B.6a (AS)

B.6.8.4 Mechanistic studies

Refer to Volume 3, B.6a (AS)

B.6.9 Medical data and information

Refer to Volume 3, B.6a (AS)

B.6.10 References relied on

Refer to Volume 3, B.6a (AS)