Bayer CropScience

Document Title

Summary of the toxicological and metabolism studies for Propineb

Amendment

Data Requirements

EU Regulation 1107/2009 & EU Regulation 283/2013
Document MCA
Section 5: Toxicological and metabolism studies

According to the guidance document, SANCO 10181/2013, for preparing dossiers for the approval of a chemical active substance

Date

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Author(s)

[redacted]

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Version history

Date	Data points containing amendments or additions[1] and brief description	Document identifier and version number
07/07/2014	Original document	M-491277-01-1
15/04/2015	Amendments done for -introduction, page 9 -12 -CA 5.6.2, page 79, -CA 5.8, pages 96, 149-153, 160-176, 189, 191, 197, 202-204, ,	M-491277-02-1

[1] It is suggested that applicants adopt a similar approach to showing revisions and version history as outlined in SANCO/10180/2013 Chapter 4 How to revise an Assessment Report

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

CA 5.1 Studies on absorption, distribution, metabolism and excretion in mammals

CA 5.1.1 Absorption, distribution, metabolism and excretion by oral route

The absorption, distribution, metabolism and excretion of propineb have already been described in the original submission for authorization by the EU according to the Council Directive 91/414/EEC.

In an "Addendum to the Monograph prepared in the context of the inclusion of Propineb in Annex I of the Council Directive 91/414/EEC and of regulation 3600/92 (2002)" the Ministero della Sanita, Rome of the RMS Italy established an Evaluation Table on Propineb "Doc. 7575/VI/97 rev. 9 (30.08.2002)" with a summary of the "Biokinetics and metabolism in rats" based on the reports of [redacted]; [redacted] (1995; M-052831-01-1) and [redacted] (1997; M-052747-01-1) under point Annex II, 5.1/02. The main results of this summary are given in the following:

Absorption
Based on the urinary excretion alone, at least 50 % of the administered oral dose was absorbed. Following administration of 1 mg [^{14}C]propineb/kg bw, mean concentrations of total radioactivity in whole blood were generally higher in female rats. The mean whole blood concentration time curve, however, followed a similar pattern in both male and female rats. At both dose levels (1 mg and 100 mg/kg bw), absorption was rapid with the mean maximum concentration of total radioactivity in whole blood at 2 - 4 hours post dose in male and female rats.

Excretion
Following administration at the low dose (1 mg/kg bw), urinary excretion accounted for a mean of approx. 50 % and 53 % of the orally administered dose during 168 hours in male and female rats, respectively. During the same period, a mean of approx. 46 % and 45 % was recovered in faeces in male and female rats, respectively. Expired $^{14}CO_2$ was a minor route of elimination in male and female rats accounting for a mean of 1.5 % and 2 % of the administered dose, respectively, over the first 48 hours post dose. Excretion was rapid and essentially complete by 48 hours post dose with the routes and rates of excretion independent of gender.

At the high dose level (100 mg/kg bw), urinary and faecal excretion during 168 hours post dose accounted for a mean of approx. 50 % and 41 % of the administered dose, respectively. Expired $^{14}CO_2$ was a minor route of elimination accounting for a mean of approx. 3 % of the administered dose over the first 48 hours post dose. The mean total amount excreted during 168 hours post dose was approx. 99 % of the oral dose.

The excretion with the bile was low (ca 3% of the dose, 0 – 24 hours) following an oral administration of 50 mg/kg bw according to a biokinetics report of [redacted]; (1975; M-102846-01-2).

Distribution
The radioactivity levels (TRR) in tissues of female and male Wistar rats at 168 hours post dose of 1 or 100 mg [^{14}C]propineb/kg bw was very low accounting for less than 1 % of the administered dose. In

the low dose groups, highest mean TRR were found in the thyroid glands, accounting for 3.315 and 2.853 µg equ/g in male and female rats, respectively. All other tissues and organs contained mean TRR levels lower than in whole blood (0.007 µg equ/g), except kidneys, adrenals, liver and lungs. The tissue distribution of total radioactivity was independent of gender.

In the high dose group (100 mg/kg bw), highest mean concentrations of TRR were found in the thyroid glands of male rats representing 113.75 µg equ/g. All other tissues and organs investigated contained mean TRR levels lower than in whole blood (0.57 µg equ/g), except kidneys, skin, adrenals, bone marrow, liver and lungs.

Although no repeated dosage was performed in these experiments, based upon the low residues in the individual tissues and organs of the body after single dosage (with the exception of the increased concentration in the thyroid) - there is no risk of a significant bioaccumulation after repeated dosage.

Metabolism

Orally absorbed [^{14}C]propineb was extensively metabolized with the metabolite profiles in native urine independent of gender at the low dose. Co-chromatography in 3 TLC systems suggested PTU (propylene thiourea, M01), PU (propylene urea, M02) and 4-methylimidazoline (MI, M03) as major metabolites. 2-Methylthio-4-methylimidazoline (MMMI, 2-methylmercapto-4-methylimidazoline, M08), N-formyl-PDA (M07) and PTU-S-trioxide (2-sulfonyl-4-methylimidazoline, SMI, M06) were suggested to be minor metabolites. Other unknown metabolites were also present. At the high dose, native urine showed additionally PDA (M04) as a major metabolite. At the low dose about 50-56% of the analyzed radioactivity was identified, at the high dose 69%.

Faeces were extensively extracted with methanol and acid resulting in an extraction efficiency of about 68-85%. PTU (M01), PU (M02) and 4-methylimidazoline (M03) were identified.

The degradation of [^{14}C]propineb proceeds mainly via PTU (M01) and also via PDA (M04). Whereas PDA (M04) seems to be an end-product in urine and faeces, PTU (M01) appeared to be further transformed through 3 pathways. The first one leads to PU (M02), a small part of which is methylated to 2-methoxy-4-methylimidazoline (M09), the second one leads to 2-methylthio-4-methylimidazoline (M08), a minor methylated metabolite of PTU (M01). The third pathway transforms PTU (M01) by stepwise oxidation of the sulfur via 2-sulfonyl-4-methylimidazoline (M06) to 4-methylimidazoline (M03) and finally to N-formyl-PDA (M07). The pathways leading to PU (M02) and N-formyl-PDA (M07) are assumed to be the major routes of PTU (M01) degradation.

The composition of metabolites in urine and faeces is shown in Table 5.1.1- 1. The proposed metabolic pathway of propineb in rat and also in goat is presented in Figure 5.1.1- 1.

Table 5.1.1- 1: [^{14}C]Propineb: balance of the radioactive metabolites excreted with urine and faeces 24 h after oral administration to male and female rats at low and high dose level

(as shown in the Evaluation Table on Propineb, Doc 7575/VI/97 rev. 9 (30.08.2002); names of metabolites adjusted to the current reporting names)

Excretion Balance[1] (% of administered dose)										
		Low dose (1 mg/kg bw)						High dose (100 mg/kg bw)		
		Male			Female			Male		
		Urine	Faeces	Total Excreted	Urine	Faeces	Total excreted	Urine	Faeces	Total excreted
Total excreted[2]		49.4	46.4	95.7	52.5	44.7	97.2	50.2	40.8	91.0
Reporting name	No.									
PTU	M01	12.4	1.9	14.3	16.3	2.6	18.9	12.3	1.3	13.6
PU	M02	3.9	1.4	5.3	4.2	1.1	5.3	7.0	1.5	8.5
AUP[3]	M13		0.7	0.7		0.6	0.6		0.9	0.9
MMMI[4]	M08	0.8		0.8	1.2		1.2	0.7		0.7
Formyl-PDA	M07	0.6		0.6	1.5		1.5	1.0		1.0
MI	M03	5.9	3.7	9.6	4.9	2.6	7.5	2.5	5.1	7.6
PDA	M04		1.2	1.2		1.5	1.5	10.1	1.6	11.7
PTU-S-trioxide[5]	M06	1.0		1.0	1.1		1.1	1.1		1.1
Total metabolites		24.6	8.9	33.5	29.2	8.4	37.6	34.7	10.4	45.1
unknowns		24.8	37.5	62.2	23.3	36.3	59.6	15.5	30.4	45.9

[1] except $^{14}CO_2$

[2] data from [redacted] and [redacted], 1995 (rounded)

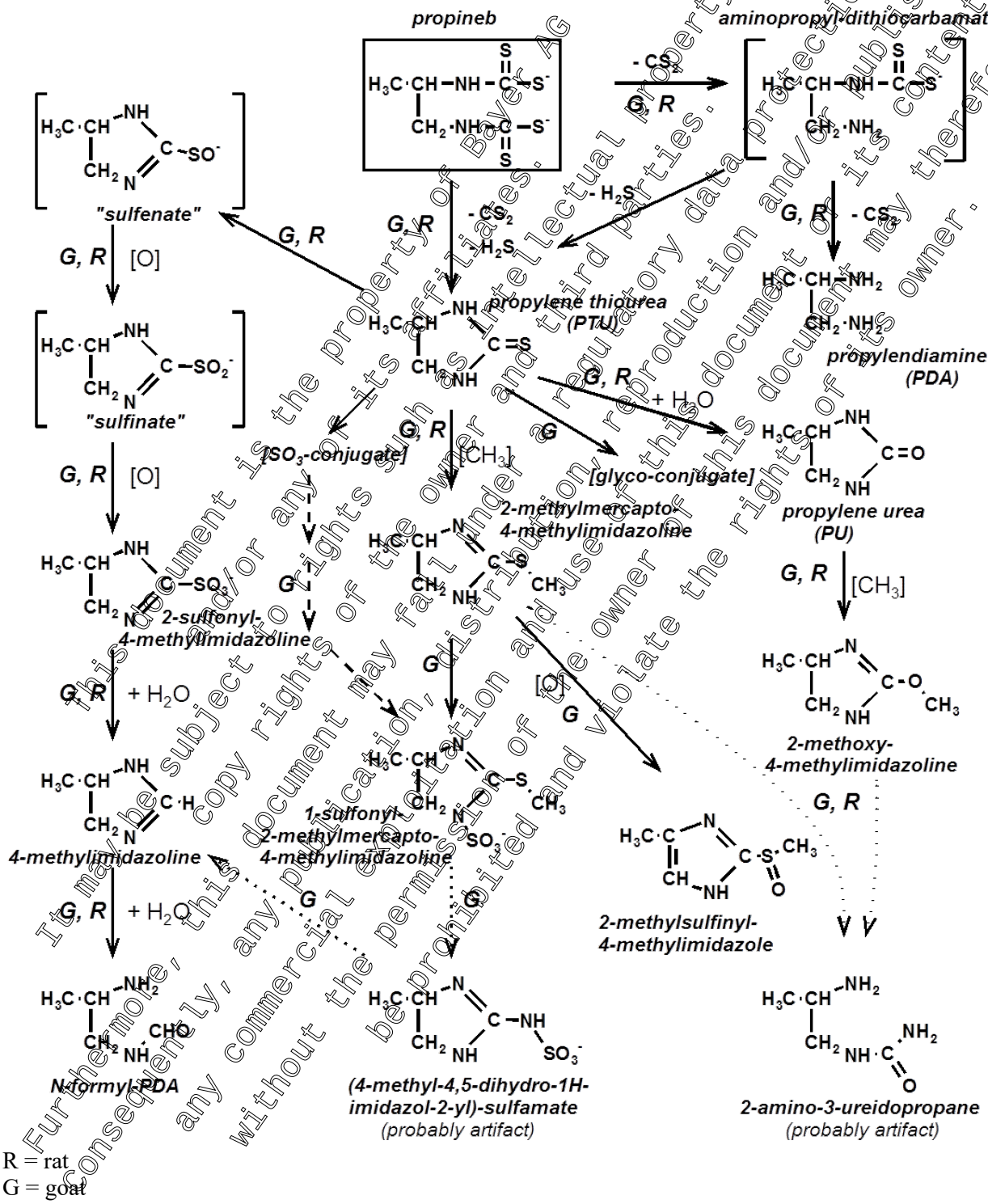
[3] 2-amino-3-ureidopropane (probably artifact)

[4] 2-methylthio-4-methyl-imidazoline, mentioned as 2-methylmercapto-4-methyl-imidazoline (MMMI) in the 2002 version

[5] mentioned as SMI, 2-sulfonyl-4-methylimidazoline, in the 2002 version

Figure 5.1.1- 1: Proposed metabolic pathway of propineb in rat and goat

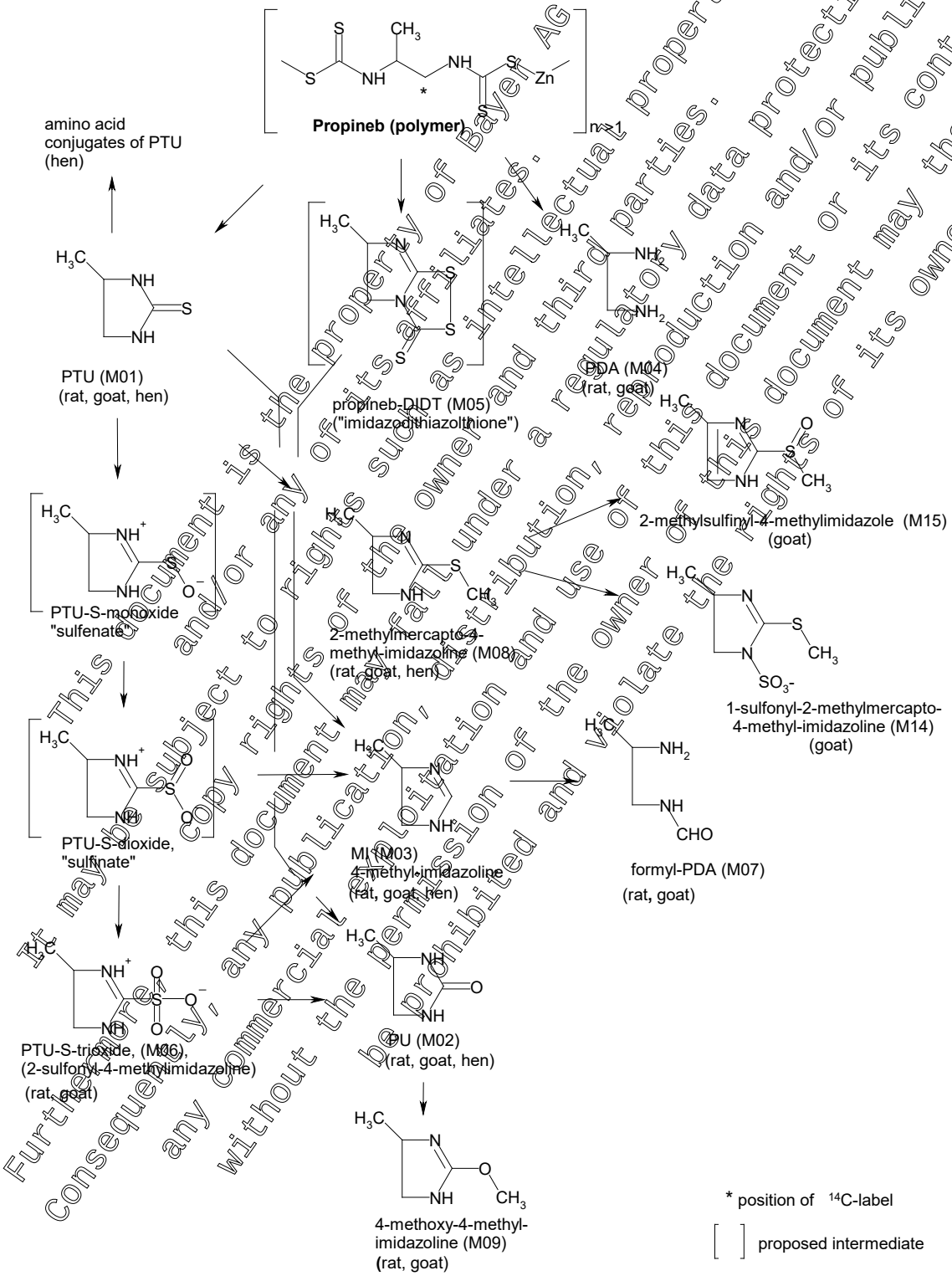
(as shown in the Evaluation Table on Propineb, Doc 7575/VI/97 rev. 9 (30.08.2002)



R = rat
G = goat

In order to include the additional metabolism of propineb in laying hens a common metabolic pathway for rat, goat and hen has been established including the metabolite numbers as defined in the list of metabolites (Document N 3). This common pathway is shown in Figure 5.1.1- 2.

Figure 5.1.1- 2: Proposed common metabolic pathway of propineb in rat, goat and hen



For comparsion with the plants a common metabolic pathway for representative uses of propineb in apple, grape and tomato is shown in Figure 5.1.1- 3. To show the commonality or non-commonality of the plant and animal metaboblites the quantitative appearance of the metabolites in plants and the rat is shown in Table 5.1.1- 2.

Figure 5.1.1- 3: Proposed metabolic pathway of propineb in plant (apple, grape, tomato)
(as shown in the Evaluation Table on Propineb, Doc 7575/VI/97-rev. 9 (30.08.2002)

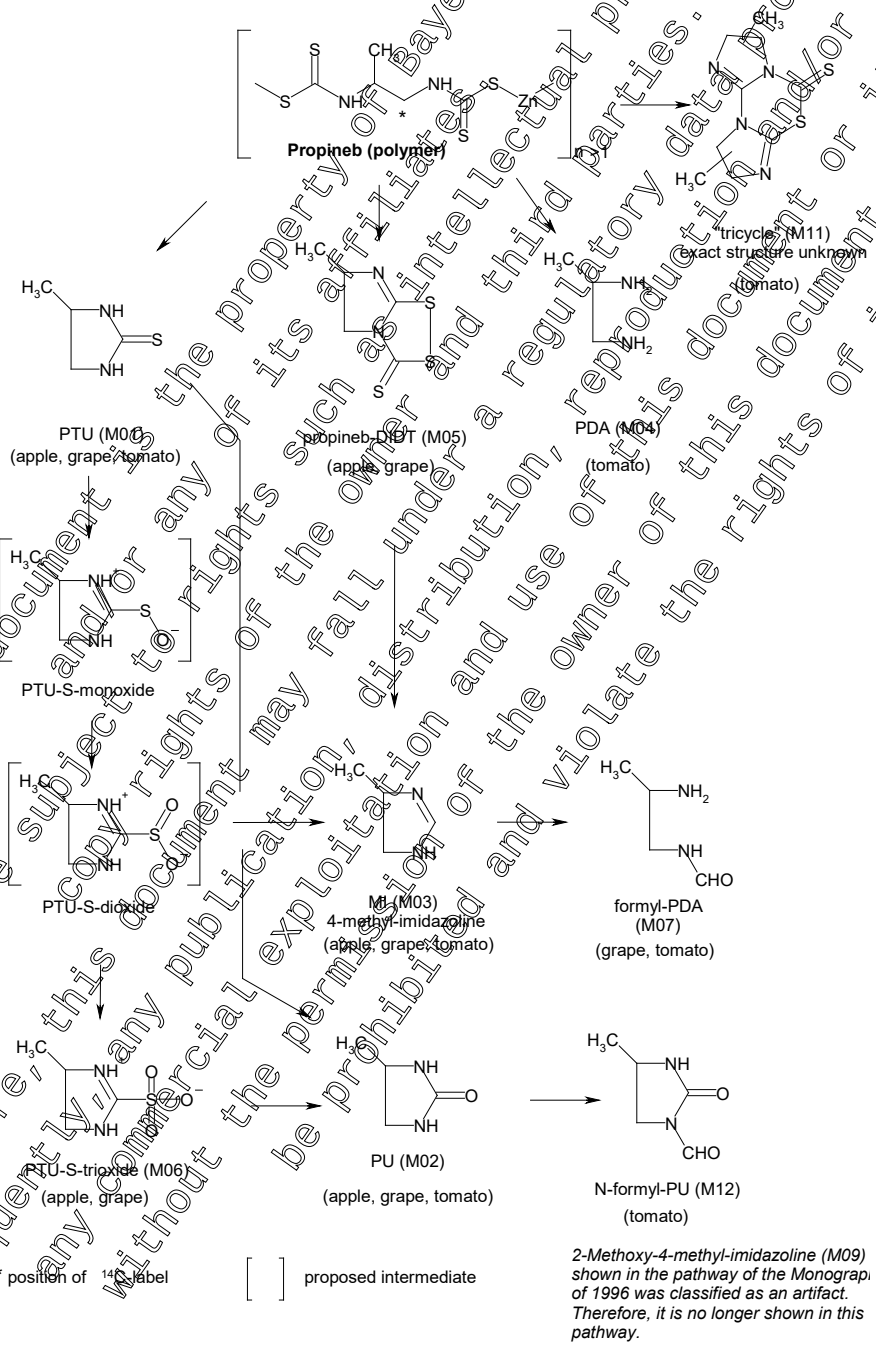
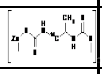
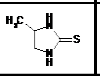
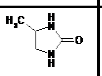
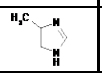
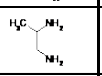
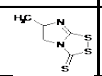
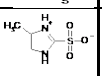
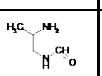
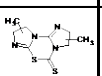
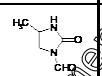
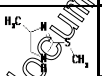
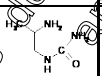


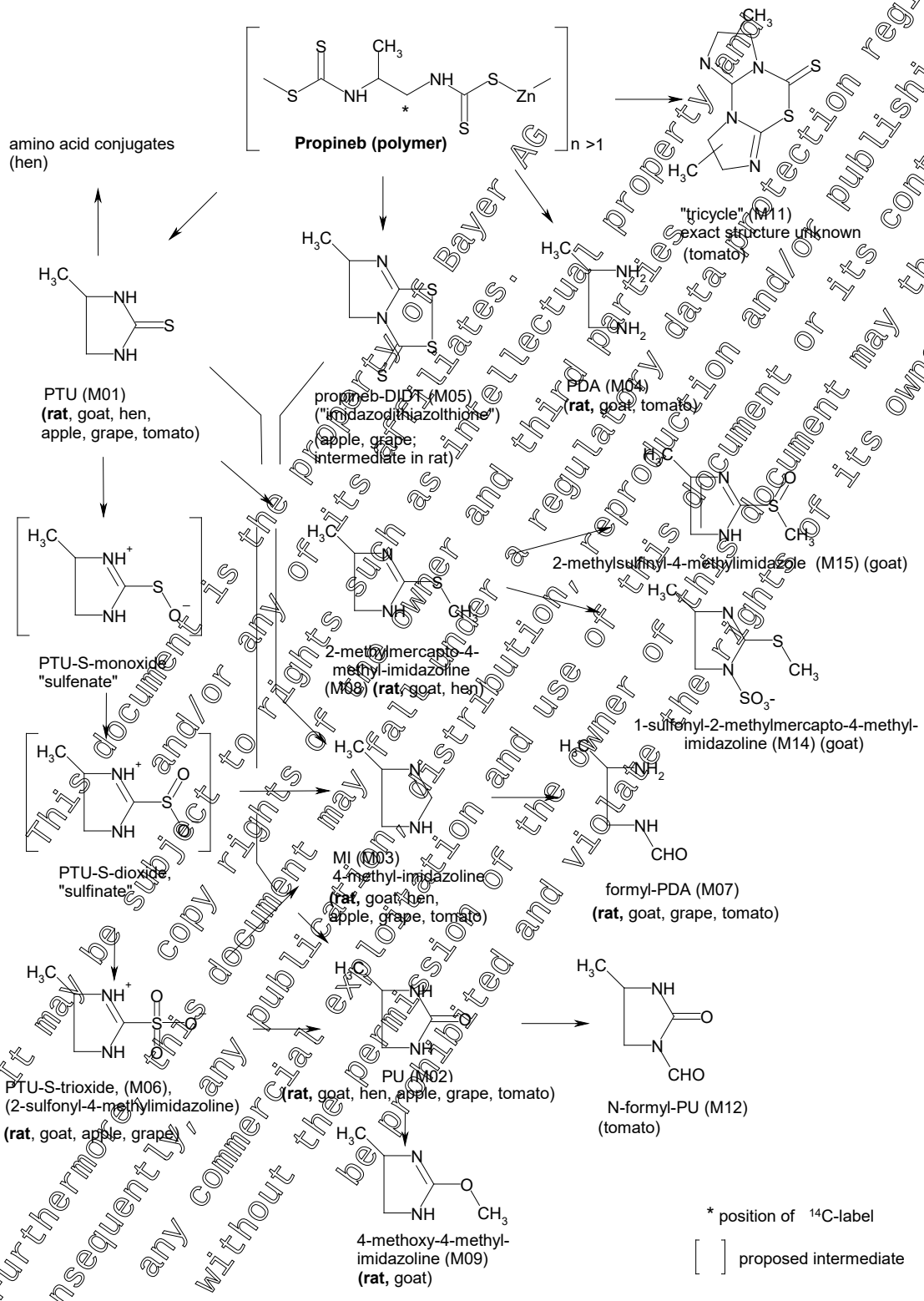
Table 5.1.1- 2: Propineb metabolites in the urine of the rat and in apple, grape and tomato

Propineb metabolites in rat and plants		Rat 1 or 100 mg/kg bw	Apple TRR ≈ 2,7 ppm equ 3 appl., PHI 14 d		Grape no TRR 3 appl., PHI 21 d		Grape TRR = 31 ppm equ 3 appl., PHI 43 d		Grape TRR=1,13 ppm equ 2 appl., PHI 100 d		Tomato TRR=1,189 ppm equ 4 appl. PHI 7 d	
Report name	**Structure**	% of dose in urine (max)**	ppm*)	% TRR	ppm*)	% TRR	ppm*)	% TRR	ppm-equ	% TRR	ppm-equ	% TRR
Propineb		-	0,40	15,0	11,6	-	12,60	40,6	0,02	1,9	0,134	11,3
M01, PTU		16,3	0,08	8,0	2,60	-	1,10	3,5	-	-	0,354	29,8
M02, PU		7,0	0,04	5,0	0,49	-	0,50	1,6	0,03	2,2	0,079	6,7
M03, MI		5,9	0,07	10,0	0,74	-	0,64	2,1	-	-	0,060	5,0
M04, PDA		10,1	-	-	-	-	-	-	-	-	0,050	4,2
M05, Propineb-DIDT		-	0,14	8,0	3,10	-	1,85	6,0	-	-	-	-
M06, PTU-S-trioxide, SMI		1,1	0,06	5,0	0,70	-	0,78	2,5	-	-	-	-
M07, Formyl-PDA, NFPDA		1,5	-	-	0,36	-	0,18	0,6	0,02	2,0	0,077	6,5
M11, Tricycle		-	-	-	-	-	-	-	-	-	0,051	4,3
M12, Formyl-PU		-	-	-	-	-	-	-	-	-	0,025	2,1
M08, MMMI		1,2	-	-	-	-	-	-	-	-	-	-
M13, AUP		only in feces	-	-	-	-	-	-	-	-	-	-
Metabolism report		Somers, Speirs, 1995 M-052831-01-1; Knoell, Ecker, 1997 M-052747-01-1	Dreze, Vogeler, 1995 M-102767-02-2		Vogeler et al. 1995 M-062013-02-2				Stork, 1998 M-102754-01-1		Miebach, Clark, 1997 M-061969-01-1	

*) These metabolite levels were measured as absolute values, not as parent equivalents
**) systemic metabolites in the rat (maximum from low & high dose, male & female rats)

A common metabolic pathway in animals (rat, goat, hen) and plants (apple, grapes, tomato) is shown in Figure 5.1.1-4.

Figure 5.1.1- 4: Common metabolic pathway of propineb in rat, goat, hen and apple, grapes and tomato



CA 5.1.2 Absorption, distribution, metabolism and excretion by other routes

No ADME study on propineb following administration by other routes (e.g. intravenous, dermal) was conducted.

CA 5.2 Acute toxicity

The acute toxicity package of propineb comprises several studies already submitted and evaluated during the AnnexI inclusion. However, as some of those studies were not run under GLP, the acute oral and dermal toxicity studies in the rat and the eye and skin irritation studies in the rabbit have been carried out following a request from non-European Authorities.
In addition, complementary information for assessing the acute inhalation effects of propineb is submitted. This information was submitted and discussed during the classification and labelling process. Based on the results of the acute inhalation studies, propineb is classified in category 4, H332: harmful if inhaled.

Due to the new data requirements a photoxicity study is required if the molar extinction coefficient is higher than 10 L x mol^{-1} x cm^{-1}. This is the case for propineb so a photoxicity study has been conducted and results showed that propineb does not possess phototoxic potential.

A suammry of the relevant Acute toxicity studies is presented in table 5.2-1.

Table 5.2-1: Summary of acute toxicity (new studies not yet submitted highlighted in bold)

Type of test	Species	Results	References
Acute oral toxicity	Rat (♂&♀)	LD_{50} > 5000 mg/kg	[redacted].; 1978 M-116192-01-1
Acute oral toxicity	**Rat (♂&♀)**	**$LD_{50\ cut\ off}$ > 5000 mg/kg**	[redacted].; **2010. M-370055-01**
Acute dermal toxicity	Rat (♂&♀)	LD_{50} > 5000 mg/kg	[redacted].; 1978 M-116192-01-1
Acute dermal toxicity	**Rat (♂&♀)**	**LD_{50}> 5000 mg/**	[redacted].; **2010. M-370058-01**
Acute inhalationl toxicity	Rat (♂&♀)	LC_{50} > 2420 mg/m^3 (♂) LC_{50} ~ 983 mg/m^3 (♀) ***Category 4, H332: harmful if inhaled.***	[redacted].; 1998 M-062776-01-1
Skin irritation	Rabbit (♂&♀)	Non-irritant	[redacted].; 1978 M-116192-01-1
Skin irritation	**Rabbit (♂)**	**Non-irritant**	[redacted].; **2010. M-370061-01**
Eye irritation	Rabbit (♂&♀)	Non-irritant	[redacted].; 1978 M-116192-01-1
Eye irritation	**Rabbit (♂)**	**Non-irritant**	[redacted].; **2010. M-370060-01**
Skin sensitization Bühler	Guinea-pig (♂)	Not a sensitizer	[redacted].; 1989. M-053646-01-1
Skin sensitization M&K	Guinea-pig (♂)	Sensitizer	[redacted], K.J.; 1987. M-053641-01-1
Phototoxicity in vitro	**BALB/c 3T3 c31 cells**	**Non-phototoxic**	[redacted]., **2014. M-490042-01-1**

CA 5.2.1 Oral

In addition to the acute oral toxicity studies already available in the Monograph and the presented Baseline Dossier a new acute oral toxicity study was conducted in 2010 in order to support a registration in Thailand.

Report:	[redacted];[redacted];2010;M-370055-01
Title:	Propineb (AE F074263) - Acute oral toxicity in rats - Acute toxic class method
Report No:	36610 TAR
Document No:	M-370055-01-1
Guidelines:	**OECD Guideline No. 423, 17th December 2001; Commission Regulation (EC) No. 440/2008, B.1tris, 30 May 2008; US EPA OPPTS Guidelines 870.1100;deviation not specified**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: Propineb (AE F074263)

Article no.:	04804643
Description:	Whitish powder
Lot/Batch no:	EDF0911415
Purity:	84.2% (w/w)
Stability of test compound:	The test item preparation was made freshly on the morning of administration by the laboratory Pharmacy

2. Vehicle: 0.5% (w/v) Methylcellulose 0.4% (w/v) Tween 80 in purified water.

3. Test animals:

Species:	Rat
Strain:	Sprague-Dawley rat, Rj: SD (IOPS Han).
Age:	8 weeks approximately
Weight at dosing:	323-363 g males and 201-226 g females
Source:	[redacted], France.
Acclimatisation period:	at least 5 days
Diet:	[redacted] R/M-H pelleted maintenance diet ([redacted], Germany)
Water:	tap water, ad libitum

Housing:	The animals were housed in polycarbonate cages with stainless steel lid (48 cm x 27 cm x 20 cm). Each cage contained one to seven animals of the same sex during the acclimation period and three rats of the same sex and group during the treatment period. Each cage contained autoclaved sawdust (SICSA, [redacted], France).
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 30 το 70% Air changes: Approximately 12 changes per hour Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

12 to 31 March 2010

2. Animal assignment and treatment

Propineb was administered by oral route (gavage) to 2 groups of six fasted Sprague-Dawley rats (three males and three females) at the dose-level of 2000 mg/kg under a dosage-volume of 10 mL/kg. The test item was prepared in 0.5% (w/v) Methylcellulose/0.4% (w/v) Tween 80 in purified water.
Mortality, clinical signs were checked dayly for a period of up to 14 days following the single administration of the test item. Body weights were recorded on days 1, 8 and 15.
On completion of the observation period, the animals were sacrificed then subjected to a macroscopic post-mortem examination.

3. Statistics

The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

Details are provided in Table 5.2.1-01. The dose of 2 000 mg/kg bw induced no mortality.
The oral LD_{50} cut-off was 5 000 mg /kg bw according to OECD guideline 423.

Table 5.2.1-01: Doses, mortality /clinical signs/ animals treated

Dose (mg/kg bw)	Toxicological results*		Occurrence of signs	Mortality (%)
2 000 (1st)	Males 0/0/3	Females 0/0/3	-	0
2 000 (2nd)	Males 0/0/3	Females 0/0/3	-	0

* : number of animals which died spontaneously and/or were sacrificed in moribund state/number of animals with signs of toxicity/total number of animals used per group

B. Clinical observations

No clinical signs were observed.

C. Body weight

When compared to the laboratory historical control data, lower body weight gain was noted during the first week in 1/3 females of the first assay and in 2/3 females of the confirmatory assay. The body weight gain of the other animals was not considered to be affected by treatment with the test item.

D. Necropsy

No abnormalities were observed at gross necropsy.

III. Conclusions

The oral LD_{50} cut off of propineb in aqueous 0.5% (w/v) methylcellulose/0.4% (w/v) tween 80 in in 0.5% aqueous carboxymethylcellulose-sodium was 5000 mg/kg bw (GHS Category 5).

CA 5.2.2 Dermal

In addition to the acute dermal toxicity study already available in the Monograph and Baseline Dossier a new acute dermal toxicity study was conducted in 2010 in order to support a registration in Thailand.

Report:	[redacted]p; [redacted]2010;M-370058-01
Title:	Propineb (AE F074263)- Acute dermal toxicity in rats
Report No:	36611 TAR
Document No:	M-370058-01-1
Guidelines:	**OECD Guideline No. 402, 24th February 1987; Commission Regulation (EC) No. 440/2008, Part B.3, 30 May 2008;deviation not specified**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: Propineb (AE F074263)

Article no.:	04804643
Description:	Whitish powder
Lot/Batch no:	EDFU941415
Purity:	84.2% (w/w)
Stability of test compound:	The test item was administered in its original form

2. Vehicle: Not applicable

3. Test animals:

Species:	Rat
Strain:	Sprague-Dawley rat, Rj: SD (IOPS Han).
Age:	8 weeks approximately
Weight at dosing:	372-387 g males and 217-242 g females

Source:	[redacted], France.
Acclimatisation period:	at least 5 days
Diet:	[redacted] R/M-H pelleted maintenance diet ([redacted], Germany)
Water:	tap water, ad libitum
Housing:	During the acclimation period, one to seven animals of the same sex were housed in polycarbonate cages with stainless steel lid (48 cm x 27 cm x 20 cm). During the treatment period, the animals were housed individually in polycarbonate cages with stainless steel lid (35.5 cm x 23.5 cm x 19.3 cm). Each cage contained autoclaved sawdust (SICSA, [redacted], France).
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 30 to 70% Air changes: Approximately 12 changes per hour Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

9 to 24 March 2010

2. Animal assignment and treatment

On the day before treatment, the dorsal area of each animal was clipped (i.e. approximately 5 cm x 7 cm for males and 5 cm x 6 cm for females) using an electric clipper. Only animals with healthy intact skin were used for the study.

A single dose of 2000 mg/kg of the test item in its original form was placed on a hydrophilic gauze pad (pre-moistened with 2 mL of purified water) and then applied to an area of the skin representing approximately 10% of the total body surface of the animals, calculated according to Meeh's formula (i.e. approximately 5 cm x 7 cm for the males and 5 cm x 6 cm for the females).

The test item and the gauze pad were held in contact with the skin for 24 hours by means of an adhesive hypoallergenic aerated semi-occlusive dressing and a restraining bandage. This dressing prevented ingestion of the test item by the animal. On removal of the dressing, any residual test item was removed using a moistened cotton pad.

The dose applied to each animal was adjusted according to the body weight determined on the day of treatment.

The animals were observed frequently during the hours following administration of the test item, for detection of possible treatment-related clinical signs. Thereafter, observation of the animals was made at least once a day until day 15. Type, time of onset and duration of clinical signs were recorded for each animal individually.

From day 2, any local cutaneous reaction was recorded.

The animals were weighed individually just before administration of the test item on day 1 and then on days 8 and 15.

On completion of the observation period, all animals were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital and sacrificed by exsanguination. All study animals were subjected to a macroscopic examination.

3. Statistics
The data did not warrant statistical analysis.

II. Results and discussion

A. Mortality

Details are provided in Table 5.2.2-01. No mortalities occurred at 2000 mg/kg bw, the only dose level tested.

The dermal LD_{50} for males was > 2000 mg/kg bw
for females was > 2000 mg/kg bw
for the combined sexes was > 2000 mg/kg bw

Table 5.2.2-01: Doses, toxicological results* / animals treated

Dose (mg/kg bw)	Male	Female	Combined
2000	0/0/5	0/0/5	0/0/10

***** : number of animals which died spontaneously and/or were sacrificed in moribund state/number of animals with signs of toxicity/total number of animals used per group

B. Clinical observations

No systemic clinical signs were noted in any animal.
A yellow coloration of the skin was noted in all animals between day 2 and day 15. This coloration masked the evaluation of cutaneous reactions in all animals on day 2, in 2/5 males and 1/5 females from day 3 until day 5 and in one male until day 9. An erythema was observed in 1/5 males on day 3 and 1/5 females from day 3 until day 5.

C. Body weight

When compared to the laboratory historical control data, a lower body weight gain was noted in 1/5 females between day 1 and day 8 (9 g *vs*. 25 ± 11 g, in control data base) and in 4/5 males between day 1 and day 8 (17 to 37 g *vs*. 47 ± 7 g, in control data base) and between day 8 and day 15 (36 to 42 g *vs*. 51 ± 8 g, in control data base). The body weight gain of the other animals was not affected by treatment with the test item.

D. Necropsy

No abnormalities were observed at gross necropsy.

III. Conclusions

The dermal LD50 of the test item, Propineb, was higher than 2000 mg/kg in male and female rats (GHS category 5, unclassified).

CA 5.2.3 Inhalation

The acute inhalation toxicity studies with propineb have been submitted in the Baseline Dossier. Results of the studies showed that propineb LC_{50} is of 983 mg/m^3 air in females and of 2420 mg/m^3 air in males, which would lead to a classification in category 3 (Toxic if inhaled) for inhalation toxicity according to the criteria of the Regulation (EC) No 1272/2008 on classification, labeling and packaging of substances and mixtures. However, additional investigations were conducted to analyze the proportion of the respirable particles generated in the experimental aerosol vs. those generated during the handling of the plant protection products containing propineb. Results indicate that the aerosol generated under the optimized condition of the acute inhalation bioassay are not representative of the conditions generated under normal handling and use of the plant protection product and classification for acute inhalation is unwarranted.
Based on these arguments, propineb is classified in category 4, (harmful if inhaled).
A summary of these analyses is provided here below as supportive information on the discussion that took place during the decision for claasification of propineb acute inhalation toxicity in EU.

Report:	[redacted]d;[redacted];[redacted];2003;M-102670-02; Amended: 2003-08-13
Title:	Particle sizes of Antracol U techn.
Report No:	A0304156/2
Document No:	M-102670-02-1
Guidelines:	**US EPA OPPTS 830.7520;Deviation not specified**
GLP/GEP:	**no**

Executive summary
In order to obtain information on the particle sizes existing in the delivered state, various batches of the product Antracol in the commercial "U technical" form were to be analyzed to determine the mass distribution of aerodynamic particle sizes of the secondary particles present.

Classical sieve analyses have shown that this method of analysis alters the existing particles in some cases. For this reason the particle size distribution was measured by laser diffraction, using a gentle method of metering and particle dispersion. This made it possible to determine the sizes of the secondary particles (clusters of primary particles) present in the powder during free fall.

Results showed that propineb samples consist of agglomerated or clustered primary particles.
If the deviation of the particle shape in the powder agglomerates from spherical geometry is ignored, the aerodynamic particle sizes can be approximately calculated from the determined equivalent sizes.

Results showed that the fraction of fines (smaller than 100 µrn) in propineb technical ranges between 2 - 3 %. The fraction of material smaller than 50 µrn ranges between 0.3 % and 0.5.

Report:	[redacted]g; [redacted];2003;M-102587-01
Title:	Propineb (LH30/Z) (Antracol U Technical) - Acute inhalation toxicity and particle-size
Report No:	MO-03-009319
Document No:	M-102587-01-1
Guidelines:	**-/-**
GLP/GEP:	**no**

Executive summary
In this position paper, the author evaluated the results of the analysis of the average particle-size of 8 different batches of propineb to understand which proportion of the propineb technical material represents the highest of respirable particles.
Results are presented in figure 5.2.3-1.

The proportion of the 'thoracic fraction', in relation to propineb technical as it is handled and used, converges against 0.00%. With respect to directive 94/79EEC the particle mass < 50 µm was also 0.5% (assuming a log-normal distribution of particle mass). Thus, this particle-size analysis indicate how low is the proportion of respirable particles when handling plant protection products conating propineb. Therefore, the results obtained with the micronized test article under the experimental conditions of the acute inhalation toxicity study (which use additional technologies to maximize the concentration of respirable particles) have limited relevance humans.

CA 5.2.4 Skin irritation

In addition to the skin irritation study already available in the Monograph and the presented Baseline Dossier a new skin irritation study was conducted in 2010 in order to support a registration in Thailand.

Report:	[redacted]g; [redacted];2010;M-370061-01
Title:	Propineb (AE F074263) - Acute dermal irritation in rabbits
Report No:	36613 TAL
Document No:	M-370061-01-1
Guidelines:	**OECD Guideline No. 404, 24th April 2002; Commission Regulation (EC) No. 440/2008, B.4, 30 May 2008**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: Propineb (AE F074263)

Article no.:	04804643
Description:	Whitish powder
Lot/Batch no:	EDFU911415
Purity:	84.2% (w/w)

Stability of test compound:	The test item was administered in its original form

2. Vehicle: Not applicable

3. Test animals:

Species:	Rabbit, males only
Strain:	Esd:NZW
Age:	2 to 4 months old
Weight at dosing:	2.8 to 3.2 kg
Source:	[redacted] S.A.S., [redacted], France
Acclimatisation period:	at least 5 days
Diet:	110 Pelleted diet ([redacted] France), *ad libitum*
Water:	tap water, *ad libitum*
Housing:	The animals were housed individually in Pajon cages (50 cm x 57 cm x 75 cm).
Environmental conditions:	Temperature: 18 ± 3°C Humidity: 30 to 70% Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

9 to 26 March 2010.

2. Animal assignment and treatment

The day before treatment, both flanks of each animal were clipped using electric clippers and just before treatment, the skin of each animal was examined in order to check the absence of any signs of skin irritation.

The test item was first evaluated on a single animal (No. 972). The durations of exposure were 3 minutes, 1 hour and 4 hours.

Since the test item was neither severely irritant nor corrosive on this first animal, it was then applied sequentially for 4 hours to two other animals (No. 970 then No. 971).

Doses of 500 mg of the test item in its original form were placed on a gauze pad moistened with purified water, which was then applied to an area of approximately 6 cm^2 of the anterior left flank (application for 3 minutes), the anterior right flank (application for 1 hour) or the posterior right flank (application for 4 hours) of the animals. The gauze pad was held in contact with the skin by means of an adhesive hypoallergenic aerated semi-occlusive dressing and a restraining bandage. The untreated skin served as control. After removal of the dressing, any residual test item was wiped off by means of a dry or moistened cotton pad.

Each animal was weighed at the beginning (before treatment) and at the end of the observation period.

At the end of the observation period, the animals were not sacrificed. They were used subsequently for the evaluation of the ocular irritation potential on the same test item.

The skin was examined approximately 1 hour, 24, 48 and 72 hours after removal of the dressing. Since there were persistent irritation reactions at 72 hours, the observation period was extended up to their complete reversibility (day 8).
The degree of erythema/eschar formation and oedema formation was recorded as specified by DRAIZE and any serious lesions or toxic effects other than dermal irritation were also recorded and fully described.

II. Results and discussion

A. Findings

After a 3-minute exposure (first animal): no cutaneous reactions were observed.

After a 1-hour exposure (first animal): a very slight or well-defined erythema (grade 1 or 2) was noted from day 1 until day 4. Mean score for erythema between 24 and 72 hours: 1. Dryness of the skin was observed from day 2 until day 7.

After a 4-hour exposure (three animals): a very slight or well-defined erythema (grade 1 or 2) was noted in all animals from day 1 until day 3 (two animals) or day 4 (one animal). A very slight edema (grade 1) was noted in another animal on day 1. Dryness of the skin was noted in 1/3 animals from day 4 until day 7. Mean scores over 24, 48 and 72 hours for each animal were 1.7, 0.7 and 1.0 for erythema and 0.0, 0.0 and 0.0 for edema.

Table 5.2.4-01: Individual skin irritation scores for erythe according to the Draize scheme on the first animal

Duration of exposure	3 minutes		1 hour	
	Erythema	Oedema	Erythema	Oedema
1 hour	0	0	2	0
24 hours	0	0	1S	0
48 hours	0	0	1S	0
72 hours	0	0	1S	0
Mean score 24-72 hours	**0**	**0**	**1**	**0**
No positive response: mean scores < 2 = - Positive response : mean scores ≥ 2 = +				

Table 5.2.4-02: Individual and mean skin irritation scores after 4 hour exposure according the Draize scheme

	Erythema and eschar			Oedema		
Animal number (body weight in kg)	**1 (2.8)**	**2 (3.0)**	**3 (3.2)**	**1 (2.8)**	**2 (3.0)**	**3 (3.2)**
1 hour	2	1	2	0	0	1
24 hours	2	1	2	0	0	0
48 hours	2	1	1	0	0	0
72 hours	1S	0	0	0	0	0
120 hours	0	-	-	0	-	-
Mean score 24-72 hours	**1.7**	**0.7**	**1.0**	**0**	**0**	**0**

No positive response: mean scores < 2 = -
Positive response : mean scores ≥ 2 = +

III. Conclusions

Under the experimental conditions of this study, the test item Propineb (AE F074263) was slightly irritant when applied topically to rabbits. According to the classification criteria laid down in Council Directive 67/548/EEC (and subsequent adaptations) on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances, the Propineb does not trigger classifictaion as irritating to the skin.

CA 5.2.5 Eye irritation

In addition to the eye irritation study already available in the Monograph and Baseline Dossier a new eye irritation study was conducted in 2010 in order to support a registration in Thailand.

Report:	[redacted]d; [redacted];2010;M-370060-01
Title:	Propineb (AE F074263) - Acute eye irritation in rabbits
Report No:	36612 TAL
Document No:	M-370060-01-1
Guidelines:	**OECD Guideline No. 405, 24th April 2002; Commission Regulation (EC) No. 440/2008, B.5, 30 May 2008;deviation not specified**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: Propineb (AE F074263)

Article no.: 04804643

Description: Whitish powder

Lot/Batch no: EDFU911415

Purity: 84.2% (w/w)

Stability of test compound:	The test item was administered in its original form
2. Vehicle:	Not applicable

3. Test animals:

Species:	Rabbit, males only
Strain:	Esd:NZW
Age:	2 to 4 months old
Weight at dosing:	3.2 to 3.4 kg
Source:	[redacted] S.A.S., [redacted], France
Acclimatisation period:	at least 5 days
Diet:	110 Pelleted diet ([redacted], France), *ad libitum*
Water:	tap water, *ad libitum*
Housing:	The animals were housed individually in Pajon cages (50 cm x 57 cm x 75 cm).
Environmental conditions:	Temperature: 18 ± 3 °C Humidity: 30 to 70% Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

26 March to April 10 2010.

2. Animal assignment and treatment

Just before treatment, the eyes of each animal were examined in order to check the absence of any signs of ocular irritation, ocular defects or pre-existing corneal injury.

The test item was first administered to a single animal (No. 972). Since the test item was not severely irritant on this first animal, it was then evaluated sequentially on two other animals (No. 970 then No. 971).

A single dose of 100 mg of the test item in its original form was introduced into the conjunctival sac of the left eye after gently pulling the lower lid away from the eyeball. The lower and upper eyelids were held together for about one second to avoid any loss of test item. The right eye, which remained untreated, served as control.

The eyes were not rinsed after administration of the test item.

The eyes were examined approximately 1 hour, 24, 48 and 72 hours after administration of the test item. Since there were persistent ocular reactions at 72 hours, the observation period was extended up to their complete reversibility (day 5).

Conjunctival reactions, iritis and corneal opacification were evaluated daily for each animal.

For the evaluation of corneal opacification (presence or absence, affected area), the eyes were examined under a UV lamp after instillation of one or two drops of 0.5% sodium fluorescein solution

(a clear fluorescence is visible in the areas of opacification). This evaluation was performed on day 2 and repeated thereafter whenever necessary.
The degree of ocular lesions was recorded as specified by DRAIZE and any serious lesions or toxic effects other than ocular lesions were also recorded and fully described. Each animal was weighed at the beginning (before treatment) and at the end of the observation period.

II. Results and discussion

A. Findings

A slight or moderate chemosis (grade 1 or 2) was noted from day 1 in all animals, persisting until day 2 in one animal and until 4 in another one.
A slight to severe redness of the conjunctiva (grades 1 to 3) was observed in all animals on days 1 and 2, persisting in one of them until day 4.
A clear discharge was noted on day 1 in all animals, persisting in two of them on day 2. An iritis (grade 1) was noted in 1/3 animals on day 1.
Mean scores calculated for each animal over 24, 48 and 72 hours were 1.0, 0.7 and 0.0 for chemosis, 2.0, 0.7 and 0.3 for redness of the conjunctiva, 0.0, 0.0 and 0.0 for iris lesions and 0.0, 0.0 and 0.0 for corneal opacity.

Table 5.2.5-01: Eye irritation scores according to the Draize scheme

	Cornea			Iris			Conjunctiva-redness			Conjunctiva-chemosis		
Animal number (body weight in kg)	**972 (3.2)**	**970 (3.2)**	**971 (3.4)**	**972 (3.2)**	**970 (3.2)**	**971 (3.4)**	**972 (3.2)**	**970 (3.2)**	**971 (3.4)**	**972 (3.2)**	**970 (3.2)**	**971 (3.4)**
Time of observation												
1 hour	0	0	0	1	0	0	3	2	3	2	2	2
24 hours	0	0	0	0	0	0	2	2	1	1	2	0
48 hours	0	0	0	0	0	0	2	0	0	1	0	0
72 hours	0	0	0	0	0	0	2	0	0	1	0	0
120 hours	0	0	0	0	0	0	0	0	0	0	0	0
Mean scores 24-72 hours	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**2.0**	**0.7**	**0.3**	**1.0**	**0.7**	**0**

III. Conclusions

Under the experimental conditions of the study, the test item Propineb (AE F074263) was slightly irritating when administered by ocular route to rabbits.
According to the classification criteria laid down in Council Directive 67/548/EEC (and subsequent adaptations) on the approximation of the laws, regulations and administrative provisions relating to the classification, packaging and labeling of dangerous substances, the test item propineb should not be classified as irritating to the eyes

CA 5.2.6 Skin sensitisation

All the available studies have been submitted and evaluated during the process of Annex I inclusion. Based on the results of a skin sensitization study according to Magnusson and Kligman, propineb is considered to be a skin sensitizer and it is classified in category 1 for skin sensitization, H317: May cause an allergic skin reaction.

CA 5.2.7 Phototoxicity

Report:	[redacted]:;[redacted];2014;M-490042-01
Title:	Propineb technical: Cytotoxicity assay in vitro with balb/c 3t3 c31cells: Neutral Red (NR) test during simultaneous irradiation with artificial sunlight
Report No:	1612100
Document No:	M-490042-01-1
Guidelines:	**Commission Regulation (EC) No. 440/2008 B 41; Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on Photosafety testing, EMEA, CPMP/SWP/398/01; OECD 432**
GLP/GEP:	**yes**

Executive Summary

The study was performed to assess the phototoxic potential of Propineb Technical. The test was performed using BALB/c 3T3 c31 cells.

The experiment was performed twice. The first experiment served as a range finding experiment (RFE), the second one was the main experiment (ME).

The following concentrations of the test item solved in DMSO (further diluted in EBSS, final concentration of DMSO in EBSS was 1% (v/v)) were tested:

RFE in presence and absence of light: 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125 µg/mL

ME in presence of light: 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 75.0, 100 µg/mL

ME in absence of light: 1.0, 2.5, 5.0, 10, 15, 20, 25, 30 µg/mL

As solvent control EBSS containing 1% (v/v) DMSO was used.

Chlorpromazine was used as positive control. The following concentrations were applied:

- without irradiation: 6.25, 12.5, 25, 37.5, 50, 75, 100, 200 µg/mL
- with irradiation: 0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0 µg/mL

One test group of cells treated with the test item was irradiated with artificial sunlight for 50 minutes with 1.65 mW/cm^2 UVA, resulting in an irradiation dose of ~ 5 J/cm^2 UVA. Another test group of test item treated cells were kept in the dark for 50 minutes.

Summary of Results

	Substance	ED_{50} (+UV) [µg/mL]	ED_{50} (-UV) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated versus non-irradiated plate
RFE	Propineb Technical	6.74	30.66	4.55	0.095	93.8
	Positive control	0.18	16.71	91.81	0.717	97.0
ME	Propineb Technical	7.21	5.01	0.70	0.005	92.1
	Positive control	0.39	11.44	29.31	0.606	119.0

The acceptance criteria were met.

A dose dependent cytotoxicity was observed after treatment of cells with Propineb Technical in the presence and absence of irradiation with artificial sunlight in both experiments. In the range finding experiment the ED_{50} value of the test item under irradiation was 6.74 µg/mL and 30.66 µg/mL in the absence of the artificial sunlight. In the main experiment selected the concentration range of Propineb Technical tested in absence of irradiation with artificial

sunlight was narrowed around the ED50 values observed in the RFE in order to calculate the ED50 values and therefore, the PIF more precisely.

The main experiment confirmed the cytotoxic effects both in presence and absence of the artificial sunlight. The ED_{50} value of the test item under irradiation was 7.21 µg/mL (similar to the RFE) but only 5.01 µg/mL in the absence of the artificial sunlight. The PIF of the test item was, therefore, 4.55 in the RFE (according to the evaluation table in chapter 3.9: probable phototoxic potential), and 0.70 in the ME (no phototoxic potential). The MPE values were calculated as 0.095 and 0.005, respectively. Both MPE values indicate a lack of phototoxicity. Since the refined concentration selection in the ME is more precise than in the RFE, it can be stated that in the study described and under the experimental conditions reported Propineb Technical **did not** have a phototoxic effects on BALB/c 3T3 cells.

I. MATERIALS AND METHODS

A. MATERIALS:

1. Test Material: Propineb Technical (AE F074263)

- **Description:** Light yellow solid
- **Lot/Batch #:** EDFU911415
- **Purity:** 81.6 % w/w (dose calculation was adjusted to purity)
- **CAS #:** 52918-63-5
- **Stability of test compound:** The test material is stable at room temperature, protected from light

2. Vehicle and/or positive control: **Vehicle:** EBSS (Earle's Balanced Salt Solution) containing 1% (v/v) DMSO
Positive control: Chlorpromazine: from 6.25 to 200 µg/mL in absence of irradiation; from 0.125 to 4.0 µg/mL in presence of irradiation

3. Test Cells: BALB/c 3T3 cells clone 31 8 supplied by Dr. Liebsch, Zebet, Berlin, Germany).

4. Culture Medium: Large stocks (Master Cell Stock) of the BALB/c 3T3 31 cell line are stored in liquid nitrogen in the cell bank of [redacted] CCR. A working cell stock is produced by multiplying from the master cell stock. Thawed stock cultures were propagated at 37 ± 1.5 °C in 75 cm² plastic flasks. Seeding was done with about 1 × 10^6 cells per flask in 15 mL of Dulbecco's Minimal Essential Medium (DMEM), supplemented with 10% newborn calf serum. The cells were sub-cultured twice weekly. The cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

5. Test compound concentrations:

RFE in presence and absence of light: 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125 µg/mL
ME in presence of light: 1.56, 3.13, 6.25, 12.5, 25.0, 50.0, 75.0, 100 µg/mL
ME in absence of light: 1.0, 2.5, 5.0, 10, 15, 20, 25, 30 µg/mL

6. Solar simulator: The irradiation was performed with a Dr. Hönle Sol 500 solar simulator. The filter H1 was used to keep the UVB irradiation as low as possible. The produced wavelength of the solar simulator with the filter was > 320 nm. Due to the inhomogeneous distribution of irradiation intensity the UVA intensity was measured at the complete area with a UV-meter. The homogeneous area was marked and the cultures were irradiated in this area. The solar simulator was switched on about 30 minutes prior to the start of experiment. The absorption spectrum of the test item was determined in the range from 270-800 nm. The test item showed absorption maxima in the range of 272.9 to 278.0 nm.

B. TEST PERFORMANCE

1. In life date

The experimental phase of the study was performed at [redacted] GmbH - [redacted] CCR ([redacted], Germany) from April 14th to May 23rd, 2014.

2. Seeding of the Cultures

2 x 10^4 cells per well were seeded in 100 µL culture medium (two plates, one was exposed to artificial sunlight, one was kept in the dark).

2. Treatment

24 hours after seeding the cultures were treated with the test item. The treatment was performed according to the OECD guideline as follows:

- the cultures were washed with EBSS;
- 8 dilutions of the solved test item were tested on two 96-well plates (100 µL/well);
- both plates were pre-incubated for 1 hour in the dark;
- after one hour one 96-well plate was irradiated through the lid at 2.4 – 2.55 mW/cm^2 (7.2 – 7.65 J/cm^2), for 50 ± 2 min at 20 - 30 °C, the other plate was stored for 50 ± 2 min at 20 - 30 °C in the dark;.
- after irradiation the test item was removed and both plates were washed twice with EBSS;
- fresh culture medium was added and the cells were incubated for 21.5 hours at 37 ± 1.5 °C and 7.5 ± 0.5% CO2.

3. Determination of Neutral Red Uptake

The medium was removed and 0.1 mL serum free medium containing 50 µg Neutral Red / mL were added to each well. The plates were returned to the incubator for another 3 hours to allow uptake of the vital dye into the lysosomes of viable cells. Thereafter, the medium was removed completely and the cells were washed with EBSS. Then 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well to extract the dye. After additional approx. 10 min at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.

4. Data Recording

The data generated were recorded in the laboratory raw data file. The results are presented in tabular form, including experimental groups with the test item, solvent, and positive control. Arithmetic means ±standard deviations were calculated for every test group.

The ED_{50} values, the Photo-Irritancy-Factor (PIF), as well as the Mean Phototoxic Effect (MPE) were calculated using the software Phototox (Version 2.0) (distributed by ZEBET, 12277 Berlin, Germany, and recommended by the OECD guideline).

The ED_{50} values (effective dose where only 50% of the cells survived) were determined by curve fitting by the software.

The PIF is defined by the following equation:

$$PIF = \frac{ED_{50}(-UV)}{ED_{50}(+UV)}$$

If a chemical is only cytotoxic +UV and is not cytotoxic when tested –UV, the PIF cannot be calculated, although this result indicates a phototoxic potential. In such cases, a > PIF value can be calculated if the (-UV) cytotoxicity test is performed up to the highest test concentration (C_{max}) and this value is used for calculation of the > PIF:

$$> PIF = \frac{C_{max}(-UV)}{ED_{50}(+UV)}$$

Since the > PIF is not an exact numerical value, no biostatistical procedure can be applied to determine the optimum cut-off. Consequently, the classification rule has to be:

If only a > PIF can be obtained, then any value > 1 predicts a phototoxic potential.

The Mean Phototoxic Effect (MPE) is based on comparison of the complete concentration response curves. It is defined as the weighted average across a representative set of photo effect values.

$$MPE = \frac{\sum_{i=1}^{n} w_i PEc_i}{\sum_{i=1}^{n} w_i}$$

The photo effect (PEc) at any concentration (C) is defined as the product of the response effect (REc) and the dose effect (DEc) i.e. PEc = REc x DEc. The response effect (REc) is the difference between the responses observed in the absence and presence of light, i.e. REc = Rc (-UV) – Rc (+UV). The dose-effect is given by:

$$DEc = \left| \frac{C/C^* - 1}{C/C^* + 1} \right|$$

where C* represents the equivalence concentration, i.e. the concentration at which the +UV response equals the -UV response at concentration C. If C* cannot be determined because the response values of the +UV curve are systematically higher or lower than Rc (-UV) the dose effect is set to 1. The weighting factors w_i are given by the highest response value, i.e. w_i = MAX {Ri (+UV), Ri (-UV)}. The concentration grid Ci is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of

MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10%. If this maximum concentration is higher than the highest concentration used in the +UV experiment the residual part of the +UV curve is set to the response value "0". Depending on whether the MPE value is larger than a properly chosen cut-off value (MPE = 0.15) or not, the chemical is classified as phototoxic.

5. Evaluation of Results

Based on the results obtained, the test item is evaluated as follows:

If **PIF < 2 or MPE < 0.1**: no phototoxic potential predicted.
If **PIF > 2 and < 5 or MPE >0.1 and <0.15** a probable phototoxic potential is predicted.
If **PIF > 5 or MPE > 0.15** a phototoxic potential predicted

6. Acceptability of the Assay

The assay meets the acceptance criteria:

- if after irradiation with a UVA dose the cell viability of the solvent control is > 80% of non-irradiated cells.
- if for the positive control Chlorpromazine the factor (PIF) between the two ED_{50} values is > 6 and if the mean OD_{540} of solvent controls is > 0.4.

II. RESULTS AND DISCUSSION

Two experiments were performed. The first experiment served as range finder (RFE), the second experiment (ME) was the confirming experiment.

Range Finding Experiment (RFE)
A dose dependent cytotoxicity was observed after treatment of cells with the test item in the presence and absence of irradiation with artificial sunlight in both experiments. The ED50 value with artificial sunlight (6.74 µg/mL) was lower than the ED50 value (without artificial sunlight) 30.66 µg/mL with a PIF value of 4.55 suggesting a probable phototoxic effect.
However, the MPE values indicated a non-phototoxic potential.

Table 5.2.7-01: Treatment of BALB/c 3T3 with Propineb in the RFE

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.$_{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.$_{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.5552*	0.0299	100.00	**Solvent Control**	0.5918*	0.0312	100.00
0.98	0.6092	0.0178	109.72	**0.98**	0.6596	0.0237	111.45
1.95	0.5974	0.0179	107.60	**1.95**	0.5974	0.0150	100.94
3.91	0.5298	0.0394	95.42	**3.91**	0.5593	0.0237	94.50
7.81	0.1922	0.0429	34.62	**7.81**	0.5344	0.0239	90.30
15.6	0.0778	0.0057	14.00	**15.6**	0.4548	0.0297	76.84
31.3	0.0735	0.0056	13.23	**31.3**	0.3466	0.0457	58.57
62.5	0.0675	0.0041	12.15	**62.5**	0.0770	0.0074	13.01
125	0.0679	0.0031	12.23	**125**	0.0608	0.0040	10.27

* mean O.D.$_{540\ nm}$ out of 12 wells
ED$_{50}$ value (with artificial sunlight) = 6.74 µg/mL
ED$_{50}$ value (without artificial sunlight) = 30.66 µg/mL
PIF = 4.55
MPE = 0.095
Mean OD$_{540\ nm}$ solvent control value (≙ viability) irradiated versus non-irradiated group: 93.8%

The concurrent positive control Chlorpromazine showed a marked decrease of survival after irradiation with artificial sunlight with an ED50 value with artificial sunlight of 0.18 µg/mL vs. an ED50 value without artificial sunlight of 16.71 µg/mL, PIF = 91.81 and MPE = 0.717.

Table 5.2.7-02: Treatment of BALB/c 3T3 with the positive control (Chlorpromazine) in the RFE

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.$_{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.$_{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**
Solvent Control	0.6011*	0.0434	100.00	**Solvent Control**	0.6195*	0.0275	100.00
0.125	0.4381	0.0225	72.88	**6.25**	0.5985	0.0065	96.60
0.250	0.1465	0.0584	24.38	**12.50**	0.4786	0.0155	77.26
0.500	0.0909	0.0165	15.11	**25.00**	0.0999	0.0071	16.13
0.750	0.0704	0.0039	11.70	**37.50**	0.0631	0.0048	10.18
1.000	0.0718	0.0086	11.95	**50.00**	0.0569	0.0034	9.18
1.500	0.0748	0.0043	12.44	**75.00**	0.0590	0.0054	9.52
2.000	0.0729	0.0048	12.12	**100.00**	0.0553	0.0038	8.93
4.000	0.0747	0.0036	12.43	**200.00**	0.0586	0.0069	9.46

* mean O.D.$_{540\ nm}$ out of 12 wells
ED$_{50}$ value (with artificial sunlight) = 0.18 µg/mL
ED$_{50}$ value (without artificial sunlight) = 16.71 µg/mL
PIF = 91.81
MPE = 0.717
Mean OD540 nm solvent control value (≙ viability) irradiated versus non-irradiated group: 97.0%

Main Experiment (ME)

In the main experiment the concentration range of Propineb Technical tested in absence of irradiation with artificial sunlight was narrowed around the ED50 values observed in the preliminary study (RFE) in order to calculate the ED50 values and the PIF more precisely.

Table 5.2.7-03: Treatment of BALB/c 3T3 with Propineb in the ME

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.$_{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.$_{540\ nm}$ Mean Value**	**Standard Deviation**	**% of Solv. Control**
Solvent Control	1.0717*	0.0938	100.00	**Solvent Control**	1.1641*	0.0661	100.00
1.56	0.9007	0.0852	84.04	**1.0**	1.0130	0.0648	87.04
3.13	0.7629	0.0457	71.18	**2.5**	0.9078	0.0278	77.98
6.25	0.6317	0.0439	58.94	**5.0**	0.3748	0.0918	32.20
12.5	0.5305	0.0451	49.50	**10**	0.1205	0.0423	10.35
25.0	0.2825	0.0316	26.36	**15**	0.0689	0.0062	5.92
50.0	0.0716	0.0048	6.68	**20**	0.0627	0.0019	5.39
75.0	0.0615	0.0019	5.74	**25**	0.0644	0.0036	5.53
100	0.0622	0.0041	5.81	**30**	0.0658	0.0031	5.65

* mean O.D.$_{540\ nm}$ out of 12 wells

ED$_{50}$ value (with artificial sunlight) = 7.21 µg/mL
ED$_{50}$ value (without artificial sunlight) = 5.01 µg/mL
PIF = 0.70
MPE = 0.005
Mean OD$_{540\ nm}$ solvent control value (≙ viability) irradiated versus non-irradiated group: 92.1%

The main experiment confirmed the cytotoxic effects both in presence and absence of the artificial sunlight.
The ED$_{50}$ value of the test item under irradiation was 7.21 µg/mL but only 5.01 µg/mL in the absence of the artificial sunlight. The PIF of the test item was 0.70 (no phototoxic potential) and the MPE was 0.005 indicating a lack of phototoxicity.

Since the refined concentration selection in the ME is more precise than in the RFE, it can be stated that in the study described and under the experimental conditions reported Propineb Technical **did not** have a phototoxic effects on BALB/c 3T3 cells.

The concurrent positive control Chlorpromazine showed a marked decrease of survival after irradiation with artificial sun light with an ED50 value with artificial sunlight of 0.39 µg/mL vs. an ED50 value without artificial sunlight of 11.44 µg/mL , PIF = 29.31 and MPE = 0.606, confirming the validity and the sensitivity of the experimental conditions of the photoxicity tests.

Table 5.2.7-04: Treatment of BALB/c 3T3 with the positive control (Chlorpromazine) in the ME

With artificial sunlight				Without artificial sunlight			
Conc. [µg/mL]	**O.D.$_{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**	**Conc. [µg/mL]**	**O.D.$_{540\ nm}$ Mean Value***	**Standard Deviation**	**% of Solv. Control**
Solvent Control	1.1218*	0.0610	100.00	**Solvent Control**	0.9428*	0.0841	100.00
0.125	0.9862	0.1025	87.91	**6.25**	0.7960	0.0893	84.53
0.250	0.8035	0.0568	71.62	**12.50**	0.4056	0.0642	43.02
0.500	0.3973	0.0463	35.41	**25.00**	0.0905	0.0149	9.60
0.750	0.0636	0.0022	5.67	**37.50**	0.0552	0.0010	5.86
1.000	0.0838	0.0507	7.47	**50.00**	0.0559	0.0020	5.93
1.500	0.0695	0.0062	6.19	**75.00**	0.0547	0.0021	5.81
2.000	0.0867	0.0186	7.73	**100.00**	0.0540	0.0017	5.72
4.000	0.1213	0.0193	10.81	**200.00**	0.0577	0.0026	6.12

* mean O.D.$_{540\ nm}$ out of 12 wells

ED$_{50}$ value (with artificial sunlight) = 0.39 µg/mL
ED$_{50}$ value (without artificial sunlight) = 11.44 µg/mL
PIF = 29.31
MPE = 0.606
Mean OD$_{540}$ nm solvent control value (≙ viability) irradiated versus non-irradiated group: 119.0%

III. CONCLUSIONS

In conclusion, it can be stated that in this study and under the experimental conditions reported, the test item Propineb does not possess any phototoxic potential.

CA 5.3 Short-term toxicity

All the existing subchronic toxicity studies have been discussed at EU level either during the European process for the inclusion in Annex I and during the discussion for updating Propineb references values after the Annex I inclusion. However a summary of the relevant studies is presented here below and in tables 5.3-1 (oral route) and 5.3-2 (inhalation and dermal route).

Two subchronic dietary studies are available in the **rat**.
During discussion for the Annex I inclusion, the first study (carried out in 1969) was considered of poor quality because not GLP and not in line. Therefore, a 90-day dietary toxicity study was performed in agreement with OECD guideline 408 (1998) and in compliance with GLP requirements. The purpose of the study was to provide robust data regarding the effects of propineb in the rat after subchronic exposure via the diet and to complete the toxicology package of relevant studies for setting the Acceptable Operator Exposure level (AOEL).

In the first study, groups of 15 male and female SPF Wistar rats were fed Propineb at dietary concentrations of 0, 5, 10, 25, 50 or 100 ppm (equivalent to approximately to 0, 0.95, 1.90, 4.82, 9.58, and 19.05 mg/kg bw/day for males and 0, 0.74, 1.45, 3.53, 7.14, and 14.3 mg/kg bw/day for the females over a period of 3 months. Main effects consisted of increase of the activity of enzymes used as biomarkers of hepatotoxicity (sorbitol and lactate dehydrogenase).
In the second study, group of Wistar rats (10 animals/sex/dose groups) received propineb at dietary levels of 0, 10, 25, 100 or 400 ppm (equivalent to 0, 0.73, 1.91, 7.60 and 31.52 mg/kg bw/day and 0, 0.89, 2.42, 10.25 and 40.61 mg/kg bw/day for males and females respectively) over 14 weeks. In addition 10 rats of each sex were treated for the same period at 0 and 400 ppm followed by a 4 week treatment-free period (recovery groups).
Dietary administration of propineb via the diet at 400 ppm provoked effects on the skeletal muscle of hind limbs in both sexes, females being affected more severely. Observed clinical signs (stepping gait, slow hind limb retraction and dragged hind limbs) were correlated with gross and histopathology findings of the skeletal muscle in the thigh (fiber atrophy, increased fatty tissue, nerve fiber swelling) and in the skeletal muscle adjacent to the spinal cord, the sternum and in the skin.

In this study the effects of Propineb on thyroid hormones were measured using well described methods and at 400 ppm (31, 52 mg/kg bw/day) there was a decrease of T4 in males and females. There were no effects on thyroid weight and morphology at any dose levels.
The study NOAEL was 100 ppm, equivalent to 7.60 and 10.25 mg/kg bw/day in males and females, respectively and this is considered to be overall NOAEL for the subchronic toxicity of propineb in the rats.

Two **dog** subchronic toxicity studies are available: one performed in 1967 and one in 1999.
In the first dog study, groups of two male and two female Beagle dogs were administered Propineb at concentrations of 0, 100, 400 or 1600 ppm in their diet (equivalent to 0, 2.67, 11.49 and 45.96 and 0, 2.77, 9.37 and 44.68 mg/kg bw/day in males and females, respectively) over four months.

The main effects were observed at the top dose and consisted of increase spleen weight. The NOAEL was set at 400 ppm (11.5 and 9.4 mg/kg bw/day in males and females respectively).
In the second study propineb was administered to groups of Beagle dogs (four animals/sex/dose group) at 0, 150, 1500 and 5000 ppm (equivalent 0, 4.6, 46.4, 150.4 and 0, 4.3, 41.4 and 149.8 mg/kg bw/day for males and females, respectively). Additionally three males per treatment level were used as recovery animals. Body weight was affected at the two top dose levels, partly due to decreased food consumption. Neurological findings like propreoceptive deficits and hind-limbs wheel-barrowing were observed at the two higher dose levels in both sexes. However, neurological clinical signs were not accompanied by histopathological changes in the skeletal muscle or nervous fibers. The slight variation of thyroid hormones levels at doses higher than 1500 ppm was accompanied by increased of relative thyroid weights but not with histopathological changes. Similarly, the observed increase of liver enzyme activities and cholesterol levels at the top dose levels was accompanied by increased liver weight, but there were no microscopic changes in the liver. The NOAEL was set at 150 ppm (4.6 and 4.3 mg/kg bw/day in males and females respectively).
The same LOAEL of 45-46 mg/kg bw/day was observed in the two studies. However, the NOAEL of the second study is lower than that observed in the first study, just because of the dose selection.

Therefore, taking the two studies together, the overall NOAEL in the dogs is considered to be 9.37 mg/kg bw/day, i.e. in the same range of that observed in the rats.

Table 5.3 – 1 Summary of dietary toxicity study

Study/Reference	NOAEL (mg/kg bw/day)	Effects at LOAEL and higher doses
Dietary 90-day Wistar Rat. 0, 5, 10, 25, 50 or 100 ppm 0 - 0.95, 1.90, 4.82 , 9.58, 19.05 mg/kg bw/day (♂) 0, 0.74, 1.45, 3.53, 7.14, 14.3 mg/kg bw/day (♀) [redacted].; 1969. M-017114-01-1 **Included in the Baseline Dossier**	4.82 (♂) - 7.14(♀)	14.3-19.05 mg/kg bw/day - Both sexes ↑sorbitol dehydrogenase activities in males ↑lactate dehydrogenase in males and females 9.58 mg/kg bw/day - Males: ↑sorbitol dehydrogenase activities in males
Dietary 90-day Wistar Rat. 0, 10, 25, 100, 400 ppm 0, 0.73, 1.91, 7.60, 31.52 mg/kg/day (♂) 0, 0.89, 2.42, 10.25, 40.61 mg/kg/day (♀) [redacted].;2003. M-108777-01-1 * **Included in the EU monograph addendum**	7.6 (♂) – 10.25 (♀)	41 mg/kg bw/day: Females: clinical signs (High-stepping gait, dragging hind limbs, no retraction of hind limbs on touch, reduced grip strength, emaciation, flaccid abdominal muscles,) histopathology (muscle atrophy, nerve fiber swelling) ↓body weight and ↓ T4. 31 mg/kg bw/day Males: Tail held erect , ↓T4
Dietary 90-day dog 0, 100, 400 or 1600 ppm 0, 2.67, 11.49, 45.96 mg/kg bw/d (♂) 0, 2.77, 9.37 and 44.68 mg/kg bw/d (♀) [redacted]1967. M-060983-01-1 **Included in the Baseline Dossier**	11.49 (♂) - 9.37 (♀)	44.7-46 mg/kg bw/day - both sexes ↑ spleen weight
Dietary 90-day dog 0, 150, 1500, 5000 ppm 0, 4.6, 46, 150.4 mg/kg/day (♂) 0, 4.3, 41.4, 149.8 mg/kg/day (♀) [redacted].; 1999. M-009667-01-1 **Included in the Baseline Dossier**	4.6 (♂), 4.3 (♀)	149 mg/kg bw/day - Both sexes ↓ bodyweight, anemia. ↓T3 and T4, ↑TSH in both sexes 46 mg/kg bw/day - Both sexes ↓T3 and T4, ↑TSH in both sexes No sex sensitivity noted.

*: study was evaluated on EU level for Annex I inclusion under 91/414 but is filed in the **Supplemental Dossier** for technical reasons.

Effects on the skeletal muscle (flaccidity and paralysis of hind legs, reduced motility and grip strength and atrophy of muscle fibers) were also observed following the inhalation route of exposure in rats. Mortality occurred in animals that, due to severe effects on motility, had no access to feeder and water. In addition to the effects on the skeletal muscle, exposure via the inhalation route provoked local p[redacted]onary irritation due to deposition of particle and Zn.

Following the dermal route no treatment related effects were observed in the rabbit after daily dermal application (7-hour/day) of up to 250 mg propineb/kg bw/day for 15 consecutive days.

Table 5.3 – 2 Summary of toxicity study following inhation or dermal exposure

Inhalation exposure		
Study	**NOAEL**	**Effects at LOAEL and higher doses**
Nose-only exposure for 6-hour/day for 5 days over 3-weeks. TNO W74 Rat. 0, 5, or 44 mg/m^3 0, 8, or 29 mg/m^3 [redacted]U.; 1979. M-062735-01-1 **Included in the Baseline Dossier**	8 mg/m^3	44 mg/m^3- Both sexes Mortality. Apathy, paralysis of hindlimbs, flaccidity ↓ Bodyweight <u>29 mg/m^3 - Males:</u> Paralysis of hindlimbs
Nose-only exposure for 6-hour/day for 5 days over 4-weeks. Wistar Rat. 0, 3.97, 11.2 or 21.95 mg/m^3 [redacted].; 2000. M-023867-01-1 **Included in the Baseline Dossier**	*None*	21.95 mg/m^3- Females Mortality. Paralysis of hindlimbs, flaccidity, reduced motility. Effects on grip strength and on foot splay ↓Bodyweight <u>11.2 mg/m^3 - Females:</u> flaccidity, reduced motility. Effects on grip strength and on foot splay At all dose levels increased number of foamy macrophages with increased intra-alveolar material and focal septal thickening
Nose-only exposure for 6-hour/day for 7 consecutive days. Female Wistar rats. 0, 1.12, 5.52 or 25.8 mg propineb/m^3 6.9 mg ZnO/m^3 [redacted]., [redacted]. 2001 M-039913-01-1 **Included in the Baseline Dossier**	1.12 mg/m^3	25.8 mg propineb /m^3 Paralysis of hindlimbs, flaccidity, reduced motility, ↓Bodyweight ↑Lung weight and accumulation of Zn Local p[redacted]onary effects causally related to Zn dissociation <u>5.52 mg propineb/m^3</u> ↑metallothionein (MT)-positive macrophages- induction Local p[redacted]onary effects causally related to Zn dissociation
Dermal exposure		
Study	**NOAEL**	**Effects at LOAEL and higher doses**
Dermal application to intact and scarified skin 7-hours/day for 15 days. White New Zealand rabbits. 0, 50 & 250 mg/kg bw/day [redacted].; 1979. M-116181-01-1 **Included in the Baseline Dossier**	250 mg/kg bw/day	No adverse effects up to the highest dose tested.

Based on the effects observed after repeated exposure in the neuromuscular system and in the thyroid Propineb has been classified for specific target organ toxicity after repeated exposure – STOT-RE category 2, H373: May cause damage to organs (thyroid peripheral nervous system) through prolonged or repeated exposure if inhaled or swallowed.

CA 5.3.1 Oral 28-day study

All the range finding studies were already presented in the Baseline Dossier. Overall they do not provide any valuable information compared to the subchronic toxicity studies. Moreover some of these studies (e.g. the 8 week dietary study in young cockerels and the 10 weeks dietary study in the hen) were considered to be not acceptable by the RMS.

CA 5.3.2 Oral 90-day study

All the dietary 90-day studies in rats and dogs were already submitted in Baseline Dossier and in the Monograph addendum.

CA 5.3.3 Other routes

Subacute inhalation and dermal toxicity studies have been already included in the Baseline Dossier.

CA 5.4 Genotoxicity testing

A complete battery of mutagenicity studies have been conducted with Propineb, submitted in the Baseline Dossier and assessed during the Annex I inclusion.

A new Ames test was performed thereafter to support the current specification and it is summarized in this section. Results showed no concern for mutagenicity.
In addition, it was decided to submit a chromosome aberration test run in Japan in 1988, which was not previously submitted. Results of this *in-vitro* study showed effects on chromosome aberration at cytotoxic doses. However, concern for propineb clastogenic and/or cytogenetic potential was dismissed by two bone marrow micronucleus tests in mice following oral and intra-peritoneal injection, already summarized in the Baseline Dossier.

In conclusion, propineb is not genotoxic.

Table 5.4 –1 Summary of Genotoxicity studies

Test system	Concentration	Results	Reference
In vitro studies			
Ames test - Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102	3 - 5000 µg/plate	Negative	[redacted].;2012 M-437298-01 **New study**
Ames test - S. typhimurium strains TA 98, TA 100, TA 1535, TA 1537	20 - 12,500 µg/plate	Negative	[redacted].; 1980 M-050169-01-1
Reverse mutation test Escherichia coli WP2 hcr S. typhimurium strains TA 98, TA 100, TA 1535, TA 1537, TA 1538	0.09 - 864 µg/plate	Negative	Anonymous (Hatano Institute), 1978 M-104050-01-1
Chromosomal aberration test in Chines Hamster Lung (CHL) cells	10 - 70 µg/mL (-S9) 20 - 80 µg/mL (+S9)	Positive	[redacted].;1989 M-001021-01 **Not included in the Baseline Dossier**
CHO-HGPRT assay Chinese hamster ovary cells	0.16 - 40 µg/mL (-S9) 0.11 - 60 µg/mL (+S9)	Negative	[redacted].; 1988 M-104115-01-1
Rec assay Bacillus subtilis H17, Rec$^+$, M45 Rec$^-$	0.9 - 864 µg/plate	Negative	Anonymous, 1978, report MO-03-012179
Unscheduled DNA synthesis (UDS) test Rat, primary hepatocytes	5 - 30 µg/mL	Negative	[redacted].;1987, M-050149-01-1
In vivo studies			
Micronucleus test Male and female NMRI-mice;	2 x 1000 or 2 x 2000 mg/kg bw, po	Negative	[redacted]1982 & 1994 M-050154-02-1
Dominant-lethal test Male NMRI-mice	500 mg/kg bw, po	Negative	[redacted].; 1974, M-105181-01-1

CA 5.4.1 In vitro studies

Report:	[redacted]n; [redacted];2012;M-437298-01
Title:	Salmonella typhimurium reverse mutation assay with propineb technical
Report No:	1481500
Document No:	M-437298-01-1
Guidelines:	**OECD 471; Commission Regulation (EC) No. 440/2008, B13/14; US-EPA 712-C-98-247, OPPTS 870.5100, deviations: none.**
GLP/GEP:	**yes**

Executive Summary

In this in vitro study, propineb technical was tested to assess its potential to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate.

In the pre-experiment/experiment I the test item was tested at the following concentrations:3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate and in experiment II at the following concentrations: 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 experiment I and

with metabolic activation in experiment II. In experiment II without S9 mix reduced background growth was observed in strains TA 1535, TA 1537, TA 98, and TA 100 at 5000 µg/plate.
No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation in experiment I and with metabolic activation in experiment II. In experiment II without S9 mixtoxic effects, evident as a reduction in the number of revertants (below the indication factorof 0.5) were observed in all strains at 5000 µg/plate.
No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with propineb pechnical 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.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.
In conclusion, propineb technical did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

I. Materials and Methods

A. Material

1. Test Material:

Description:	White powder
Lot/Batch:	EDFU911415
Purity:	82.3% Concentrations were adjusted to purity
CAS:	9016-72-2
Stability of test compound:	Stable at room temperature for the study duration

2. Control materials:

Negative:	Culture medium
Solvent:	deionised water
Positive:	Sodium azide (SERVA) for TA 1535, TA 100 at 10 □g/plate in deionized water without S9 mix
	4-nitro-o-phenylene-diamine, 4-NOPD ([redacted]) for TA 1537 at 50 µg/plate in DMSO, TA 98 at 10 µg/plate in DMSO without S9 mix
	methyl methane sulfonate, MMS ([redacted]) for TA102 at 3 µg/plate in deionised water without S9 mix
	2-aminoanthracene, 2-AA (SERVA) for TA 1535, TA 1537, TA 98, TA 100, at 2.,5 µg/plate in deionized water and for TA 102 at 10 µg/plate in deionized water with S9 mix

3. Test organisms:

Species:	Salmonella typhimurium LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, and TA 98
Source:	Strains obtained from [redacted] GmbH ([redacted], Germany)

4. Test compound concentrations:

Range-finding	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate
Pre-incubation assay:	For all strains with or without S9 mix: 33; 100; 333; 1000; 2500; and 5000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed between May 21 to June 21, 2012 at [redacted] GmbH - [redacted] CCR ([redacted], Germany).

1. Experimental performance

To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates.
For each strain and dose level, including the controls, three plates were used.
The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL Test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL Bacteria suspension (cf. test system, pre-culture of the strains),
- 2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution (solvent control), or reference mutagen solution (positive control), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacteria suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After preincubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.
After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark

2. Acceptability of the Assay

The Salmonella typhimurium reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of the laboratory historical data
- the positive control substances should produce a significant increase in mutant colony frequencies
- a minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed (3).
A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (2). An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

Toxicity of the test item can be evident as a reduction in the number of spontaneous revertants or a clearing of the bacterial background lawn.
The pre-experiment is reported as main experiment I, since there were evaluable plates (>0 colonies) at five concentrations or more in all strains used.
No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation in experiment I and with metabolic activation in experiment II. In experiment II without S9 mixtoxic effects, evident as a reduction in the number of revertants (below the indication factorof 0.5) were observed in all strains at 5000 µg/plate.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with Propineb Technical 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.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

Table 5.4.1-1 Mean mutants values - Pre-experiment and Experiment I

Test item	Concentration µg/plate	Revertant colony plate (Mean ± SD)				
		TA1535	*TA1537*	*TA98*	*TA100*	*TA 102*
Without metabolic activation (S9)						
Deionized water		15 ± 6	13 ± 3	24 ± 4	171 ± 27	425 ± 9
Untreated		12 ± 6	15 ± 3	30 ± 3	157 ± 11	409 ± 24
Propineb Technical	3	16 ± 7	11 ± 1	21 ± 5	174 ± 4	422 ± 20
	10	14 ± 2	13 ± 1	25 ± 4	185 ± 5	404 ± 26
	33	13 ± 4	18 ± 4	28 ± 11	166 ± 16	433 ± 16
	100	17 ± 2	15 ± 2	27 ± 7	175 ± 12	490 ± 11
	333	17 ± 3	14 ± 2	26 ± 2	158 ± 10	464 ± 21
	1000	13 ± 2	15 ± 1	22 ± 2	153 ± 5	404 ± 24
	2500	15 ± 5[P]	15± 2[P]	29 ± 4[P]	144 ± 22[P]	340 ± 49[P]
	5000	15 ± 1[P]	10 ± 3[P]	27 ± 3[P]	112 ± 5[P]	273 ± 15[P]
NaN_3	10	1804 ± 46			1808 ± 108	
4-NOPD	10			253 ± 14		
	50		170 ± 6			
MMS	2.0					3177 ± 46
With metabolic activation (S9)						
Deionized water		21 ± 7	20 ± 2	42 ± 4	217 ± 23	624 ± 34
Untreated		22 ± 4	19 ± 4	51 ± 7	205 ± 2	576 ± 49
Propineb Technical	3	21 ± 6	21 ± 2	41 ± 7	173 ± 16	532 ± 16
	10	21 ± 7	24 ± 6	43 ± 11	200 ± 23	550 ± 21
	33	22 ± 9	23 ± 6	45 ± 10	206 ± 14	561 ± 17
	100	23 ± 9	21 ± 5	42 ± 2	207 ± 21	592 ± 23
	333	22 ± 1	20 ± 1	43 ± 2	183 ± 30	551 ± 12
	1000	20 ± 1	19 ± 6	32 ± 8	184 ± 18	538 ± 13
	2500	18 ± 4[P]	22 ± 7[P]	35 ± 5[P]	194 ± 9[P]	520 ± 16[P]
	5000	16 ± 3[PM]	19 ± 5[PM]	29 ± 3[PM]	189 ± 14[PM]	539 ± 9[PM]
2-AA	2.5	463 ± 20	291 ± 29	1983 ± 200	2740 ± 206	
	10.0					2041 ± 115

P = Precipitate M = Manual count

Table 5.4.1.2: Mean mutant values Experiment II

Test item	Concentration µg/plate	Revertant colony plate (Mean ± SD)				
		TA1535	*TA1537*	*TA98*	*TA100*	*TA 102*
Without metabolic activation (S9)						
Deionized water		13± 3	17 ± 4	31 ± 4	155 ± 12	430 ± 24
Untreated		9 ± 1	21 ± 2	27 ± 2	143 ± 8	371 ± 8
Propineb Technical	3	13 ± 3	16 ± 4	29 ± 2	152 ± 13	419 ± 12
	100	14 ± 5	20 ± 5	27 ± 6	154 ± 9	455 ± 19
	333	16 ± 4	17 ± 4	34 ± 10	131 ± 6	456 ± 38
	1000	15 ± 4	15 ± 1	27 ± 4	167 ± 22	384 ± 21
	2500	11 ± 3[P]	14 ± 6[P]	20 ± 4[P]	98 ± 8[P]	320 ± 10[P]
	5000	0 ± 0[PMR]	1 ± 1[PMR]	0 ± 0[PMR]	0 ± 0[PMR]	179 ± 16[P]
NaN_3	10	2016 ± 46			2237 ± 82	
4-NOPD	10			381 ± 17		
	50		93 ± 4			
MMS	2.0					3483 ± 224
With metabolic activation (S9)						
Deionized water		22 ± 6	27 ± 4	41 ± 6	186 ± 6	463 ± 10
Untreated		19 ± 2	28 ± 3	42 ± 7	170 ± 15	525 ± 34
	33	26 ± 2	29 ± 7	47 ± 14	193 ± 16	525 ± 43
	100	23 ± 5	29 ± 2	39 ± 7	172 ± 14	555 ± 37
	333	19 ± 4	28 ± 8	42 ± 4	151 ± 7	517 ± 35
	1000	24 ± 2	30 ± 4	41 ± 3	154 ± 13	541 ± 55
	2500	17 ± 4[P]	28 ± 8[P]	30 ± 9[P]	150 ± 9[P]	460 ± 8[P]
	5000	10 ± 3[PM]	17 ± 3[PM]	20 ± 3[PM]	109 ± 3[PM]	484 ± 21[PM]
2-AA	2.5	273 ± 11	184 ± 9	1338 ± 200	1513 ± 63	
	10.0					1781 ± 386

P = Precipitate M = Manual count R = Reduced background growth

III. Conclusions

The mutagenic activity of test substance Propineb was evaluated to be negative for the reverse mutation test in bacterial system.

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Report:	[redacted]g; [redacted]; 1989-M-001021-01
Title:	Chromosomal aberration test of propineb using cultured mammalian CHL cells
Report No:	DT-54
Document No:	M-001021-01-1
Guidelines:	**US EPA OPPTS 870.5375**
GLP/GEP:	**yes**

Executive summary

A chromosme aberratio tets was carried out with Propineb in Chinese hamster lung fibroblasts (CHL cells) Citomycin C (MMC) and cyclophosphamide (CPA) as positive controls for the tests without and with metabolic activation, respectively.

Tests on inhibition of cell growth and cell division were carried out to determine the dose levels of the test article. The foolowing dose were tested for the chromosomal aberration test: 17.5, 35 and 70 µg/mL for 24-hour treatment and 10, 20 and 40 for 48-hour treatment without metabolic activation

and 20, 40 and 80 µg/mL fr the 24-hour treatment with metabolic activation. MMC doses were 0.05 µg/mL for both the 24-hour and 48-hour treatments. CPA concentration was 10 µg/mL.

There were no effects on chromomal aberration in the 48-hour treatment without metabolic activation. However, results of the 24-hour treatment without metabolic activation and with
In the presence of S9 mix, propineb caused a statistically significant increase in the proportion of metaphase figures containing chromosomal aberrations at the highly toxic concentration of 10 mM in the first test, when compared with the solvent control value ($P<0.001$). In the second test, at the highest level scored for aberrations, 7.5 mM, the toxicity was acceptable with a reduction in mitotic index of 55% and showed a positive response.
A quantitative analysis for polyploidy was made in cultures treated with the negative control and highest dose level. An increase in the proportion of polyploid cells was seen in both tests in both the absence and presence of S9 mix and was statistically significant in the first test in the absence of S9 mix at a highly toxic concentration.
All positive control compounds caused large, statistically significant increases in the proportion of aberrant cells, demonstrating the sensitivity of the test system and the efficacy of the S9 mix.

It was concluded that the test substance technical AE 0172747 showed evidence of clastogenic activity in this *in vitro* cytogenetic test system, at toxic concentrations only.

Material and methods

Test Material:
- Description: Propineb
- Lot/Batch: White powder
- Purity: 231804298
- CAS: 82 %
- Stability of test compound: 9016-72-2
- Stable at room temperature

Solvent used: dimethylsulfoxide (DMSO)

Control materials:
- Negative: Tissue Culture medium
- Solvent: DMSO 1%
- Positive: non- activation (-S9 mix): Mitomycin C in sterile water, concentration 0.05 µg/mL
- Positive: with activation (+S9 mix): Cyclophosphamide sterile water, concentration 10 µg/mL

Activation : S9 was preared from the liver of 7-week old male Sprague-Dawley rats given a mixture of phenobarbital and 5,6-benzoflavone as enzyme inducers.

S9 mix contained: S9 fraction (10% v/v), $MgCl_2$ (5 mM), KCl (30 mM), HEPES pH 7.2 (3 mM), glucose-6-phosphate (5 mM), NADP (4 mM). All the cofactors were filter-sterilised before use.

Test cells :
Chinese hamster lung fibroblasts (CHL) cells supplied by the National Institute of Hygienic Science (Japan).

Culture medium:
Eagle's minimum essential medium supplemented with inactivate 10% newborn calf serum (10%NCS/MEM) was used.

Test compounds concentration used (µg/mL):

	24-hours	48-hours
Non activated conditions	17.5, 35 and 70	10, 20 and 40
Activated conditions	20, 40 and 80	

Study Design and Methods:
Study performance
The study was conducted at [redacted], Japan. The experiments started on February 6th 1989 and ended on March 16th 1989.

Preliminary cytotoxicity Assay (First test)
In the test without metabolic activation for 24-hour and 48-hour treatment the following concentrations were tested: 0, 40, 50, 60, 70, 80, 90 and 100 µg/mL.
In the test without metabolic activation and 48-hour treatment the following concentrations were tested: 0, 20, 25, 30, 35, 40, 50 and 60 µg/mL.
In the test with metabolic activation for 6 hours with the test material followed by 18-hour incubation period the following concentrations were tested: 0, 40, 50, 60, 70, 80, 90 and 100 µg/mL.
Cytotoxicity was determined by assessing the concentration that inhibited 50% of growth (IC50)

Cytogenetic assay
Cell treatment:
Cells were exposed to the test compound, solvent or positive control for 24 or 48 hours under both non-activated conditions.
Under activated metabolic conditions the cells were exposed to the test compound, solvent or positive control for 6- hours and then incubated with fresh NCS/MEM for additional 18 hours.

Spindle inhibition:
Two hours before the cells were harvested; mitotic activity was arrested by addition of Colcemid® to each culture at a final concentration of 0.1 µg/mL.

Cell harvest:
Cultured cells were detached from the incubation dish by adding 025% trypsin and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded. Then , 0.075 M KCl was added and the cells kept at 37°C for 15 minutes, followed by fixation with methanol:acetic acid solution. Cell were suspended in methanol:acetic acid solution to obtained a suspension.

Slide preparation:
A few drops of the harvested cell suspensions were dropped onto pre-cleaned microscope slides which were then allowed to air-dry. The slides were then stained in 2% Giemsa.

Metaphase analysis:
The selected slides were coded. One hundred metaphase figures were examined, from each culture and were scored for structural aberrations and for numerical aberrations (polyploidy).

Evaluation criteria: .
The test substance was considered to cause a positive response if the following conditions were met:
1 Statistically significant increases (P<0.01) in the frequency of metaphases with aberrant chromosomes (excluding gaps) were observed at one or more test concentration.
2 The increases were reproducible between replicate cultures.

Statistics
The number of aberrant and polyploid metaphase cells in each treatment group was compared with the solvent control value using Fisher's exact test (Fisher, 1973).

Results and discussion
Cytotoxicity assay
In the absence of S9 mix, Propineb caused a reduction of growth below 50% from 60 µg/mL and in the 48-hour treatment already at 20 µg/mL

In the presence of S9 mix, Propineb caused a reduction of growth below 50% from concentrations of 70 µg/mL.

Table 5.4.1-3 Cytotoxicity

Without S9 Mix	24-hour	Propineb (µg/mL)	0	40	50	60	70	80	90	100
		Growth rate (%)	100	73.5	54.3	42.8	37.4	23.2	14.7	14.3
	48-hour	Propineb (µg/mL)	0	20	25	30	35	40	50	60
		Growth rate (%)	100	35.3	23.6	32.6	31.2	24.7	20.8	15.0
With S9 Mix		Propineb (µg/mL)	0	40	50	60	70	80	90	100
		Growth rate (%)	100	82.6	75.8	53.0	37.0	21.0	16.0	10.7

Mitotic metaphase of chromosome enough to make an assessment of chromosomal aberration were observed at doses lower than 70 µg/mL for the 24-hour treatment and lower than 40 µg/mL for the 48-hour treatment without metabolic activation and lower than 80 µg/mL with metabolic activation.
Therefore the highest concentration was decided to be 70 µg/mL for the 24-hour and 40 µg/mL for the 48-hour treatments without metabolic activation and 80 µg/mL for the metabolic activation methods. In addition to this two lower doses with a dilution ratio of 2 were added in each treatment.

Cytogenetic assays

In the absence of S9 mix, propineb caused a statistically significant increase in the proportion of cells with chromosomal aberrations, when compared with the solvent control (P<0.01) at the top dose tested of 70 µg/mL following 24-hour treatment.
Increased incidence in the proportion of cells with chromosomal aberration was confirmed in a second test again confined at the top dose level of 70 µg/mL.
There was no effect on polyploidy at any dose levels.

There was no effect on structural chromosomal aberration and/or polyploidy following treatment for 48 hours.

In presence of S9 mix, propineb caused a statistically significant increase in the proportion of cells with chromosomal aberrations, when compared with the solvent control (P<0.01) at the top dose tested of 80 µg/mL.

Table 5.4.1-4: Chromosme aberration test without metabolic activation (-S9)

Exposure period (hours)	Concentrations (µg/mL)		Cells with aberrations Excluding gaps Total number (%)	Cells with aberrations Including gaps Total number (%)
24	Control	Culture medium	0 (0.0)	0 (0.0)
		DMSO	0 (0.0)	2 (1.0)
	Propineb	17.5	1 (0.5)	1 (0.5)
		35	0 (0.0)	0 (0.0)
		70	22 (11.5)***	26 (13.0)***
	Control	0.05 (MMC)	81 (40.5)***	81 (40.0)***
Exposure period (hours)	**Concentrations (µg/mL)**		**Cells with aberrations Excluding gaps** Total number (%)	**Cells with aberrations Including gaps** Total number (%)
48	Control	Culture medium	0 (0.0)	0 (0.0)
		DMSO	0 (0.0)	0 (0.0)
	Propineb	10	1 (0.5)	1 (0.5)
		20	2 (1.0)	2 (1.0)
		40	0 (0.0)	0 (0.0)
	Control	0.05 (MMC)	65 (32.5)***	69 (43.5)***

***= p< 0.001

Table 5.4.1-5: Chromosme aberration test with metabolic activation (+S9)

Exposure period (hours)	Concentrations (µg/mL)		Cells with aberrations Excluding gaps Total number (%)	Cells with aberrations Including gaps Total number (%)
6	Control	Culture medium	3 (1.5)	3 (1.5)
		DMSO	1 (0.5)	2 (1.0)
	Propineb	20	1 (0.5)	1 (0.5)
		40	0 (0.0)	1 (0.5)
		80	20 (10.0)***	22 (11.0)***
	Control	10 (CPA)	177 (88.5)***	180 (90.0)***

*** = p< 0.001

Conclusion

Propineb induced structural chromosomal aberration at toxic doses, i.e. at doses inducing more that 50% inhibition of cell growth. Anyhow, the positive results of this in vitro test are discounted by the results of the in vivo chromosome aberration test.

CA 5.4.2 In vivo studies in somatic cells

No new studies.

CA 5.4.3 In vivo studies in germ cells

This type of study is not triggered.

CA 5.5 Long-term toxicity and carcinogenicity

No chronic or carcinogenity studies have been carried out since the last EU submission. In the previous submission three studies were submitted: two combined chronic and carcinogenity studies in rat, a combined chronic and carcinogenity study in the mouse and a two-year toxicity study in the dog.

In the first study 40 animals/sex/dose groups received Propineb at dietary levels of 0, 5, 10, 25, 50, 100 ppm equivalent to approximately 0.23, 0.46, 1.10, 2.50, 4.58 and to 0.2, 0.51, 1.28, 2.56, 5.0 mg/kg bw/day in males and females respectively. There were no overt signs of toxicity at any dose level, the main effects being increased activity of liver enzymes GPT and GOP and decreased protein-bound Iodine plasma levels in both sexes at 100 ppm. There was no evidence of carcinogenic potential up to 100 ppm. The study NOAEL was 50 ppm equivalent to approximately 2.5 mg/kg bw/day.
The second rat chronic/oncogenicity study was started contemporary to the first one in the same facility. In the second study twenty-five additional animals/sex dose groups were given Propineb in the diet at 1, 10, 100, 1000, 2000 and 8000 ppm. After only 5 days of feeding, the rats of the three highest dose levels of 1000, 2000, 8000 ppm, the showed severe myasthenia in the hind-limbs which gradually lead immobility and also affected the fore extremities of the animals in the 2000 and 8000 ppm groups. These symptoms developed earlier and more massively in the female rats than in the males. Therefore the achieved test material intake in expressed in mg/kg bw/day could be determined for males up to 2000 ppm (i.e. approximately 0.05, 0.48, 5.03, 57.8 and 120 mg/kg bw/day) and for females up to 1000 ppm (i.e. approximately 0.04, 0.46, 4.5 and 58 mg/kg bw/day). A further

consequence of this muscular debility was that the affected rats had considerable difficulty in eating and drinking, with consequent less food consumption, decreased in weight (from 15% in males of the 1000 ppm groups to 50%) and increased mortality. Thyroid weights were increased in males from the 100 ppm dose levels and in surviving females of the 1000 ppm group. Histopathology revealed degenerative changes of skeletal muscle (atrophy and replacement with adipose tissue) and increased incidence of thyroid tumours in males (6/25) at 1000 ppm, a dose level that exceed the MTD. The NOAEL was 10 ppm (approximately 0.5 mg/kg bw/day) based on the effect on thyroid weight at 100 ppm (approximately 45.05 and 4.5 mg/kg bw/day).
The two rat studies can be considered together for the evaluation of the oncogenic potential of Propineb in rats. In fact the two studies were conducted in the same laboratory, using the same strain of animals (Wistar rats) from the same breeder. Moreover, the choice of dose selections provide information on Propineb oncogenic potential in about 65 animals per dose group (at 10 and 100 ppm) and on 40 animals given Propineb at 5, 25, 50 ppm for up to two years. The overall NOAEL in the rat is 50 ppm equivalent to approximately 2.5 mg/kg bw/day in both sexes, based on the LOAEL of 100 ppm set in both studies (equivalent to approximately 4.5 mg/kg bw/day).

In the combined chronic oncogenicity study in the mouse, 50 males and 50 female NMRI mice were given Propineb in the diet at 0, 50, 200, 800 ppm (equivalent to of 6.8, 26.2 and 106.3 mg/kg bw/day in the males and 8.9, 36.3 and 139.8 mg/kg bw/day in the females) for 24 months. There was no evidence of treatment-related effects up to the highest dose level during the in-life phase. At necropsy, the thyroid weight was increased in the female at 800 ppm; however there was no correlation with microscopic alteration in the thyroid. The incidence of hepatocellular adenomas of top dose males was increased compared to that of the controls (19.6% vs. 6.4%). However, the incidence is still comparable to the range of the laboratory historical control data (i.e. up to 18.4%) for male mice. There were no hepatocellular adenomas in females. The NOAEL was 200 ppm equivalent to 26.2 and 36.3 mg/kg bw/day in males and females respectively.

The effects of chronic dietary exposure to propineb were evaluated in the dogs, by administering Propineb in the diet at 0, 100, 300, 1000 or 3000 ppm (equivalent approximately to 2.5, 7.5, 25.0 and 75.0 mg/kg bw/day) for periods of up to 24 months. The study was run in 1973, when GLP were not compulsory. It is submitted as complementary information. Overall there was no evidence of severe toxic effects up to the top dose level, a part from increased liver enzyme activity and relative thyroid weight in males at 3000 ppm. The NOAEL can be considered to be 1000 ppm, equivalent to 25 mg/kg bw/day.

Table 5.5-1 Summary of chronic carcinogenicity toxicity studies

Study/Reference	NOAEL (mg/kg bw/day)	Effects at LOAEL and higher doses
Chronic Carcinogenicity Wistar Rat. 0, 5, 10, 25, 50 or 100 ppm 0, 0.23, 0.46, 1.10, 2.50, 4.58 mg/kg bw/day (♂) 0, 0.2, 0.51, 1.28, 2.56, 5.0 mg/kg bw/day (♀) [redacted]; [redacted]. F.; [redacted].; 1974. M-049957-01-1	2.50 (♂) – 2.56(♀)	4.58-5.0 mg/kg bw/day - Both sexes ↑GPT and GOP ↓protein-bound iodine plasma levels in both sexes at 100 ppm
Chronic Carcinogenicity Wistar Rat. 0, 1, 100, 1000, 2000 or 8000 ppm 0, 0.05, 0.48, 5.03, 57.8 and 120 mg/kg bw/day (♂) 0, 0.04, 0.46, 4.5 and 58 mg/kg bw/day mg/kg/day (♀) Due to mortality at 2000 and 8000 ppm, it was not possible to calculate the achieved intake only up to 1000 ppm [redacted]; [redacted].. 1974. M-050009-01-1	0.48 (♂) – 0.46(♀)	58 mg/kg bw/day: Both sexes ↑thyroid, kidney and liver weights ↑thyroid hyperplasia degenerative changes of skeletal muscle (atrophy and replacement with adipose tissue) 4.8 – 4.6 mg/kg bw/day - Both sexes: Enlarged thyroid
Chronic Carcinogenicity NMRI mice 0, 50, 200, or 800 ppm 0, 6.8, 26.2 and 106.3 mg/kg bw/d (♂) 0, 8.9, 36.3 and 139.8mg/kg bw/d (♀) [redacted] U.; 1980. M-056652-02-1 *	26.2 (♂) - 36.3 (♀)	106.3 mg/kg bw/day Males ↑ liver adenomas 139.8 mg/kg bw/day Females ↑thyroid weight
Chronic Beagle Dog (2 years) 0, 100, 300, 1000 or 3000 ppm 0, 2.5, 7.5, 25.0 and 75.0 mg/kg/day (♂) & (♀) [redacted]; [redacted]; [redacted] H.; 1973. M-049991-01-1	25 (♂) & (♀)	75 mg/kg bw/day - Males ↑Liver GPT activity ↑ Relative thyroid weight 75 mg/kg bw/day - Females ↓ food consumption

*: study including raw data is presented in the **Supplemental Dossier**; version M-056652-01-1 is part of the **Baseline Dossier**

CA 5.6 Reproductive toxicity

A reproductive toxicity study and a developmental toxicity study have been carried out after the Annex I inclusion and are described in detail. In addition, an overall summary which takes into account the whole relevant studies to assess the reproductive toxicity of propineb is presented here below.

Overall, two reproductive toxicity/fertility studies have been conducted with propineb. The first was conducted in the early 70's using FB rats and dietary levels of 0, 20, 60, 200 or 600 corresponding to approximately 1,3, 3, 10 or 30 mg/kg bw/day using standard conversion factors for animals of this age. The dose levels were likely around 2-fold higher during the lactation period because nursing dams consume approximately twice as much food as non-nursing females. The 600 ppm dietary level produced clear evidence of toxicity including increased mortality in dams and statistically lower body weight and body weight gains in P-generation males and females. Clinical signs observed in P-generation animals included decreased activity (weak), myasthenia of the hind extremities that considerably hindered the rats in their mobility and uptake of food. The effects were more pronounced in the females. Treatment-related findings in P-generation animals at 200 ppm included decreased activity and mild myasthenia of the hind extremities. After one mating, gestation rate and litter size were slightly lower. There were no effects related to treatment at lower dietary levels. The NOAEL for parental toxicity and the offspring was determined to be 3 mg/kg/day and 10 for reproductive toxicity.

As recommended by US EPA in the December 2007 pre-submission meeting for Import Tolerance, a new reproduction study was performed in accordance to OPPTS guideline 870.3800 ([redacted], 2010). In this study, propineb was administered via the diet to groups of male and female rats at levels of 0, 30, 60 and 180 ppm. The mean daily intake of the test substance monitored throughout the various in life phase of the study was equivalent to 1.6, 3.0-3.2, 9.5-10 mg/kg bw/day in males and 1.7-2.1, 3.5-4.1 or 11.6-13.8 mg/kg bw/day in females. The highest dose was selected in order to avoid the onset of the neuromuscular effects observed from doses equivalent to approximately 20 mg/kg bw/day in various dietary toxicity studies with propineb and to be able to discriminate between toxic and reprotoxic effects.

Body weights of F0 and F1 adult were decreased up to 11% and body weight gain was reduced up to 47% compared to control females. These effects on body weight and gain were seen in the absence of effects on food consumption. Notwithstanding maternal toxicity, pup weight and sexual development were not affected at any dose level. There were no effects on fertility parameters at any dose level. Thus, the NOAEL for maternal toxicity was 60 ppm (equivalent to approximately 4 mg/kg bw/day); the NOAEL for offspring was 180 ppm (equivalent to 13.8 mg/kg bw/day); and the NOAEL for reproductive toxicity was 180 ppm (equivalent to 10 and 12.5 mg/kg bw/day in males and females, respectively.

In addition a new developmental toxicity study was carried out in the rat according to the current guideline, as recommended by the US EPA. In this study, groups of 23 sperm-positive female Sprague-Dawley rats were exposed to Propineb by oral gavage from gestation day (GD) 6 to 20 at doses of 0, 3, 12 and 48 mg/kg body weight/day in suspension in aqueous solution of 0.5% methylcellulose 400. Adverse effects were observed in both dams and fetuses at the dose of 48 mg/kg/day: maternal body weight parameters were decreased and retarded ossification was noted for a

few elements of the skeleton. The dose level of 12 mg/kg/day was considered to be a No Observed Effect Level (NOEL) for both maternal and fetal toxicity.

Results of the new study are in line with those of the old, non GLP one, in which dams were exposed by gavage to propineb at doses of 0, 3, 10, 30 or 100 from gestation day 6 to 15. The maternal no-observed-effect levels was 10 mg/kg bw/day for maternal toxicity (based on the occurrence of clinical signs in the dams at 30 mg/kg bw/day) day and the fetal NOAEL was 30 mg/kg body weight per day based on the occurrence of dysplasia of the long tubular bones of the extremities of the fetuses at 100

No new studies were conducted in the rabbit. In this study, where Chinchilla rabbits were given propineb by gavage at doses of 10, 30 or 100 mg/kg bw/day from the gestation day 6 to the 18. The maternal no-observed-effect levels was 10 mg/kg bw/day (due to dyspnea, ventro-lateral recumbency, inability to sit or stand and to move the extremities at 100 mg/kg bw/day) and of 30 mg/kg body weight per day for fetotoxicity (based ondecreased numbers of live fetuses at 100 mg/kg body weight per day.

Table 5.6-1 Summary of Reproductive toxicity studies (in bold new studies)

Type of study	NO(A)EL (mg/kg bw/day)	LOAEL (mg/kg/day)	Effects at LOAEL and higher doses	
Multigeneration study				
Three generation study in the rat 0, 20, 60, 200 or 600 ppm 0, 1.0, 3.0, 10 or 30 mg/kg bw/day (♂) & (♀) [redacted]; [redacted]; [redacted]. 1973 M-075529-01-1	3	10	parents progeny	From 10 mg/kg bw/day Dose-relets disturbances in general condition and myasthenia
	3	10	pup development	From 10 mg/kg bw/day ↓ decreased pup numbers of the F3a generation
	10	30	reproduction	30 mg/kg bw/day Reduce pregnancy rate and mating due to high toxicity
Two-generation rat 0, 30, 60 or 180 ppm 0, 1.6, 3.0-3.2, 9.5-10 mg/kg bw/day (♂) 0, 1.7-2.1, 3.5-4.1 or 11.6-13.8 mg/kg bw/day (♀) [redacted].;2010; M-370252-0	3.0-4.1 (60 ppm)	9.5-11.9 (180 ppm)	parents progeny	Decreased Body weight
	> 11.9 (180 ppm)	/	pup development	No effects
	> 13.8 (180 ppm)	/	reproduction	No effects
Developmental toxicity studies				
Embryotoxicity rat 0, 3, 10, 30 and 100 mg/kg bw/day [redacted].; 1973. M-053094-01-1	10	30	dam	100 mg/kg bw/day: Maternal mortality and paralysis 30 mg/kg bw/day: Clinical signs (somnolence, ruffled coat, and limpness)
	30	100	fetus	100 mg/kg bw/day: Skull, pelvis, rib abnormalities
Embryotoxicity rat 0, 3, 12, and 48 mg/kg bw/day [redacted].; 2014 M-479395-01-1	12	48	dam	48 mg/kg bw/day: decreased corrected bw
	12	48	fetus	48 mg/kg bw/day ↑ delayed ossification
Embryotoxicity rabbit 0, 10, 30 and 100 mg/kg bw/day [redacted]1988 M-050184-02-01	10	30	dam	100 mg/kg bw/day: Maternal mortality, clinical signs (dyspnea, recumbent, abnormal head position, inability to move the extremities), and 30 mg/kg bw/day: post-implantaion loss
	30	100	fetus	100 mg/kg bw/day: ↑ fetal mortality

CA 5.6.1 Generational studies

Report:	[redacted] ;[redacted];2010;M-370252-01
Title:	Technical grade propineb: A two-generation reproductive toxicity study in the Wistar rat
Report No:	08-R72-OB
Document No:	M-370252-01-1
Guidelines:	**OPPTS Guideline Number: 870.3800 Reproduction and Fertility Effects EU Guidelines on Reproductive Toxicity Studies 91/414/EEC OECD 416 Two-Generation Reproduction Toxicity Study JMAFF 12 Nousan No. 8147 Health Canada, Guideline on Reproduction Toxicity Studies.** The exception is that the homogeneity and stability of the test substance in the diet were verified after the study was completed, due to unanticipated challenges associated with developing the analytical method. This is not believed to have had an affect on the outcome or interpretation of the study, since the results verified the homogeneity and stability of the test substance in the feed, under the conditions that were used in this study.
GLP/GEP:	**yes**

Executive summary

In a two generation-reproduction study, Propineb was administered continuously in the feed to the Wistar rat (30 animals/dose/sex) at nominal dietary concentrations of 0, 30, 60 and 180 ppm. All test diets (including control) were available for ad libitum consumption; the homogeneity and stability of Propineb as a dietary admixture was confirmed. Body weight and food consumption determinations and detailed clinical examinations of each animal were conducted weekly throughout the study, as well as, an evaluation of multiple reproductive parameters. All animals placed on study were subject to a postmortem examination, which included recording all gross lesions, weighing designated organs and collecting representative tissue specimens for histopathologic evaluation.

The mean daily intake of the test substance (mg propineb/kg bw/day) throughout this two-generation reproduction study at nominal dietary concentrations of 0, 30, 60 or 180 ppm, respectively, were 1.6, 3.0-3.2 and 9.5-10.0 in the males and between 1.7 -2.1, 3.6-4.1 and 11.6-13.8 in the females.

In the parent and the adult of F1 generation the main effects consisted of decreased body weight and bodyweight gain in the females after 5 week of exposure. There were no toxicological effects in the offspring. There were no reproductive effects up to highest dose level.

The dose level of 60 ppm (equivalent to 4.0 mg/kg bw/day) in the females and 180 ppm (equivalent to 10.0 mg/kg bw/day) in the males. The dose levels of 180 ppm (equivalent to 13.8 mg/kg bw/day) was also the NOAEL for the offsprings and for reproductive toxicity.

Materials:

<u>Test Material:</u> Propineb

- Description: White yellowish powder
- Lot/Batch: EDFU711100
- Purity: From 80.5% - to 82.3% (analysed during various study phases)
- CAS: 9016-72-2
- Stability of test compound: stability checked during various phase of the study

Test animals:

Species:	Rat
Strain:	Wistar Han CRL: WI (HAN)/Rat
Age:	8-9 weeks old
Weight at start:	males, mean value range: 214.2 – 258.7 g females, mean value range: 127.4 – 175.4 g
Source:	[redacted] Laboratories Inc., [redacted], NC (USA)
Acclimation period:	one week
Diet:	[redacted] Certified Rodent Diet 5002 meal, [redacted], MO (USA)
Water:	Water from the municipal supply, *ad libitum*
Housing:	Individual hanging stainless steel cages with deotized cage board in the bedding tray. During the gestation and lactation phases, individual dams and their litters, and F1 and F2 pups were housed in polycarbonate cages with ground corn cob bedding (Bed-O- COBs).
Environmental conditions –	
Temperature:	18-26°C
Humidity:	30-70%
Ventilation:	at least 10.67 air changes per hour
Photoperiod:	Alternating 12-hour light and dark cycles

Study Design :

In life dates

The study was initiated on July 7th 2008 and the in-life phase was completed on April 30th 2009. The study was carried out at Bayer CropScience, LP, Toxicology [redacted], KS,(USA) which changed to [redacted], KS (USA) on April 1st 2009.

Animal assignment and treatment

Four groups of 30 male and 30 female rats each were given 0, 30, 60, and 180 ppm of proprineb in the diet seven days/week throughout the entire study. These rats were designated the *P*-Generation. After 10 weeks, each male was cohabited with a female in the same group, the females were allowed to litter, and wean their offspring. The offspring were designated the F1 Generation.

After weaning, F1-pups were maintained for approximately six weeks prior to initiation of the second generation. 30 male and 30 female rats from each group were selected for growth and subsequent mating to produce the F2 Generation. F2-pups were sacrificed at weaning on lactation day 21.

Table 5.6.1-1 Study design and animal assignment

Test group	Test substance	Dose levels ppm	Number of animals P-Generation	
			Males	Females
1	0	0	30	30
2	Propineb	30	30	30
3		60	30	30
4		180	30	30

Diet preparation and analysis

The test substance was dissolved in acetone and then mixed with the feed. Treated diet was mixed at room temperature; aliquots of the chemical were taken from the original test batch and transferred to the mixing area. The control test diet was prepared in the same manner as chemically-treated test diet, excluding only the test substance. A sample of each batch of feed mixed was taken and retained in the freezer until the study was complete and the analytical data deemed satisfactory. Replacement admixtures for each treatment group were prepared weekly (or at greater intervals depending on freezer stability) and stored under freezer conditions until presented to the animals the following week (or weeks).

The concentration of the test substance in the feed for the females only was adjusted during the lactation period (Days 0-21) by 50%. Samples from the first batch of adjusted feed for each dietary level was analyzed to measure the concentration. During the lactation phase a substantial increase in food consumption is observed in all dams which results in greatly increased intake of test substance (normal occurrence). A decrease in the dietary concentration of the test substance offsets this increased food consumption, thereby maintaining an approximately constant test substance intake (mg/kg body weight/day) throughout the study.

The concentration of Propineb in the various test diets was verified for batches intended for weeks 1, 2, 3, and at monthly intervals thereafter (Bayer CropScience LP, Environmental Research, [redacted], [redacted], KS). Test diets intended for the first week of lactation were alsoanalyzed. The homogeneity and stability of Propineb when mixed in the rodent feed was characterized.

Mean analytical concentrations for each dose group were 24.7, 49.1, and 153 ppm, ranging from 82-85% of the corresponding nominal concentrations of 30, 60, and 180 ppm, respectively. During lactation, the concentration of the test substance in the feed for the females was adjusted by 50%. Mean analytical concentrations for each dose group during lactation were 13.0, 25.9, and 78.9 ppm, ranging from 86-88% of the corresponding nominal concentrations of 15, 30, and 90 ppm, respectively. The AI of the test substance was not detected in the control diet. Mean recovery was 77% and ranged from 72-86% for rodent ration spiked with 14.8 ppm of Propineb and mean recovery was 102% and ranged from 92 110% for rodent ration spiked with 180 ppm of Propineb.

The mean concentrations of Propineb in the feed, sampled from three distinct layers in the mixing bowl and containing a nominal concentration of either 15- or 180- ppm, were determined to be 13.3 ppm (range 12.3-14.3 ppm; %RSD = 5.3) and 150 ppm (range 141-156 ppm; %RSD = 3.0), respectively. Based on a %RSD. to10% Propineb was judged to be homogeneously distributed in the feed over a concentration range of 15-180 ppm.

Following 7 days of room temperature storage, the analytically-determined concentration of the AI of the test substance in the 15- or 180-ppm admixture was determined to be 12.7 ppm (13.6 ppm on Day 0) and 155 ppm (146 ppm on Day 0), respectively. Following 28 days of freezer storage, the analytically-determined concentration of the AI of the test substance in the 15- and 180-ppm admixtures was determined to be 13.4 ppm (13.3 on Day 0) and 161 ppm
(150 on Day 0), respectively. Propineb mixed in rodent ration was judged to be stable at room temperature for at least seven days and following freezer storage for a minimum of 28 days, over a concentration range of 15-180 ppm.

Methods

Observations

Females and males were observed (cageside) for clinical signs twice daily during the working week and at least once on weekends and holidays. Cageside observations, mortality, morbundity, behavioural changes, signs of difficult or prolonged delivery, and overt toxicity by viewing the animal in the cage were conducted. A detailed evaluation of clinical signs, and a physical examination was conducted once per week.

Body weight

Parental animals (P and F1) body weights were recorded weekly for both males and females during the premating period. During the mating period and until sacrificed, body weights for the males were recorded once per week. During gestation, dam body weights were recorded on Days 0, 6, 13, and 20 and during lactation, on Days 0, 4, 7, 14, and 21.

Food consumption and compound intake

Food consumption was recorded once per week for both males and females parental animals (P and F1) during the premating periods. During gestation, dam food consumption was recorded on Days 0, 6, 13, and 20 and on lactation days 0, 4, 7, 14, and 21.

Urine collection

Prior to sacrifice, urine was collected from 10 control and 10 high dose adult males. Urine was not collected from the females. Males were individually housed throughout the day in cages fitted with urine collection trays, with food and water available. Urine was collected on ice over a one to six hour period and samples were transferred to the ultralow freezer (≤ -80°C) as soon as possible after collection. After urine collection, males were transferred back to their appropriate gang cage.

The PND 21 pup urine was collected overnight with up to 2 pups per sex if available from the control and high dose groups. Pups were housed throughout the day in cages fitted with urine collection trays, with food and water available. Urine was not collected on ice for the weanlings. After urine collection, pups were transferred back to their appropriate nesting cage.

Oestrus cycle evaluation

The oestrus cycle was determined by examining daily vaginal smears over a three-week period prior to mating of the P- and F1-Generation females, immediately prior to the cohabitation period. Additionally, the estrous cycle stage was determined for all females just prior to termination.

Sperm analysis

Sperm was collected from one testis and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively at sacrifice for all P- and F1-Generation males. In addition, an evaluation of the morphology and motility was performed on sperm sampled from the distal portion (closest to the urethra) of the *vas deferens* on males of the control and high dose groups of both generations. Sperm motility and counts were conducted using the Integrated Visual Operating System (IVOS, Hamilton-Thorne Research, 1998).

Mating

Mating was accomplished by co-housing one female with one male for up to 14 consecutive days. During the mating phase, vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemination was observed in the vaginal smear was designated Day 0 of gestation for that female. In order to evaluate those females which might have been inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all remaining females were placed in polycarbonate nesting cages, following the 14-day mating period.

Parturition and lactation

Beginning on gestation day 21, each P or F1 female was examined twice per day for signs of parturition or dystocia. The number of live and stillborn pups (both F1 and F2 Generations) was recorded for each litter. As soon as possible after parturition was judged complete, each pup was examined, weighed and individually identified by tattoo of the paws. Dead pups were necropsied and the lungs floated to determine if the pup died after delivery or was stillborn.

Offsprings

The size of each litter was adjusted on lactation Day 4 to yield, as closely as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment was made (e.g., three females and five males). No adjustment was made for litters of fewer than eight pups. Adjustments were made by random selection of the pups using software provided by SAS.10. Grossly abnormal pups underwent a gross internal and external examination, and all culled pups were discarded.

The F1- and F2-pups not culled on lactation Day 4 were maintained with the dam until weaning on lactation Day 21. On lactation Day 21, a sufficient number of randomly selected F1- pups/sex/litter were maintained to produce the next generation. F1-pups not selected to become parents of the next generation were sacrificed, examined macroscopically and had organs weighed. One randomly selected pup/sex/litter for each generation had tissues collected and evaluated for any structural abnormalities or pathological changes.

Random selection of pups for selection to go to next generation and those for organ weight collection was performed using software provided by SAS.

Gross Necropsy

a) Adults

All surviving parental males were sacrificed as soon as possible after the last litters were produced. Maternal animals were sacrificed following the weaning of their respective litters (lactation Day 21). F1 adult males were sacrificed after the beginning of the delivery phase for the F1-females.

Terminal body weights were taken and the abdomen and thoracic cavities were opened, a gross internal examination was performed, and the uterus was excised and the former implantation sites, if present, were counted. In addition, patency of the cervical/uterine os in these females was examined via flushing of the uterine horns with 10% buffered formalin.

The following tissues were collected and weight: brain, pituitary, liver, kidneys, spleen, thyroid, thymus, adrenals, epididymis, ovary, prostate, seminal vesicles with coagulating gland, testis, uterus with cervix, vagina. All paired organs were weighed individually.

The following tissues were also collected and fixed in 10% buffered formalin for histopathology examination: brain, pituitary, liver, kidneys, spleen, thyroid, adrenals, epididymis, ovary, oviduct, prostate, seminal vesicles with coagulating gland, testis, uterus with cervix, vagina, physical identifier, gastrocnemius muscle and gross lesions. The ovaries as well as one testicle (the side not utilised for sperm analysis) were collected and fixed in Bouin's fixative.

b) Offspring

The F1-offspring not selected as parental animals and all F2-offspring were sacrificed at 21 days of age. These animals were subjected to postmortem examinations (macroscopic and/or microscopic examination).

The following tissues from 21-day weanlings were collected and weighed: brain, spleen, thymus, uterus.

The following tissues from 21-day weanlings were collected and micropathology was performed: gross lesions, thyroid, gastrocnemius muscle, uterus, ovary, vagina, cervix, oviduct, testis, epidydimis, prostate, coagulating gland, seminal vesicle.

Pups found dead or terminated in a moribund condition underwent a gross necropsy for possible defects and/or cause of death.

Statistics

Parametric data (including body weight gain and food consumption) were analyzed using a univariate Analysis of Variance (ANOVA), and if significant differences were observed, a Dunnett's Test was performed. Nonparametric data (e.g., number of estrous cycles, litter size, and number of implantation sites) were first analyzed by the Kruskal-Wallis test and then subjected to Dunn's Test if significant differences were identified. Nonparametric dichotomous data (e.g. fertility and gestation indices) were initially analyzed by the Chi-Square Test and if significance was observed between groups then by the Fisher's Exact Test with the Bonferroni adjustment. To the extent possible, the frequency of gross lesions were first examined visually, then, in the event of questionable distribution, by statistical analysis using the Chi-square and Fisher's exact tests. Sperm parameters were analyzed using ANOVA, single factor. Differences between the control and test compound-treated groups were considered statistically significant when $p < 0.05$ or $p < 0.01$.

Indices

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

$$\text{Mating Index (\%)} = \frac{\text{\# inseminated females}^{a} \times 100}{\text{\# of females co-housed}}$$

$$\text{Fertility Index (\%)} = \frac{\text{\# of pregnant females}^{b} \times 100}{\text{\# of inseminated females}}$$

$$\text{Gestation Index (\%)} = \frac{\text{\# of females with live pups} \times 100}{\text{\# of pregnant females}}$$

[a] Includes pregnant females not observed sperm positive or with an internal vaginal plug.

[b] Includes females which did not deliver, but had implantation sites.

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

Birth Index (%) = total # of pups born/litter x 100 / total # of implantation sites/litter

Livebirth Index (%) = # of live pups born/litter x 100 / total # of pups/litter

Viability Index (%) = # of live pups/litter on day 4 (pre-culling) x 100 / # of live pups born/litter

Lactation Index (%) = # of live pups/litter on day 21 x 100 / # of live pups/litter on day 4 (post-culling)

Gestation Length = Number of whole days from day in which insemination is observed in the vaginal smear (designated Day 0 of gestation) to Lactation Day 0 (delivery of pups and entry in computer system).

Historical control

Historical control data are provided in the report were obtained from reproduction studies performed in the testing laboratory (1998 -2008) in the Wistar rat.

Results And Discussion

A. Achieved intale

Animals were administered propineb at nominal dietary dosages of 0, 30, 60 or 180 ppm.

The mean daily intake of the test substance (mg propineb/kg body weight/day) calculated from food consumption, body weight and diet analysis data, is presented in the following table. The test substance active ingredient was not detected in the control diet.

Table 5.6.1-2 Achieved intake

Phase of Study	30 ppm in mg/kg/day[a]	60 ppm in mg/kg/day[a]	180 ppm in mg/kg/day[a]
Premating (*P*-gen) - Male	1.6	3.2	10.0
Premating (F_1-gen) - Male	1.6	3.0	9.5
Premating (*P*-gen) - Female	2.0	4.0	12.5
Premating (F_1-gen) - Female	1.9	3.6	11.9
Gestation (*P*-gen) - Female	1.7	3.5	11.6
Gestation (F_1-gen) - Female	1.8	3.5	12.5
Lactation (*P*-gen) - Female	1.9	4.1	13.3
Lactation (F_1-gen) - Female	2.1	4.0	13.8

[a] Individual values were based on the means for each particular phase

B. General Observations

Clinical signs

There were no test substance-related mortalities or clinical observations observed during the course of this study at any dietary level tested in either generation.

Body weights and food consumption

a) Premating males

No test substance-related findings were observed on body weight, body weight gain, or food consumption during the study for the *P*-generation males at any dietary level tested.

The F1-generation males of the 180 ppm dose group exhibited slight declines in body weight throughout premating (overall mean decline of 5.0%) with significance observed weeks 7 and 8 (mean decline for these two weeks of 6.0% relative to controls). No effect on body weight gain was observed in this dose group.

No test substance-related findings were observed on body weight or body weight gain during the study for the *F1*-generation males at any other dietary level tested.

b) Premating females

The P-generation females of the 180 ppm dose group exhibited significant body weight declines for weeks 5, 9, and 10 of the premating phase. At week 10, body weights were declined 7.3%, relative to controls. A decline in body weight gain was also observed in the 180 ppm dose group (declined 32.6%, relative to controls).

Food consumption was unaffected by treatment at any dietary level tested .

Table 5.6.1-3 Body weights (g), body weight gains (g), and food consumption (g/rat/day) in Parent animals (selected intervals)

Observations/study week	Dose Group			
	0 ppm	30 ppm	60 ppm	180 ppm
P Generation Males				
Mean body weight (g) - Week 17 S.E.	468.4 6.94	469.2 6.86	462.1 7.55	460.4 8.59
Mean weight gain (g) Weeks 1-17	198.2	199.6	186.9	185.3
Mean food consumption (g/kg/day) Weeks 1-10	65.7	66.1	65.3	65.1
P Generation Females - Pre-mating				
Mean body weight (g) - Week 10 S.E.	226.5 3.17	230.0 3.68	229.8 2.82	209.9** 3.17
Mean weight gain (g) Weeks 1-10	58.5	62.4	60.5	39.4
Mean food consumption (g/kg/day) Weeks 1-10	82.0	82.5	81.8	81.7
F1 Generation Males				
Mean body weight (g) - Week 15 S.E.	447.3 9.76	432.0 7.26	440.2 9.24	432.4 6.45
Mean weight gain (g) Weeks 1-15	149.4	150.7	149.6	141.8
Mean food consumption (g/kg/day) Weeks 1-10	64.3	64.2	62.0	62.1
F1 Generation Females - Pre-mating				
Mean body weight (g) - Week 10 S.E.	231.7 4.00	228.1 3.48	233.7 3.24	206.1** 3.21
Mean weight gain (g) Weeks 1-10	49.3	47.8	46.9	26.3
Mean food consumption (g/kg/day) Weeks 1-10	77.1	77.4	73.6	78.1

**Statistically different from control, p<0.01

Gestation

P-generation - In the 180 ppm dose group, significant declines in body weight (mean decline Days 0-20 of 6.5%) were observed. There were no test substance-related findings observed on absolute body weight at any other dietary level tested. There were no effects observed on body weight gain or food consumption at any dietary level tested.

F1-generation- In the 180 ppm dose group, significant declines in body weight (mean decline Days 0-20 of 9.3%) was observed. There were no test substance-related findings observed on absolute body weight at any other dietary level tested. There were no effects observed on body weight gain at any dietary level tested. A slight increase in food consumption on a g/kg/day basis was observed in the females of the 180 ppm dose group (significant week 13- 20).

Test substance-related effects on food consumption were not observed at any other dietary level tested.

Table 5.6.1-4 Maternal body weights (g), body weight gains (g), and food consumption (g/rat/day) in Females during gestation (selected intervals)

P Generation Females - Gestation				
Observations/study week	Dose Group			
	Control 0 ppm	LDT 30 ppm	MDT 60 ppm	HDT 180 ppm
Mean body weight (g) - Day 0 S.E.	226.8 3.41	234.3 3.97	233.3 3.38	210.5** 2.81
Mean body weight (g) - Day 6 S.E.	244.4 3.67	251.9 3.60	251.5 3.63	222.9** 3.67
Mean body weight (g) - Day 13 S.E.	266.8 3.83	276.6 4.03	273.7 3.86	251.7* 3.31
Mean body weight (g) - Day 20 S.E.	328.1 4.93	332.5 5.44	332.7 5.52	311.3* 4.03
Mean weight gain (g) - Days 0-20 S.E.	101.2 2.26	98.3 3.42	99.4 3.10	100.9 2.44
Mean food consumption (g/kg/day)Days 0-20	71.8	70.9	70.3	75.8

F_1 Generation Females - Gestation				
Observations/study week	Dose Group			
	0 ppm	30 ppm	60 ppm	180 ppm
Mean body weight (g) - Day 0 S.E.	233.3 4.51	230.2 3.25	235.6 3.62	205.2** 3.44
Mean body weight (g) - Day 6 S.E.	246.6 4.40	243.6 3.49	252.5 3.83	218.7** 3.52
Mean body weight (g) - Day 13 S.E.	266.6 4.61	264.9 3.59	272.3 4.09	242.7** 3.62
Mean body weight (g) - Day 20 S.E.	320.6 6.26	319.9 5.24	331.7 5.03	300.5* 4.62
Mean weight gain (g) - Days 0-20 S.E.	87.1 3.28	89.7 3.11	96.1 2.35	95.2 3.19
Mean food consumption (g/kg/day) Days 0-20	73.6	74.9	71.8	81.5

* Statistically different from control, $p \leq 0.05$; ** Statistically different from control, $p \leq 0.01$

c) Lactation

P-generation- There were no significant body weight declines during lactation at any dietary level tested. There were no effects on food consumption considered to be test substance-related at any dietary level tested.

F1-generation- In the 180 ppm dose group, significant declines in body weight (mean decline Days 0-21 of 5.5%) was observed. Body weight effects were not observed at any other dietary level tested. There were no effects on food consumption considered to be test substance-related at any dietary level tested.

Table 5.6.1-5 Body weights (g), body weight gains (g), and food consumption (g/rat/day) in Females during lactation (selected intervals)

P Generation Females - Lactation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 30 ppm**	**MDT 60 ppm**	**HDT 180 ppm**
Mean body weight (g) - Day 0 S.E.	252.6 3.81	259.5 3.90	257.6 3.74	241.2 3.22
Mean body weight (g) - Day 4 S.E.	260.9 3.75	265.8 3.49	265.5 4.12	253.1 4.00
Mean body weight (g) - Day 7 S.E.	268.4 3.86	270.5 3.69	272.2 4.19	258.8 4.03
Mean body weight (g) - Day 14 S.E.	283.8 4.28	284.4 3.92	285.9 4.21	276.4 4.70
Mean body weight (g) - Day 21 S.E.	270.9 5.47	273.0 3.69	278.0 4.67	266.2 4.42
Mean food consumption (g/kg/day) Days 0-21	159.1	147.5	155.3	168.0
F1 Generation Females - Lactation				
Observations/study week	**Dose Group**			
	Control 0 ppm	**LDT 30 ppm**	**MDT 60 ppm**	**HDT 180 ppm**
Mean body weight (g) - Day 0 S.E.	254.2 3.93	252.6 3.41	256.3 4.62	234.9** 3.94
Mean body weight (g) - Day 4 S.E.	266.1 4.35	264.2 3.79	270.7 4.06	248.8* 3.78
Mean body weight (g) - Day 7 S.E.	270.6 3.99	269.0 3.61	273.7 4.09	255.6* 3.79
Mean body weight (g) - Day 14 S.E.	284.0 3.82	282.6 4.22	288.7 3.84	272.6 3.62
Mean body weight (g) - Day 21 S.E.	278.0 3.23	270.9 3.75	281.0 4.27	266.2* 3.60
Mean food consumption (g/kg/day) Days 0-21	163.8	156.6	153.5	174.8

Reproductive parameters

There were no compound-related effects on any parameter (e.g., mating, fertility, or gestation indices, days to insemination, gestation length, or number of implants) in either generation at any dietary level tested. There were no test article-related effects on oestrus cycle length or the number of cycles at any dietary level in either generation.

There were no effects considered to be test-substance-related on any sperm parameter evaluated at any dietary level tested for either generation. Epididymal counts for the F1- males of the 180 ppm dietary group appear low compared to the controls but are not considered to be a result of treatment with the test substance based on the wide variability with epididymal counts, on the absence of reproductive consequences, and micropathology evaluation did not show any effect on the epididymis.

Table 5.6.1-6 Sperm analysis in Parent and F1 males

Sperm Analysis		Dose Group (ppm)			
		Control 0 ppm	LDT 30 ppm	MDT 60 ppm	HDT 180 ppm
P Generation Males					
Sperm Motility	% Motile	83.1	81.4	82.0	81.9
	% Progressive	56.7	56.1	56.8	56.7
Sperm Counts (sperm/gram)	Testis	34.6	N/A	N/A	39.1
	Epididymis	176.9	N/A	N/A	180.4
Sperm Morphology (mean total number)	Normal	198.3	N/A	N/A	197.8
	Abnormal	1.4	N/A	N/A	2.1
	Detached Head	0.2	N/A	N/A	0.03
F_1 Generation Males					
Sperm Motility	% Motile	80.8	84.6	85.0	84.2
	% Progressive	55.3	57.5	58.7	58.4
Sperm Counts (sperm/gram)	Testis	33.0	N/A	N/A	34.4
	Epididymis	137.9	150.9	135.5	116.2
Sperm Morphology (mean total number)	Normal	197.3	N/A	N/A	197.7
	Abnormal	2.2	N/A	N/A	2.2
	Detached Head	0.5	N/A	N/A	0.1

a Data obtained from Table 23 in the study report.
N/A = Not Applicable

There were no test substance-related effects observed on the mean primordial (preantral) follicles, antral follicles, or corpora luteal counts for the F1-females at any dietary level tested

Litter Parameters (F1 and F2 generation)

a) Clinical signs

There was no test substance-related effects observed on the viability of the pups at any dietary level tested. There were no test substance-related clinical observations observed in either generation at any dietary level tested.

b) Growth and developmental parameters

In both generations, pup body weights at birth for all three treated groups were comparable to the control group. There were no test substance-related effects on pup body weight or body weight gain observed during the lactation period at any dietary level tested.

There were no effects observed on either vaginal patency or balanopreputial separation for the F1-pups at any dietary level tested. Therefore, anogenital distance measurements were not deemed necessary for F2 pups.

Necropsy

a) P-generation and F1 Adults

There were no terminal body weight or organ weight effects observed at any dietary level tested in the males.

A decline of 5.4% of terminal body weight was observed in the 180 ppm dose group compared to controls in F1 females. However, organ weights were not affected at this and all the other dose levels.

There were no test substance-related gross necropsy and/or microscopic findings at any dietary level tested in either generation.

b) Pups

There were no test substance-related gross necropsy or microscopic findings at any dietary level tested in either the F1- or F2-pups.

Conclusion

The parental male systemic NOAEL is 180 ppm (10.0 mg Propineb/kg bw/day).

The parental female systemic NOAEL is 60 ppm (4.0 mg Propineb/kg bw/day), based on decreased body weight and/or body weight gain during premating (P and F1), gestation (P and F1) and lactation (F1) at 180 ppm (11.9 mg Propineb/kg bw/day).

The reproductive NOAEL is 180 ppm in both the males and females (10.0 mg Propineb/kg bw/day for males and 12.5 mg Propineb/kg bw/day for females) based on no test-substance- related reproductive findings observed at the highest dose tested.

The offspring NOAEL is 180 ppm (13.8 mg Propineb/kg bw/day) based on no test substance-related findings observed in the pups.

CA 5.6.2 Developmental toxicity studies

Report:	KCA 5.6.2 /06; [redacted]. 2014 ; M-479395-01-1
Title:	Technical grade propineb: A two-generation reproductive toxicity study in the Wistar rat
Report No:	SA12220
Document No:	M-479395-01-1
Guidelines:	**OECD guideline 414 (January, 2001), EEC Directive 2004/73/EC, Method B.31 (April, 2004), US EPA OCSPP Guideline number 870.3700, MAFF IN Japan notification 12 Nousan N°8147 (November, 2000)**
GLP/GEP:	**yes**

Executive Summary

In this study, groups of 23 sperm-positive female Sprague-Dawley rats were exposed to Propineb (AE F074263, batch number EDFU911415, a light yellow solid, 80.4% w/w purity), by oral gavage from gestation day (GD) 6 to 20. The sperm-positive day was GD 0. The doses given were 0, 3, 12 and 48 mg/kg body weight/day in suspension in aqueous solution of 0.5% methylcellulose 400. The volume of administration was 10 ml/kg based on the most recent body weight recorded.

Clinical observations were recorded daily. Maternal body weights were recorded for all females on GD 0, 6, 8, 10, 12, 14, 16, 18 and 21. Food consumption was also measured for all the females during the intervals GD 1-6, 6-8, 8-10, 10-12, 12-14, 14-16, 16-18 and 18-21. At scheduled sacrifice, on GD 21, a macroscopic examination of the visceral organs was performed, the gravid uterine weight was recorded and the dams were evaluated for number of corpora lutea, number and status of implantations (resorptions, dead and live fetuses). In addition, the liver was weighed at scheduled sacrifice for all pregnant females. Live fetuses were removed from the uterus, counted, weighed, sexed and examined externally. Approximately half of the live fetuses from each litter were fixed in Bouin's solution and subsequently dissected for internal examination. The remaining half were eviscerated, skinned, fixed in absolute ethanol and stained according to a modification of the TYL and MARR technique (1977), (1), for skeletal examination of bone and cartilage.

Pregnancy rate was unaffected by treatment. There were no treatment-related mortalities, clinical signs or changes in mean food consumption throughout the study in dams. At necropsy, there were no treatment-related macroscopic findings in dams and mean liver weight was unaffected by treatment. At cesarean section, the following litter parameters were unaffected by treatment: number of live fetuses, number of implant sites per dam, percentages of pre- and post-implantation losses, number of early and late resorptions, fetal death status and percentage of male fetuses. There were no treatment-related malformations at the external, visceral and skeletal fetal examination.

At 48 mg/kg bw/day, there was evidence of toxic effects on maternal body weight parameters. Mean body weight gain of all pregnant females surviving to terminal sacrifice was reduced by 51% between GD 6 and 8 ($p \leq 0.01$) and by 12% between GD 18 and 21 ($p \leq 0.05$), when compared to controls. Throughout other intervals, mean maternal body weight gain was similar to controls. Overall between GD 6 and 21, mean maternal body weight gain was reduced by 10% (not statistically significant) compared to the control group. Mean maternal corrected body weight change (maternal body weight change between GD 0 and 21 independent of the uterine weight at cesarean section) was reduced by 20% ($p \leq 0.05$) compared to the control group.

At cesarean section, a 3% reduction in mean fetal body weight (combined and per sex), compared to controls (not statistically significant), although means values remained within the range of in-house historical control data.
At the external and visceral fetal examinations, there were no treatment-related variations.
At the skeletal fetal examination, an overall evidence of delayed ossification suggestive of a slight delayed development secondary to maternal toxicity was observed. The changes consisted of an increased incidence of 4 variations at the fetal and litter levels: "Frontal (uni/bi) and/or parietal (uni/bi) and/or interparietal: incomplete ossification", "7th cervical centrum: unossified", "Forepaw(s): 3rd and/or 4th proximal phalanx: unossified" and "1st metatarsal: unossified". In addition, the incidence was outside the range of in-house HCD at the fetal and litter levels for two of these variations ("Frontal (uni/bi) and/or parietal (uni/bi) and/or interparietal: incomplete ossification" and "Forepaw(s): 3rd and/or 4th proximal phalanx: unossified").
At 12 and 3 mg/kg/day, there was no treatment-related effect on any of the parameters assayed.

In conclusion, the dose level of 12 mg/kg/day was considered to be a No Observed Effect Level (NOEL) in terms of maternal toxicity and in terms of fetal toxicity.

MATERIALS AND METHODS

I. MATERIALS

A. Test Material: Propineb Technical

Description:	Light yellow solid
Lot/Batch:	EDFU91-415
Purity:	80.4%
CAS:	12071-83-9
Stability of test compound:	Stable at room temperature for the study duration

B. Vehicle and or positive control: 0.5% aqueous methylcellulose 400

C. Test Animals

Species:	Rat
Strain:	Crl:CD (SD) Sprague-Dawley
Age:	11-13 weeks (females)
Weight:	Females: 247 - 329 g
Source:	[redacted] Laboratories, [redacted], Germany.
Acclimation period:	At least 10 days
Diet:	A04C-10 pelleted rodent diet from [redacted]., *ad libitum.*
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum.*
Housing:	Individual housing of pregnant females in suspended stainless steel wire mesh cages.

Environmental conditions

Temperature:	20 – 24 °C
Humidity:	40 - 70 %
Air change:	10 to 15 air changes per hour
Photoperiod:	12 h dark/ 12 h light (7 am- 7 pm)

I. STUDY DESIGN

1. **In life dates:** the experimental in life period was from 23 May 2013 to 4 July 2013. The study was carried out at the Toxicology Research Centre of bayer CropScience, [redacted] (France).

2. **Mating**
One hundred and twenty adult nulliparous female rats were obtained from the supplier. Females were mated on a one-to-one basis with stock males of the same strain and same supplier. Each morning following pairing, those female rats showing spermatozoa in a vaginal smear or sperm plug *in situ* were considered as pregnant animals. The day where evidence of mating was found, was designated as gestation Day 0 (GD 0).

3. **Animal assignment and treatment**
The females were assigned to control and treated groups using a body weight procedure for each day of pairing, a computerized randomization procedure (Pristima, version 6.3.2 build 17, Xybion Corp.) for each day of pairing. If possible, those females having been paired with the same male were not allocated to the same group. The acceptable body weight was ±20% of the mean body weight on the day of randomization. Body weight means were checked after the mating period to ensure similar means among all groups. Permanent identification numbers were assigned to animals within each group. Each animal was identified by a cage card and an ear tag bearing a unique number. The dose groups are indicated in the below table.

Table 5.6.2-1 Study design

Group	Test Substance	Dose levels (mg/kg/day)	Concentrations* (g/L)	Volume (mL/kg)	Number of animals
1	Vehicle	0	0	10	23
2	Propineb (AE F074263)	3	0.3	10	23
3		12	1.2	10	23
4		48	4.8	10	23

The range of doses has been selected in agreement with the Sponsor Representative and based on results obtained in a range-finding study where pregnant rats received Propineb (AE F074263) at 0, 10 and 40 mg/kg/day from GD 6 to 20 (M-456814-01-1). In this study, a dose level of 40 mg/kg/day caused slight maternal toxicity as evidenced by reduced mean

maternal body weight gain between GD 6 and 8 (-57% compared to controls, p.0.05) and overall mean maternal body weight gain between GD 6 and 21 (-9% compared to controls, not statistically significant). Mean food consumption was also slightly decreased between GD 6 and 8 (-12% compared to the controls, p.0.05). At this dose level, no treatment-related changes were noted for litter parameters or at the external and skeletal fetal examinations. A dose level of 10 mg/kg/day did not induce any treatment-related maternal or fetal effects.

4. Diet preparation and analysis

The appropriate amount of test item was suspended (w/w) extemporaneously each day in an aqueous solution of methylcellulose 400 ([redacted], France) at 0.5%, protected from light and stored at ambient temperature until use. The suspensions were mixed continuously before and during dosing with an electromagnetic stirrer.

Homogeneity of the suspensions was checked on the second and sixteenth formulations (F2 and F16) for all concentrations. The mean values obtained from the homogeneity check were used as measured concentrations of the second and sixteenth formulations. In addition, concentrations of the tenth and twenty-third formulations (F10 and F23) used in the study were checked. Data were recorded and analyzed using Empower 3 (Build 3471).

Since test item suspensions were prepared extemporaneously, no analysis for stability was performed.

Homogeneity and concentration analysis: The mean concentrations of the 4 formulations checked ranged between 91 and 96% of nominal concentrations, which was within the in-house target range of 90 to 110% of nominal concentration.

Individual checks for homogeneity ranged between 67 and 102% of nominal concentrations. Concentrations below the in-house target range of 90 to 110% were noted in formulation F2 at the three dose levels. As this was observed only for the formulation F2 and as the mean concentrations of the 4 formulations checked were within the in-house target range of 90 to 110%, it was considered to have no impact on the reliability of the study.

5. Dosage administration

All doses were administered once daily, from GD 6 to 20 inclusive, orally, by gavage, in a volume of 10 mL/kg body weight/day. Dose volumes were calculated on the basis of the animal's most recently recorded body weight. Control animals received an equivalent volume of vehicle alone (aqueous solution of 0.5% methylcellulose 400).

The suspensions were mixed continuously before and during treatment with an electromagnetic stirrer. They were stored at approximately 5°C (±3°C) when not in use.

III. METHODS – MATERNAL OBSERVATIONS AND EVALUATIONS

A. Observations

All clinical signs were recorded for individual animals. All animals were examined daily from GD 0 through GD 21.

All cages were checked for dead or moribund animals twice daily, once in the morning and again in the afternoon (except at weekends and public holidays when checking was carried out once daily).

B. Body weight and food consumption

Body weights were recorded on GD: 0, 6, 8, 10, 12, 14, 16, 18 and 21.
Full feeder weights were measured on GD: 1, 6, 8, 10, 12, 14, 16 and 18.
Empty feeder weights were measured on GD: 6, 8, 10, 12, 14, 16, 18 and 21.
From these records the mean daily consumption was calculated. Food spillage was also noted.

C. Cesarean sections

Scheduled sacrifice

On GD 21, all surviving females were sacrificed by inhalation of carbon dioxide, for examination of uterine content. Each female was first subjected to macroscopic examination of the visceral organs. The liver of all pregnant females was weighed.
All maternal, litter and fetal data were recorded without knowledge of treatment group.
The reproductive tract was weighed (gravid uterine weight), except for animals showing total litter resorption, dissected out and the following parameters recorded:

1. Number of corpora lutea,
2. Number of implantations,
3. Number of resorptions (classified as early and late),
4. Number of live and dead fetuses,
5. Sex of live fetuses,
6. Individual weights of live fetuses.

Uterine horn(s) without visible implantations were immersed in a 10% solution of ammonium sulfide according to the Salewski method (1964), in order to visualize any sites which were not apparent. Then, tissues and carcass of dams were discarded.

Intra-uterine death was classified according to Gleich and Frohberg (1977) as:

- Early resorptions: macroscopic discrimination between fetal residues and placental material not possible,
- Late resorptions: distinct macroscopic discrimination between fetal and placental remains possible.

Dead fetuses: defined as dead conceptuses showing distinct digits on fore and hind-paws.

IV. METHODS – FETAL EXAMINATIONS

All fetal examinations were recorded without knowledge of treatment group.
All the live fetuses were sacrificed by subcutaneous injection (0.02 mL/fetus) of Dolethal® (18.22 g/100 mL, sodium pentobarbital) and subjected to an external examination. Approximately half of the live fetuses from each litter were immersed in Bouin's fluid for subsequent internal examination following free-hand sectioning. The remaining half were skinned, eviscerated and then placed in absolute ethanol before staining with alizarin red S and alcian blue according to a modified staining

technique for skeletal examination of bones and cartilages. All specimens were archived.

Structural deviations were classified as follows:
Malformations:
A permanent structural change that is likely to adversely affect the survival or health.
Variations:
A change that occurs within the normal population under investigation and is unlikely to adversely affect survival or health (this might include a delay in growth or morphogenesis that has otherwise followed a normal pattern of development).

Where appropriate color photographs were prepared showing a representative sample of fetal findings and are retained in the study file.

D. Data Analysis

1. Statistical Analysis
The following variables were analysed:
Maternal endpoints: maternal body weight changes, corrected body weight change, carcass, liver and uterus weights, food consumption

Litter based endpoints: number of corpora lutea, number of implantation sites, number of resorptions (early, late, total), pre- and post-implantation loss percentages, fetal body weight (combined sexes and per sex).
Fetal endpoints: fetal sex (male vs. female, described in terms of percent male fetuses) , fetal death status (live vs. dead, described in terms of number of dead fetuses, and number of litters with dead fetuses), selected fetal observation data (external, visceral or skeletal)

Mean and standard deviation for all maternal, litter and fetal parameters were calculated for each group.
Statistical analyses were performed in the first instance for all pregnant females that survived to the scheduled sacrifice. Where relevant, statistical analyses were conducted for all pregnant females with live fetuses only or including pregnant females that did not survive to the scheduled sacrifice
Body weight changes calculated according to interval periods, calculated corrected body weight changes, carcass, liver and uterus weights, number of corpora lutea, number of implantation sites and number of resorptions (early, late) pre- and post-implantation loss percentages were evaluated using the Bartlett Test and then depending on the calculated statistical difference using either the ANOVA or Kruskal-Wallis Test. If the difference was significant then the Dunnett and Dunn Test, respectively, were used.

Average food consumption calculated according to interval periods, was evaluated using the Bartlett Test. If the differences were not significant the parameters were further analysed using the ANOVA and Dunnett test. If the differences were significant then the data were analysed using the ANOVA followed by the Dunnett test (if the differences were not significant) and using the Kruskal-Wallis Test followed by the Dunn test (if the differences were significant).

Fetal body weight (combined sexes and per sex) were evaluated using the Levene Test. If the differences were not significant the parameters were further analysed using the ANOVA and Dunnett test. If the differences were significant then the data were transformed and analysed again with the Levene Test. The parameters were analysed using the ANOVA followed by the

Dunnett test (if the differences were not significant) and using the Kruskal-Wallis Test followed by the Dunn test (if the differences were significant).

For fetal sex (male vs. female fetuses) and fetal death status (live vs. dead fetuses) endpoints, control group and each exposed group were compared using the Chi-square test for fetal sex parameter, using the Fisher Exact test (2-sided) for fetal death status parameter. Death status was analyzed both using the fetus as the statistical unit and using the litter as the statistical unit. Group means were compared at least at the 5% level of significance.

In addition, selected fetal observation data (external, visceral or skeletal) of control group and each exposed group were compared using the Fisher Exact test (2-sided).

Statistical analyses, as described below, were performed on the following parameters using Pristima, version 6.3.2 build 17, Xybion Corp., except for fetal body weight and selected fetal observation data (external, visceral or skeletal) which were anlysed using SAS Software Release 9.2.

2. Indices

Data from non-pregnant animals were not included in group mean calculations of any maternal parameters. Data from pregnant animals that died or were prematurely sacrificed were included in body weight and food consumption calculations up to the last day data was recorded.

The following indices (endpoints) were calculated for each dam.

- Maternal body weight (BW) changes for interval periods were calculated as follows:
  Body Weight Changes (GD 6 to 8) = BW on GD 8 – BW on GD 6

- Corrected body weight change (CBWC) was calculated as follows:
  CBWC = (BW on GD 29 – BW on GD 6) – (gravid uterine weight)

- Average food consumption (FC) was calculated during intervals in g/day as follows:

$$\text{Food Consumption (GD 6 to 8)} = \frac{\text{FC GD 8}}{2}$$

The following endpoints were calculated for each litter (dam).

- Pre-implantation loss was calculated per litter as a percentage according to the formula:

$$\left(\frac{\text{Number of corpora lutea} - \text{Number of implantations}}{\text{Number of corpora lutea}}\right) \times 100$$

- Post implantation loss was calculated per litter as a percentage according to the formula:

$$\left(\frac{\text{Number of implantations} - \text{Number of live fetuses}}{\text{Number of implantations}}\right) \times 100$$

- Number of live fetuses was calculated as the sum of number of live fetuses per litter
- Number of dead fetuses was calculated as the sum of number of dead fetuses per litter
- Percentage of dead fetuses per litter was calculated according to the formula:

$$\frac{\text{Number of dead fetuses}}{\text{Total number of fetuses}} \times 100$$

- Percentage of male fetuses per litter was calculated according to the formula:

$$\frac{\text{Number of live male fetuses}}{\text{Total number of live male and female fetuses}} \times 100$$

- Mean fetal body weight per litter was calculated according to the formula:

$$\frac{\text{Sum of individual weights of live fetuses}}{\text{Number of weighed live fetuses}}$$

The following endpoints were calculated per group.

- Mean fetal body weight was calculated according to the formula:

$$\frac{\text{Sum of mean fetal body weight of live fetuses per litter}}{\text{Number of litters with weighed live fetuses}}$$

- Mean fetal body weight per sex was calculated according to the formula (example for male fetuses):

$$\frac{\text{Sum of mean fetal body weight per litter of live male fetuses}}{\text{Number of litters with weighed live male fetuses}}$$

For external, visceral and skeletal fetal findings, the percentage of fetuses affected per group for a given parameter was calculated using the following formula:

1. Percentage of fetuses affected per group:

$$\frac{\text{Sum of live fetuses affected}}{\text{Number of live fetuses examined}} \times 100$$

- The percentage of litters affected per group was calculated using the following formula:

$$\frac{\text{Sum of litters with live fetuses affected}}{\text{Number of litters with live fetuses examined}} \times 100$$

- RESULTS AND DISCUSSION

1. MATERNAL OBSERVATIONS

- Mortality

There were no treatment-related mortalities during the study.
One low dose female (2F1739) was prematurely sacrificed on GD 12 due to markedly reduced body weight and food consumption which were attributed to a dental abnormality.

- Clinical observations

There were no treatment-related clinical signs during the study.

- Body Weights

At 48 mg/kg/day, mean body weight gain all pregnant females surviving to terminal sacrifice was reduced by 51% between GD 6 and 8 ($p \leq 0.01$) and by 12% between GD 18 and 21 ($p \leq 0.05$), when compared to controls. Throughout other intervals, mean maternal body weight gain was similar to controls. Overall between GD 6 and 21, mean maternal body weight gain was reduced by 10% (not statistically significant) compared to the control group. Mean body weight was marginally affected with a maximum decrease of 4% on GD 21 compared to controls. Moreover, mean maternal corrected body weight change (maternal body weight change between GD 0 and 21 independent of the uterine weight at cesarean section) was reduced by 20% ($p \leq 0.05$) compared to the control group.

At 12 mg/kg/day, mean maternal body weight parameters were unaffected by treatment. The decrease in body weight change noted between GD 6 and 8 (-39% compared to controls) was considered not to be a treatment-related effect, as it was not statistically significant and because the value remained within the range of in house historical control data (HCD).
There was no effect on mean maternal body weight parameters at 3 mg/kg/day. The slight decrease in mean body weight gain noted between GD 14 and 18 (12%, $p \leq 0.05$) in comparison to the control group was considered to be incidental as it was observed in isolation and with no dose-relationship.

Table 5.6.2-2 Maternal body wheights

Mean (± SD) maternal body weight gain (g) and corrected body weight gain of all pregnant females				
Interval	**Dose level of Propineb (AE F074269) in mg/kg/day**			
	0	3	12	48
Number of dams (pregnant surviving to terminal sacrifice)	21	21	23	23
Pretreatment, GD 0-6:	40.4 ± 7.45	38.0 ± 7.85	38.5 ± 6.72	35.5 ± 9.82
Treatment, GD 6-8:	7.2 ± 3.67	6.2 ± 3.96	4.4 ± 4.24	3.5** ± 3.95
Treatment, GD 8-10:	9.6 ± 4.33	9.7 ± 4.51	8.2 ± 4.08	10.2 ± 3.68
Treatment, GD 10-14:	20.9 ± 4.74	21.9 ± 6.42	21.9 ± 6.07	20.7 ± 4.54
Treatment, GD 14-18:	48.3 ± 7.37	42.5 * ± 9.26	47.2 ± 6.00	43.8 ± 6.44
Treatment, GD 18-21:	57.2 ± 8.19	52.7 ± 11.10	56.8 ± 6.95	50.6 * ± 9.05
Treatment, GD 6-21	143.4 ± 18.22	132.6 ± 24.86	138.5 ± 16.77	128.7 ± 16.27
Corrected body weight gain, GD 0-21:	71.3 ± 15.43	73.9 ± 16.72	69.6 ± 17.85	56.8 * ± 16.70

* Statistically different ($p \leq 0.05$) from the control.
** Statistically different ($p \leq 0.01$) from the control.

- **Food consumption**

There was no effect on mean food consumption at any dose level.

- **Gross pathology and organ weights**

At necropsy of dams, there were no treatment-related macroscopic findings. The macroscopic findings observed occurred in one animal only or with no dose-relationship and were thus considered to be incidental.

There was no effect on mean maternal liver weight at 48, 12 or 3 mg/kg/day.

2. CESAREAN SECTION DATA

The pregnancy rate was 96% in the control and low dose groups, and 100% in the mid and high dose group.
At 48 mg/kg/day, mean fetal body weight (combined and per sex) was slightly reduced by 3% compared to controls (not statistically significant), although mean values remained within the range of in-house HCD. Other litter parameters including the number of live fetuses, number of implant sites per dam, percentages of pre- and post-implantation losses, number of early and late

resorptions, fetal death status, percentage of male fetuses and fetal body weight for combined and separate sexes were unaffected by treatment.

At 12 and 3 mg/kg/day, litter parameters were unaffected by treatment. The statistically significant decrease in the number of implantation sites and increase in pre-implantation loss noted in the low dose group in comparison to controls was incidental (mainly attributable to a unilateral pregnancy for 2 dams).

Table 5.6.2-3 Observations at cesarean section

Observation	Dose level of Propineb (AE F074263) in mg/kg/day				HCD[e]
	0	**3**	**12**	**48**	
Maternal data:[a]					
No. Animals assigned	23	23	23	23	NA
No. Animals pregnant	22	22	23	23	NA
Pregnancy rate, %	96	96	100	100	NA
No. Animals non-pregnant	1	1	0	0	NA
Maternal wastage					
No. intercurrent death or sacrifice (total)	0	1	0	0	NA
No. intercurrent death or sacrifice (pregnant)	0	1	0	0	NA
No. premature delivery	0	0	0	0	NA
No. intercurrent death or sacrifice (non pregnant)	0	0	0	0	NA
Uterine data at scheduled sacrifice:[b]					
Total No. corpora lutea[c] **Corpora lutea / dam**	386 17.5 ± 2.32	373 17.8 ± 2.68	401 17.4 ± 3.34	414 18.0 ± 2.30	NA 15.74-17.86
Total No. implantations[c] **Implantations / dam**	351 16.0 ± 1.73	284 13.5** ± 3.33	347 15.1 ± 1.53	355 15.4 ± 2.02	NA 14.38-16.04
Total No. litters[c]	22	21	23	23	NA
Total No. live fetuses[c] **Live fetuses / dam**[c]	329 15.0 ± 1.81	270 12.9 ± 3.44	334 14.5 ± 1.44	341 14.8 ± 1.90	NA 13.52-14.95
Total No. dead fetuses **Dead fetuses / dam**[c] **(%)**	1 0.0 ± 0.21	0 0.0 ± 0.00	0 0.0 ± 0.00	0 0.0 ± 0.00	NA 0.000-0.043
Total No. early resorptions[c] **Total No. late resorptions**[c] **Early resorptions / dam** **Late resorptions / dam** **Litters with total resorptions**[c,d]	20 1 0.9 ± 1.06 0.0 ± 0.21 0	14 0 0.7 ± 0.86 0.0 ± 0.00 0	12 1 0.5 ± 0.79 0.0 ± 0.21 0	13 2 0.6 ± 0.84 0.1 ± 0.42 0	NA NA 0.474-2.042 0.000-0.217 NA
Mean fetal weight, combined (g)	5.59 ± 0.321	5.51 ± 0.259	5.48 ± 0.332	5.40 ± 0.300	5.30-5.57
Mean fetal weight, males (g)	5.74 ± 0.353	5.73 ± 0.247	5.64 ± 0.360	5.56 ± 0.274	5.45-5.70
Mean fetal weight, females (g)	5.43 ± 0.321	5.33 ± 0.318	5.32 ± 0.335	5.25 ± 0.333	5.16-5.42
Sex ratio (% males)	51.9 ± 12.81	48.1 ± 13.90	49.1 ± 14.82	50.5 ± 9.49	46.5-53.5
Preimplantation loss per litter (%)	8.63 ± 6.393	23.36** ± 17.034	11.88 ± 10.865	13.70 ± 10.887	3.32-11.88
Postimplantation loss per litter (%)	6.16 ± 6.839	5.20 ± 7.683	3.60 ± 4.967	3.98 ± 5.895	3.95-13.15

c Statistical analysis was not conducted on this endpoint.
d Also includes litters with dead fetuses only or dead fetuses and resorptions.
e In-house Historical Control Data range (lowest – highest) of main uterine parameters
NA Not applicable.
* Statistically different (p <0.05) from the control.
** Statistically different (p <0.01) from the control.

3. DEVELOPMENTAL TOXICITY / FETAL EVALUATION

1. Fetal malformations

There were no treatment-related malformations noted at the fetal examination at the three dose levels.

Nine fetuses were observed with malformations. Since they were evenly distributed between the groups and as the malformations occurred as isolated cases in each treated group (the 2 cases of retinal fold were observed in the controls), they were considered to have occurred spontaneously.

Table 5.6.2-4 Incidence of fetal malformation

Dose level of Propineb (AE F074263) (mg/kg/day)	0	3	12	48	0	3	12	48
OBSERVATIONS	Number of litters examined				Number of fetuses examined			
	22	21	23	23	329	270	334	341
	Number of litters affected (Percentage of litters affected)				Number of fetuses affected (Percentage of fetuses affected)			
Occurrence of fetal malformations	3 (13.6)	2 (9.5)	1 (4.3)	2 (8.7)	3 (0.9)	2 (0.7)	1 (0.3)	3 (0.9)

2. External observations

There were no treatment-related variations noted at the external fetal examination at the three dose levels.

The external variation noted in one mid dose fetus (subcutaneous edema on abdomen and neck) occurred as an isolated finding, was observed with no dose-relationship and its incidence at the fetal and litter levels was within the range of in-house HCD. It was thus considered to have occurred spontaneously.

3. Visceral observations

The incidence of remnant thymic present (uni/bilateral) was higher than the controls both at the fetal and litter levels in the mid and low dose groups. As the increased incidences were observed with no dose-relationship and as the incidences at the fetal and litter levels remained within the range of in-house HCD, this variation was considered not to be treatment-related.

Table 5.6.2-5 Visceral fetal observations

Propineb (AE F074263)	0	3	12	48	HCD	0	3	12	48	HCD
OBSERVATIONS	Number of litters examined									
	22	21	23	23		160	129	161	165	
	Number of litters affected (Percentage of litters affected)					Number of fetuses affected (Percentage of fetuses affected)				
Variations										
Thymic remnant present (uni/bi). #	3 (13.6)	8 (38.1)	7 (30.4)	3 (13.0)	(5.3-40.9)	6 (3.8)	10 (7.8)	10 (6.2)	3 (1.8)	(2.5-10.3)

Statistical analysis was conducted on this observation

4. Skeletal observations

There was an overall evidence of delayed ossification was observed only in the high dose group.

At 48 mg/kg/day, treatment-related changes consisted of an increased incidence of 4 spontaneous variations at the fetal and litter levels (see table below), which were observed in a dose-related manner and for which the difference from controls was statistically significant at the fetal level. In addition, the incidence was outside the range of in-house HCD at the fetal and litter levels for two of these variations ("Frontal (uni/bi) and/or parietal (uni/bi) and/or interparietal: incomplete ossification" and "Forepaw(s): 3rd and/or 4th proximal phalanx: unossified").

At 12 mg/kg/day, there were no treatment-related variations. The incidence of the variation "Frontal (uni/bi) and/or parietal (uni/bi) and/or interparietal: incomplete" was slightly higher than the controls at the fetal and litter levels and was outside the HCD. However, as this change was observed in isolation at this dose level and because the increased incidence at the fetal and litter levels was of low magnitude and was not statistically significantly different from the controls, it was considered to be incidental.

At 3 mg/kg/day, there were no treatment-related variations. The incidence of the variation "1st metatarsal: unossified" was statistically significantly higher than the controls at the litter and fetal levels. However, as this finding was observed with no dose-relationship and as the incidence at the fetal and litter levels remained within the range of in-house HCD, it was considered to be incidental.

Table 5.6.2-6 Skeletal fetal variations: treatment-related effects (highlighted in yellow)

Propineb (AE F074263) (mg/kg/day)	0	3	12	48	HCD	0	3	12	48	HCD
OBSERVATIONS	Number of litters examined					Number of fetuses examined				
	22	21	23	23		169	141	173	176	
	Number of litters affected (Percentage of litters affected)					Number of fetuses affected (Percentage of fetuses affected)				
Frontal (uni/bi) and/or parietal (uni/bi) and/or interparietal : incomplete ossification.	3 (13.6)	2 (9.5)	5 (21.7)	6 (26.1)	(0.0-12.0)	3 (1.8)	2 (1.4)	9 (5.2)	14 (8.0)	(0.0-2.2)
7th cervical centrum : unossified.	3 (13.6)	5 (23.8)	6 (26.1)	7 (30.4)	(0.0-41.7)	4 (2.4)	9 (6.4)	11 (6.4)	15* (8.5)	(0.0-12.2)
Forepaw(s): 3rd and/or 4th proximal phalanx : unossified.	0 (0.0)	2 (9.5)	2 (8.7)	3 (13.0)	(0.0-9.5)	0 (0.0)	3 (2.1)	2 (1.2)	7 (4.0)	(0.0-3.8)
1st metatarsal : unossified.	0 (0.0)	4 (19.0)	2 (8.7)	2 (8.7)	(0.0-36.0)	0 (0.0)	4 (2.8)	2 (1.2)	8 (4.5)	(0.0-10.1)

The incidence of the skeletal variations listed in bold in the following table was above the range of in-house HCD. However, the increased incidences were observed with no dose-relationship and were thus considered to be incidental. Furthermore, the incidence of the findings selected for statistical analysis was not statistically significantly different from the controls.

Table 5.6.2-7 Summary of observed skeletal fetal variations

Dose level of Propineb (AE F074263) (mg/kg/day)	0	3	12	48	HCD	0	3	12	48	HCD
OBSERVATIONS	Number of litters examined					Number of fetuses examined				
	22	21	23	23		169	141	173	176	
	Number of litters affected (Percentage of litters affected)					Number of fetuses affected (Percentage of fetuses affected)				
Anterior and/or posterior fontanelles : enlarged.	0 (0.0)	1 (4.8)	1 (4.3)	1 (4.3)	(0.0-4.8)	0 (0.0)	**2 (1.4)**	2 (1.2)	1 (0.6)	(0.0-1.3)
Supraoccipital : incomplete ossification.	0 (0.0)	1 (4.8)	2 (8.7)	0 (0.0)	(0.0-8.7)	0 (0.0)	1 (0.7)	**3 (1.7)**	0 (0.0)	(0.0-1.2)
Hyoid centrum : incomplete ossification.	4 (18.2)	3 (14.3)	5 (21.7)	3 (13.0)	(0.0-23.7)	4 (2.4)	4 (2.8)	**16 (9.2)**	6 (3.4)	(0.0-3.9)
5th and/or 6th sternebrae : incomplete ossification.	10 (45.5)	10 (47.6)	**13 (56.5)**	12 (52.2)	(32.0-52.4)	26 (15.4)	21 (14.9)	23 (13.3)	17 (9.7)	(5.7-16.8)
5th sternebra : unossified.	1 (4.5)	**4 (19.0)**	3 (13.0)	2 (8.7)	(0.0-17.4)	2 (1.2)	**5 (3.5)**	3 (1.7)	2 (1.1)	(0.0-2.3)
Ribs (uni/bi) : wavy (slightly).	0 (0.0)	0 (0.0)	**3 (13.0)**	0 (0.0)	(0.0-4.8)	0 (0.0)	0 (0.0)	**3 (1.7)**	0 (0.0)	(0.0-1.3)
14th thoracic rib (uni) : short.	1 (4.5)	3 (14.3)	1 (4.3)	0 (0.0)	(0.0-14.3)	1 (0.6)	**4 (2.8)**	1 (0.6)	0 (0.0)	(0.0-2.5)
At least one thoracic centrum : bipartite / dumbbell cartilage.	0 (0.0)	0 (0.0)	**2 (8.7)**	0 (0.0)	(0.0-4.8)	0 (0.0)	0 (0.0)	**2 (1.2)**	0 (0.0)	(0.0-0.7)

All the other changes in the incidence of skeletal variations at the fetal and/or litter levels were considered to be incidental, as they were observed in isolation, or with no dose-relationship and/or remained within the range of in-house HCD. Furthermore, the incidences of the findings selected for statistical analysis were not statistically significantly different from the controls.

CONCLUSIONS

In conclusion, a dose level of 48 mg/kg/day Propineb (AE F074263) administered to the pregnant Sprague-Dawley rat by oral gavage was considered to be a Lowest Observed Adverse Effect Level (LOAEL) in terms of maternal toxicity, as evidenced by effects on mean body weight parameters and fetal weights, and in terms of fetal toxicity as a slight retarded ossification was noted for a few elements of the skeleton. A dose level of 12 mg/kg/day was considered to be a No Observed Effect Level (NOEL) in terms of maternal toxicity and in terms of fetal toxicity.

CA 5.7 Neurotoxicity studies

The neurotoxic potential of propineb was investigated in the rat after single and subchronic exposure. The studies were submitted in the addendum to the monograph. In addition a developmental neurotoxicity study was carried out upon request to US EPA.

Both studies were carried out according to OECD guideline 424 (1997) and in compliance with GLP requirements.

In the acute neurotoxicity study, a single administration of propineb at 2000 mg/kg provoked mortality and signs of severe toxicity, like red nasal stain, urine stain, pale eyes, pale body, abnormal gait (described as flat-footed with short steps), decreased rearing in the open field and decreased forelimb grip strength. At 500 mg/kg bw urine stain was observed in a female. The study NOEL was set at 200 mg/kg bw and the study NOAEL is considered to be 500 mg/kg bw/day as the presence of sting in the urine of one female, in absence of correlated findings is not evidence of an adverse effect..

In the subchronic neurotoxicity study Wistar Hanover rats were tested. Dietary administration of propineb up to the highest dose level of 300 ppm did not provoke any effect in the male rats. In females, compound-related signs included clinical signs (flat-footed, short stepped gait affecting the hind limbs), reduced motor activity and reduced body weight. Histopathology examination showed skeletal muscle atrophy in both the forelimbs and hind limbs in females receiving 300 ppm.
The study NOAEL was 300 ppm for males (equivalent to 17.11mg/kg bw/day) and 150 ppm for females (equivalent to 9.36 mg/kg bw/day).

No effects were observed in the developmental neurotoxicity study up to the high dose level tested. The dose selection for the developmental neurotoxicity study was based on the results of the rat subchronic and subchronic neurotoxicity studies, as well as on the available information on the neurologic effects observed in the rat toxicity studies, which showed effects from dose levels approaching 200 ppm or 20 mg/kg bw/day. Therefore, for the DNT study the following dose levels were selected: 0, 30, 60 and 180 ppm. This dose range is identical to that of the rat reproduction and fertility effects study. The two studies were performed contemporary in the same laboratory, using animals of the same strain and from the same breeder and administering the same diets. However in the DNT study no maternal signs of toxicity were observed at 180 ppm, a dose which produced up to 11% body weight decrease in adult females. The dose of 180 ppm (equivalent to 12.3 mg/kg bw/day) was the NOAEL for both maternal and offspring toxicity.

Table 5.7-1 Summary of Neurotoxicity studies

Study/Reference	**NOAEL (mg/kg bw/day)**	**Effects at LOAEL and higher doses**
Acute Neurotoxicity Wistar Rat. Gavage in both sexes 0, 200, 500 or 2000 mg/kg bw/day 0.48 [redacted].; 2004. M-075420-01-2 **Submitted in the addendum to the monograph**	500 (♂) & (♀)	<u>2000 mg/kg bw/day - Both sexes</u> Mortality in 3 females. Abnormal gait (persisted to day 14) and decreased forelimb grip strength in
Subchronic Neurotoxicity Wistar Rat. 0, 30, 150, or 300 ppm 0, 1.45, 7.63, 17.11 mg/kg/day (♂) 0, 1.90, 9.36, 21.21 mg/kg/day (♀) [redacted] 2004. M-066913-01-2 **Submitted in the addendum to the monograph**	17.11 (♂) – 9.36 (♀)	<u>21.21 mg/kg bw/day - Females</u> ↓ bodyweight and food consumption ↓ grip strength and rears, atrophy of skeletal muscle. No effects in males
Developmental Neurotoxicity Wistar Rat. 0, 30, 60, or 300 ppm 0, 2.3, 4.4 and 12.3 mg/kg/day (♀) [redacted]; 2010. M-370251-01-1 **New study**	12.3	No effects up to the highest dose tested

CA 5.7.1 Neurotoxicity studies in rodents

Report:	[redacted]t; [redacted];2010;M-370251-01
Title:	A developmental neurotoxicity study with technical grade propineb in Wistar rats
Report No:	08-D72-OV
Document No:	M-370251-01-1
Guidelines:	**U.S. EPA, OPPTS 870.6300 and OECD Test Guideline 426 Health Canada PMRA DACO No. 4.5.14; The exception is that the homogeneity and stability of the test substance in the diet were verified after the study was completed, due to unanticipated challenges associated with developing the analytical method. This is not believed to have had an affect on the outcome or interpretation of the study, since the results verified the homogeneity and stability of the test substance in the feed, under the conditions that were used in this study.**
GLP/GEP:	**yes**

Executive summary

Technical grade propineb was administered via the diet from gestation Day (GD) 6 through lactation Day (LD) 21 to mated female Wistar rats at nominal concentrations of 0, 30, 60 or 180 ppm with adjustment during lactation to maintain a more consistent dosage throughout the period of exposure.

The average mean daily intake of the test substance (mg propineb/kg body wt/day) based on the average dietary consumption for the last two weeks of gestation and three weeks of lactation at nominal dietary concentrations of 30, 60 or 180 ppm, respectively, was 0, 2.3, 4.4 and 12.3 mg/kg/day.
All test diets (including control) were provided for *ad libitum* consumption throughout the study, except during neurobehavioral testing. The Parental (P)-generation females were evaluated by cage-side and detailed clinical observations, body weight, food consumption and reproductive endpoints. On postnatal Day (PND) 4, litters with a minimum of seven pups, including at least three per sex, were culled to yield, as closely as possible, four males and four females. Subsets of surviving offspring, representing 19 - 20 litters per dietary level, were subjected to evaluation using the following observations and measurements - detailed clinical observations and a detailed observational battery, pupil response, surface righting, preputial separation or vaginal patency, body weight, food consumption, automated measures of activity (figure-eight maze), auditory startle habituation, learning and memory (passive avoidance after weaning and a water maze task beginning on PND 60±2 days) and an ophthalmic examination.
Neural tissues were collected from 10 rats/sex/dietary level (representing 20 litters) on PND 21 (brain only) and at study termination (approximately 75 days of age) for microscopic examination and morphometry.
There were no treatment-related effects in the dams at any dose level.
There were no test substance-related findings in the offspring at any dose level.
The maternal and developmental NOAELs were 180 ppm (equivalent to 12.3 mg/kg bw/day).

Material

Test Material: Technical grade Propineb
Description: White-yellow powder
Lot/Batch: EDFU711100
Purity: 82.3% (July 18, 2007); Expiration Date January 19, 2009
81.8% (April 3, 2009); Expiration Date April 3, 2011
CAS: 9016-72-2
Stability of test compound: Confirmed by analytical methods

Vehicle and/or positive control: The test substance was administered via the diet. Acetone served as a solvent in the diet preparation process and was allowed to evaporate prior to administration. The control diet was prepared the same way, excluding the test substance.
The study did not include positive controls, but references are made to previous studies to serve that purpose.

Test animals:
Species: Rat
Strain: Wistar Crl:WI(Han)
Age: At least 12 (females) and 15 (males) weeks of age at co-housing (based on calculated birth date provided by vendor).

Weight at dosing:	191.1 – 248.3 g range for females (± 20% weight determination); Males had no specified weight requirements.
Source:	[redacted]Laboratories, Inc. [redacted], North Carolina (USA)
Acclimation period:	at least 7 days
Diet:	Purina Laboratory Rodent chow #5002, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were individually housed in stainless steel wire mesh cages
Environmental conditions –	
Temperature:	18-26°C
Humidity:	30 to 70%
Air changes:	Approximately 12 changes per hour
Photoperiod:	Alternating 12-hour light and dark cycles

Study design:

In life dates:

The study was carried out at Bayer CropScience LP, Toxicology, [redacted], Kansas (USA) from January 12 to 24 April 2009.

Animal assignment and treatment

Approximately 120 male and 120 female (nulliparous and nonpregnant) Wistar rats were placed on study to provide a minimum of 20 acceptable litters per dietary level. Four dose groups (approximately 30 females/dietary level) were administered the test substance in the diet at nominal concentrations to of 0, 30, 60 or 180 ppm.

A pilot study was conducted to determine whether there was evidence of exposure of the offspring by the transfer of propineb through the milk during lactation. In this study, 12 timepregnant Wistar rats (provided eight suitable litters - six treated and two control dams) were exposed to a nominal concentration of 0 or 180 ppm propineb in the diet from gestation Day 6 through lactation Day 14, with adjustments in dietary level during lactation to maintain a more constant dosage (mg/kg/day) throughout exposure. Offspring from each litter were sacrificed on lactation Days 4 (culls), 10 or 14 (approximately six treated/sex/age, representing six litters at each age) to measure the concentration of propineb (or active metabolites) in the milk found in the pup's stomachs. The milk was collected into an appropriate container, pooled from each litter, divided into two samples and stored in a freezer (minimum -70°C) until analysis. Selected milk samples from control and treated PND 14 pups were analyzed with LC/MS/MS using selected reaction monitoring (SRM). The presence of the active metabolite Propylene Urea (PU) was found in the stomach contents of PND 14 pups, which demonstrated exposure of the offspring to the test substance through the milk.

In the main study, animals had not been previously treated and were at least 14 weeks (males) or 12 weeks (females) of age at cohousing. The adult males served only as "breeders" and, as such, were not exposed to the test substance or included in any tests. Mating was managed by co-housing one female with one male, for a maximum of five consecutive days, in suspended stainless-steel gang cages. All animals began cohabitation on the first day of mating by placing males and females together in suspended stainless-steel cages. Each morning during the co-habitation phase, the dams and cages

were examined for a vaginal plug and vaginal smears were taken and examined for the presence of sperm. The day on which insemination was observed in the vaginal smear was designated day 0 of gestation (GD 0) for that female. On day 0 of presumed gestation, the female was removed and housed individually in a plastic nesting cage. Typically, females that were not sperm positive were sacrificed without a necropsy examination. The treated feed was provided for consumption beginning on GD 6 and continuing through lactation day 21 (LD 21). A sample of each batch of feed mixed was taken and retained in the freezer until the study was complete and the analytical data deemed satisfactory. After day 21 of postnatal development, untreated feed was provided for consumption to all groups. A given batch of feed was available for *ad libitum* consumption for a period of one (GD 0- LD 21) or two (postweaning) weeks prior to changing, at which time any uneaten feed was collected and disposed of by incineration.

Diet preparation and analysis

Formulations were prepared weekly by mixing appropriate amounts of the test substance in the diet [redacted] Certified Rodent Diet 5002 in meal form and were stored at freezer (approximately -23.0°C) conditions.

Acetone served as a solvent in the diet preparation process and was allowed to evaporate prior to administration. The control diet was prepared the same way, excluding the test substance.

Dietary concentrations were not adjusted to correct for purity (percent active ingredient) in the test substance but were adjusted (reduced by 50%) during lactation, to maintain a more constant level of exposure (mg/kg/day) throughout the period of exposure. Dietary levels during gestation were 0, 30, 60 or 180 ppm and were then reduced during weeks 1-3 of lactation by 50% to offset the substantial increase in food consumption that is normally observed in dams during lactation (dietary levels during lacation were 0, 15, 30 and 90 ppm). The treated feed was provided for consumption beginning on GD 6 and continuing through lactation Day 21 with fresh feed provided every seven days. A sample of each batch of feed mixed was taken and retained in the freezer until the study was complete and the analytical data deemed satisfactory.

Table 5.7.1 –1 Study design – Maternal assignment

Test group	Concentration in diet (ppm)	Females assigned
1	**0**	**30**
2	**30**	**30**
3	**60**	**30**
4	**180**	**30**

After Day 21 of postnatal development, untreated feed was provided for consumption to all F1-generation animals that were retained on study. A given batch of feed was available for ad libitum consumption for a period of one (GD 0 - LD 21) or two (post-weaning) weeks prior to changing, at which time any uneaten feed was collected and disposed of.

Methods

A) Parental (F0) Generation

Observations

Following acclimation and continuing until animals were removed from the study, F0-generation males and females were observed (cage-side) for clinical signs at least once daily. A detailed evaluation of the dams for clinical signs with a physical examination was conducted once daily from the initiation of exposure (GD 6) through lactation day 21.

Body weight

Body weight and food consumption were measured once per week during gestation and lactation, as follows: Gestation days 6, 13 and 20 and lactation days 0, 7, 14 and 21. In addition, dams were also weighed on LD 4.

Food consumption and compound intake

Food consumption was measured once per week during gestation and lactation, as follows: Gestation days 6, 13 and 20 and lactation days 0, 7, 14 and 21. In addition, dams were also weighed on LD 4. Measurements of food consumption may have included consumption by the pups, especially during the third week of lactation. Fresh feed and clean feeders were provided weekly.

Ophthalmic examination

Pre-exposure and pre-terminal (week 12) ophthalmic examinations were conducted on study animals in a semidarkened room. The pupillary reflex was tested using a penlight or transilluminator and then a mydriatic agent was applied to each eye to dilate the pupil. After mydriasis, the conjunctiva, cornea and lens were examined with a slit lamp microscope (Kowa SL-2, Kowa Company, Ltd., Tokyo 103, Japan), and the vitreous humor, retina, choroid, and optic disc were examined using an indirect ophthalmoscope (All Pupil Indirect Ophthalmoscope, Keeler Instruments, Inc., Broomall, PA, USA) and a condensing lens.

Functional Observational Battery (FOB) and Motor Activity Testing

Animals that were presumed to be pregnant (approximately 30 per dietary level) were observed on GD 13 and GD 20 and a minimum 10 dams/dietary level that were maintained on study were also observed on LD 11 and LD 21 by an individual who was unaware of each animal's dose group assignment. This evaluation included observations in the home cage, during handling, and outside the home cage in an open field, using standardized procedures. This observational battery included, but was not limited to, assessments (with severity scoring) of lacrimation, salivation, piloerection, exophthalmia, urination, defecation, pupillary function, palpebral closure, convulsions, tremor, abnormal movements, unusual behaviors, posture and gait abnormalities.

Delivery and Culling

Each dam was evaluated daily for evidence of delivery from GD 20 to the completion of delivery, designated lactation day 0 (LD 0) for the dam and postnatal day 0 (PND 0) for the pups. Litter size (the number of pups delivered) and pup "status" at birth were recorded for each litter. If a dam delivered fewer than three pups per sex or if the litter size decreased to fewer than seven pups by PND 4, the dam and litter were sacrificed without necropsy examination. For litters that met the minimum

size requirements, the size of each litter was adjusted on PND 4 to yield, as closely as possible, four males and four females. Adjustments of litters were made by random selection of the pups using SAS [1] applications. If the number of male or female pups was less than four, a partial adjustment was made (e.g., three females and five males). If there were more than 23 acceptable litters for any dietary level, the surplus litters were sacrificed on PND 4 after weighing without routine necropsy. Culled dams and pups were sacrificed by carbon dioxide asphyxiation and decapitation, respectively. Dams with insufficient litters were also sacrificed by carbon dioxide asphyxiation.

Sacrifice and pathology

F0-generation males and females were sacrificed by carbon dioxide asphyxiation. A gross necropsy examination was not performed routinely on these animals.

B) Offspring (F1 Generation)

Observations

All pups were observed (cage-side) for clinical signs at least once daily. These observations were sufficient to characterize mortality, moribundity, overt toxicity and neurobehavioral changes, by viewing the animal in its cage. More detailed observations for clinical signs were made once daily (a.m.) before weaning and once weekly thereafter.

On PND 4, 11, 21, 35 (+1 day), 45 (+1 day) and 60 (+2 days), selected pups (approximately 16 (minimum 10)/sex/dietary level, representing at least 20 litters/level) assigned to Set C were observed outside the home cage by an individual who was unaware of dose group assignment. Generally, this evaluation was performed according to the procedures described for the dams (see above), using standardized procedures.

All pups were examined daily for evidence of sexual maturation by inspecting females for vaginal patency beginning on PND 29 and males for preputial separation beginning on PND 38. This corresponds to being approximately one or two days before the projected day of onset, continuing until present. The day of onset for each pup was recorded. All pups were also tested for the presence of pupil constriction on PND 21.

Body weight

Surviving pups were weighed on PND 0, 4, 11, 17, and 21, and once weekly thereafter. The individual pups were also weighed when vaginal patency or preputial separation were first evident.

Food consumption and compound intake

Food consumption was not measured after weaning on PND 21, when all animals received untreated diet. Fresh feed and clean feeders were provided at least once every 14 days.

Ophthalmic examination

At approximately 50-60 days of age, ophthalmic examinations were conducted using the males and females (a minimum of 10/sex/dietary level; representing at least 20 litters per level) that were selected for perfusion at study termination. If needed to clarify the significance of findings, the animals reserved for adult brain weight measurements were also subjected to ophthalmologic

examination. The exam took place in a semi-darkened room. The pupillary reflex was tested using a penlight or transilluminator, with a mydriatic agent applied to each eye to dilate the pupil. The conjunctiva, cornea and lens were examined with a slit lamp microscope either before or after pupillary dilatation. After mydriasis, the vitreous humor, retina, choroid, and optic disc were examined using an indirect ophthalmoscope equipped with a condensing lens.

Functional Observational Battery (FOB) and Motor Activity Testing

The test room used for motor activity, acoustic startle habituation, passive avoidance conditioning and water maze testing was a standard animal room that was maintained on the same light: dark cycle as the room in which animals were housed, with tests conducted during the light phase. The order of testing and assignment of animals to specific test devices was semi-random, such that groups were balanced across test times and devices and no animal was tested more than once in the same device. The only exception is that animals were purposely tested in the same water maze on both occasions, as per standard procedure. Males and females were generally tested on the same days at the appropriate days of age. After sexual maturation, test devices were cleaned during the ensuing interval to reduce the residual scent from the other sex.

Motor Activity (Set A). An automated test to measure activity was performed on postnatal days 13, 17, 21 and 60 (+2 days). One male and/or one female from each litter (approximately 16 (minimum 10) /sex/ dietary level, representing at least 20 litters/level) were assigned for testing on each of these four occasions.

Acoustic startle habituation was evaluated on postnatal days 22 and 60 (+2 days). One male and/or one female from each litter (approximately 16 (minimum 10)/sex/dietary level, representing at least 20 litters/level) was assigned for testing on each of these three occasions.

Acoustical Measurements. On postnatal days 22 and 29, learning, short-term retention, and long-term retention were examined in a passive avoidance test. One male and/or one female from each litter (approximately 16 (minimum 10)/sex/dietary level, representing at least 20 litters/level) were assigned for testing. Only animals that demonstrated acquisition were tested for retention.

Water Maze. One male and/or one female from each litter (approximately 16 (minimum 10)/sex/dietary level, representing at least 20 litters/level) were assigned for testing on postnatal day 60 (+2 days), and again seven days later. Only animals that demonstrated acquisition were tested for retention.

Sacrifice and pathology

a) Gross necropsy findings

Where required, the necropsy involved an examination of all organs (including the brain), body cavities, cut surfaces, external orifices and surfaces. All gross abnormalities were recorded. Gross lesions in neural tissues or skeletal muscle were appropriately sampled for microscopic examination. Other gross lesions were not collected for microscopic examination.

Animals that were selected for perfusion or for fresh brain weight determinations (approximately 10/sex/dietary level for each group) underwent a necropsy examination, with collection of gross lesions from neural and muscle tissues for possible microscopic examination. At study termination, randomly-selected animals from Sets A-C (10/sex/dietary level) were sacrificed by CO_2 asphyxiation. The brain was removed from these animals and weighed (fresh weight), and was then discarded.

Perfusion. Animals that were selected for perfusion on PND 21 (from Set D) and at study termination (from Sets A-C) were deeply anesthetized using an intraperitoneal dose of pentobarbital (approximately 50 mg/kg) and then perfused via the left ventricle with a sodium nitrite (in phosphate buffer) flush followed by *in situ* fixation using universal fixative (1.0% (w/v) glutaraldehyde and 4% (w/v) EM-grade formaldehyde) in phosphatebuffer. On PND 21, only the brain (with olfactory bulbs) was collected. At study termination, the brain and spinal cord, both eyes (with optic nerves) and selected (bilateral) peripheral nerves (sciatic, tibial and sural), the gasserian ganglion, gastrocnemius muscle, both forelimbs and physical identifier were collected. All tissues were post-fixed in 10% buffered formalin. The brain was weighed upon removal from the skull, prior to placement into formalin, and the brain:body weight ratio calculated.

Measurements. Prior to sectioning the brain for histology, a Vernier caliper was used to obtain two linear measurements (mm).

1. Anterior-to-posterior (AP) length of the cerebrum, extending from the anterior pole to the posterior pole, exclusive of the olfactory bulbs; and
2. Anterior-to-posterior (AP) length of the cerebellum, extending from the anterior edge of the cortex to the posterior pole.

b) Histopathology

The brain tissue from perfused animals, and any gross lesions collected at necropsy, were further processed for microscopic examination. After the gross measurements were taken, the brain was divided into eight coronal sections for microscopic examination. The eight brain sections were processed according to standard procedures for paraffin embedding. The tissues for morphometric analysis were processed to this (block) stage at all dietary levels, with the ones from control and high-dose animals sectioned at approximately 5 µm, and examined after staining with hematoxylin and eosin (H&E). Tissues from low- and mid-dose animals were not processed further. In addition, the brain sections reserved for morphometric measurements (levels 3-5 and 7) were stained using luxol fast blue/cresyl violet.

Additional tissues were collected for microscopic examination from animals that were perfused at study termination. This included three levels of the spinal cord (cervical, thoracic and lumbar), the cauda equina, eyes, optic nerves and gastrocnemius muscle were embedded in paraffin and stained with H&E. Dorsal root ganglia (including dorsal and ventral root fibers) from the cervical and lumbar swellings and gasserian ganglia were embedded in glycol methacrylate (GMA). GMA-embedded tissues were sectioned at 2 µm - 3 µm and stained using a modified Lee's stain. Peripheral nerve tissues (sciatic, tibial and sural nerves) were embedded in GMA resin and sectioned longitudinally. The sciatic nerve was also cut in cross section.

The tissues from high-dose animals were examined relative to those from the control groups. If no treatment-related lesions were evident, further analysis was not performed. Any region where treatment-related neuropathology was observed underwent the following semi-quantitative analysis. Sections from all dose groups were coded and examined in randomized order without knowledge of the code. The frequency of each type of lesion was determined with the severity of each lesion graded. The code was then broken and the data evaluated for dose-effect relationships. Selected brain regions underwent the following quantitative analysis, with the individual performing the measurements aware of dose group assignments. Initially, seven linear measurements were taken. If treatment-related effects were evident following this initial evaluation, then additional measurements may have been

undertaken. Two of the seven measurements involved gross measurements of the intact brain, as described above.

The other five were taken from the histologic sections using software calibrated with an ocular micrometer. These five measurements are described as follows:

- **Frontal cortex** thickness (Forebrain). This measurement was of the dorsal portion of the cerebral cortex within the coronal section passing through the region of the optic chiasm.
- **Parietal cortex** thickness (Forebrain). This measurement was of the dorsolateral portion of the cerebral cortex within the coronal section taken through the optic chiasm.
- **Caudate putamen** horizontal width (Forebrain; maximum cross-sectional width). This measurement was performed on the coronal section taken at the level of the optic chiasm.
- **Hippocampal gyrus** thickness (Midbrain). This measurement was of the full width of the hippocampal gyrus from the ventral tail of the dentate gyrus to the overlying subcortical white matter. Measurements were taken from the hippocampus from both sides of this section, and the mean value was recorded.
- **Cerebellum** height (Cerebellum / Pons). This measurement extended from the roof of the fourth ventricle to the dorsal surface.

In addition to these measurements, all brain sections from these control and high-dose male and female offspring underwent an extensive micropathologic evaluation.

<u>Statistics</u>

Group means with equal variances were analyzed further using an Analysis of Variance (ANOVA), followed by a Dunnett's test if a significant F-value was determined in the ANOVA. In the event of unequal variances, these data were analyzed using nonparametric statistical procedures (Kruskal-Wallis ANOVA followed by the Mann- Whitney U test for between-group comparisons).

- *<u>Functional Observational Battery.</u>* Continuous data were analyzed using an ANOVA, with *post-hoc* comparisons using Dunnett's test. Categorical data were analyzed using General Linear Modeling and Categorical Modeling (CATMOD) Procedures, with *post-hoc* comparisons using Dunnett's test and an Analysis of Contrasts, respectively .
- *<u>Motor and locomotor activity</u>* (total session activity and activity for each 10-minute interval) were analyzed using ANOVA procedures. Session activity data for the four test occasions were first analyzed using an ANOVA to determine whether there was a significant day by treatment interaction. For days on which there was a significant treatment effect, Dunnett's test was used to determine whether the treated group was significantly different from the control. Interval data were subjected to a Repeated- Measures ANOVA, using both test interval and test occasion as repeated measures, followed by an ANOVA to determine whether there was a significant treatment by interval interaction on each test occasion. For those test days, the data for each interval was subjected to analysis using Dunnett's test to determine whether the treated group was significantly different from the control.
- *<u>Acoustic startle</u>* response amplitude data (peak amplitude) for the three test occasions were first analyzed using an ANOVA procedure. If there was a significant group effect, Dunnett's test was used to determine whether the treated group was significantly different from control. The response amplitude data for each block of ten trials (five blocks/test session) were subjected to a Repeated-Measures ANOVA, using test block as the repeated measure. If there

was a significant group by block interaction, the values for each block were subjected to analysis using Dunnett's test to determine if the results for treated animals were significantly different from control.

- *Passive avoidance* data were analyzed as follows. Latency data were analyzed using a Wilcoxon Test for time to failure (i.e., time to cross). The number of trials-to-criterion was analyzed using Kruskal-Wallis and Wilcoxon tests for the acquisition phase and Pisher's Exact Test for retention. The number of rats failing to meet the criterion level of performance in the learning (acquisition) phase was analyzed as incidence data.
- *Water maze* results were analyzed using parametric and non-parametric tests. Latency data were analyzed by a univariate ANOVA, with post-hoc analysis using Dunnett's test.

The number of trials-to-criterion and the number of errors were analyzed using Kruskal- Wallis and Wilcoxon tests for the acquisition phase and Fisher's Exact Test for retention. The number of rats failing to meet the criterion level of performance in the learning phase was analyzed as incidence data.

Results And Discussion

Test material and diet analysis

Homogeneity and stability analysis

The homogeneity and stability of the test substance in rodent feed have been verified at dietary concentrations of 15 and 180 ppm and were determined to be homogeneous and stable for 7 days at room temperature and 28 days at freezer conditions.

Concentration analysis: During gestation, the nominal 30, 60 and 180 ppm dietary levels averaged 77-84% of the nominal concentrations. Based on these results, the average dietary levels during gestation were 0, 25.1, 46.3 and 140 ppm, respectively. For lactation, dietary levels were reduced 50% to achieve a more consistent dosage (mg/kg b.wt/day) throughout the period of exposure, since food consumption increases during this time period. During lactation, the nominal 15, 30 and 90 ppm dietary levels averaged 78-84% of the nominal concentrations. Based on these results, the average dietary levels during lactation were 0, 12.6, 25.4 and 70.1 ppm, respectively.

The identity of the active ingredient was confirmed by analytical methods. The concentration of the active ingredient in the test substance was measured in order to verify the stability of the test substance at room temperature storage conditions.

A) Maternal (F0 generation)

Reproduction parameters

Reproduction parameters were not affected by the test substance at any dietary level.

Clinical Observations During Gestation

There were no treatment-related effects.

Body Weight and Food Consumption During Gestation

There were no effects on body weight, body weight gain and food consumption during gestation and lactation at any dietary level.

Test Substance Intake

The average daily intake of the propineb (mg propineb./kg body weight/day) was calculated using weekly body weight and food consumption data

Table 5.7.1 -2 Achieved intake

Concentrations (ppm)	30 ppm	60 ppm	180 ppm
GD 6-13	2.1	3.7	10.5
GD 13-20	2.0	3.7	10.5
LD 0-7	1.9	4.0	10.3
LD 7-14	2.7	5.0	13.9
LD 14-21	3.0	5.7	16.5

Based on these results, the average daily intake of active ingredient during gestation and lactation was 0, 2.3, 4.4 and 12.3 mg/kg/day.

B. Offspring (F1 Generation)

Clinical Signs - Lactation and Postweaning

Postpartum (PND 0-21). There were no compound-related signs in males or females at any dietary level.

Postweaning. Compound-related clinical signs after weaning (when exposure was discontinued) were limited to corneal opacities in one or two mid-dose males and females and five high-dose males.

Animals Found Dead or Moribund - Post-Culling

There were no missing, found dead or moribund offspring (males and females combined) found after culling litters on PND 4

Body Weight and Body Weight Change – Lactation and postweaning

Body weight was not affected by the test substance at any dietary level in either sex.

Developmental Landmarks (Sexual Maturation) and Pupil Constriction

The ages for onset of balanopreputial separation, vaginal patency and surface righting were not affected by the test substance at any dietary level. There was a slight delay in balano-preputial separation in high-dose males (44.9 versus 43.3 for controls). This slight difference from control was not considered to be test substance-related but more likely due to normal variability since the delay was not statistically significant; was within the range (42.2-44.9 days) of historical control for the last 10 studies conducted in this laboratory

Pupil constriction in response to a penlight was apparent in all control and treated pups on PND 21. Therefore, there was no indication of a compound-related effect.

Functional Observational Battery (FOB)
There were no test substance-related findings in males or females at any dietary level.
There was a statistical increase in the mean number of rears in the open field, compared to controls for low-dose females on PND 45 (6.9 versus 4.3 for controls). This difference from control is not thought to be test substance-related since the incidence was not dose related and the mean average for rears in the control animals (4.3) is below the range for historical controls in the last ten studies conducted in the testing laboratory. There was a statistical difference in the case of removal from the home cage (increased incidence of vocalization) for high-dose female pups on PND 4 relative to controls. This difference from control is not thought to be related to the test substance since it was not dose related.

Summary Session Motor and Locomotor Activity
A comparison of interval results for control and treated animals revealed no compound related effects at any dietary level. Levels of motor and locomotor activity for treated animals were comparable to controls at all test intervals, on all test occasions.

Acoustic startle, passive avoidance, water maze
There were no differences in startle amplitude for treated males or females, relative to control, at any dietary level. The average response amplitude for treated animals for all 50 trials and the response amplitude for the five blocks of trials, which is used to assess habituation, were comparable to control at all dietary levels.

Ophthalmology
There were no test substance-related lesions in males or females at any dietary level.

Necropsy - organ weight
There were no gross observations considered test substance-related at any dietary level in either sex for perfused Day 21 or termination animals or in non-perfused termination animals.
Absolute and relative fixed brain weights for perfused Day 21 males and females were not different from control at any dietary level.

Brain Measurements (Morphometry)
Gross Measurements (Cerebrum and Cerebellum Length). There was no difference in cerebrum or cerebellum length at any dietary level at either age.

Microscopic Measurements (Brain)
There were no test substancerelated differ differences in micropathology brain measurements in high-dose termination males or females.

Table 5.7.1 -3 Brain measurements (mm), body and brain weight on offspring

Parameter	Dose (ppm in diet)			
	Control	30 PPM	60 PPM	180 PPM
Males				
Gross Measurements				
Day 21				
Ant/Post Cerebrum Length (mm)	13.67±0.27 (10)	13.86±0.28 (10)	13.70±0.32 (10)	13.68±0.14 (10)
Ant/Post Cerebellum (mm)	7.39±0.48 (10)	7.31±0.48	7.31±0.17 (10)	7.32±0.30 (10)
PND 75 (±5) (Termination - Perfused)				
Ant/Post Cerebrum Length (mm)	14.69±0.23 (10)	14.90±0.26 (10)	14.80±0.33 (10)	14.55±0.39 (10)
Ant/Post Cerebellum (mm)	8.19±0.27 (10)	8.35±0.30 (10)	8.14±0.56 (10)	8.10±0.34 (10)
Microscopic Measurements				
PND 21				
Frontal Cortex (mm)	1.832±0.010 (10)	--	--	1.804±0.005 (9)
Parietal Cortex (mm)	1.880±0.011 (10)	--	--	1.905±0.006 (9)
Caudate Putamen (mm)	2.704±0.059 (10)	--	--	2.500±0.108 (9)
Hippocampal Gyrus (mm)	1.701±0.007 (10)	--	--	1.719±0.012 (10)
Cerebellum (mm)	5.007±0.020 (10)	--	--	4.970±0.230 (10)
PND 75 (±5) (Termination - Perfused)				
Frontal Cortex (mm)	1.799±0.014 (9)	--	--	1.803±0.015 (9)
Parietal Cortex (mm)	1.985±0.015 (9)	--	--	2.000±0.014 (9)
Caudate Putamen (mm)	3.266±0.029 (9)	--	--	3.216±0.039 (9)
Hippocampal Gyrus (mm)	1.948±0.014 (9)	--	--	1.975±0.019 (10)
Cerebellum (mm)	5.575±0.144 (10)	--	--	5.576±0.088 (10)
Females				
Gross Measurements				
PND 21				
Ant/Post Cerebrum Length (mm)	13.50±0.23 (10)	13.63±0.21 (10)	13.63±0.24 (10)	13.69±0.15 (10)
Ant/Post Cerebellum (mm)	7.15±0.28 (10)	7.27±0.43 (10)	7.25±0.26 (10)	7.09±0.28 (10)
PND 75 (±5) (Termination - Perfused)				
Ant/Post Cerebrum Length (mm)	14.07±0.34 (10)	14.42±0.35 (10)	14.36±0.42 (10)	14.42±0.32 (10)
Ant/Post Cerebellum (mm)	8.15±0.40 (10)	8.28±0.28 (10)	8.02±0.31 (10)	8.25±0.30 (10)
Microscopic Measurements				
PND 21				
Frontal Cortex (mm)	1.636±0.003 (10)	--	--	**1.807±0.007* (9)**
Parietal Cortex (mm)	1.702±0.003 (10)	--	--	**1.906±0.005* (9)**
Caudate Putamen (mm)	2.539±0.039 (10)	--	--	**2.805±0.031* (9)**
Hippocampal Gyrus (mm)	1.580±0.008 (10)	--	--	1.621±0.016 (10)
Cerebellum (mm)	4.995±0.118 (10)	--	--	4.900±0.142 (10)
PND 75 (±5) (Termination - Perfused)				
Frontal Cortex (mm)	1.821±0.002 (10)	--	--	1.860±0.011 (9)
Parietal Cortex (mm)	1.955±0.003 (10)	--	--	1.948±0.014 (10)
Caudate Putamen (mm)	3.227±0.022 (10)	--	--	3.312±0.033 (10)
Hippocampal Gyrus (mm)	1.821±0.021 (10)	--	--	1.778±0.014 (9)
Cerebellum (mm)	5.073±0.068 (10)	--	--	4.958±0.176 (10)

Micropathology

There were no compound-related changes in brain sections for either day 21 pups or for perfused termination animals from the control and 1500 ppm dosage groups.

Additional Non-Brain Termination Animal Tissues:

Spinal cord (cervical, thoracic, and lumbar), cauda equina, spinal nerve roots and dorsal root ganglia (cervical and lumbar), gasserian ganglion, eyes, optic nerves, gastrocnemius muscle, sciatic nerve, tibial nerve, and sural nerves were also collected from perfused termination animals and evaluated microscopically.

There were no test substance-related microscopic lesions evident in any tissue from the termination high-dose males or females. There were a few microscopic observations of which "axonal degeneration" of individual nerve fibers in spinal cords, nerves, and dorsal root ganglions were the most common. Also, focal retinal dysplasia occurred in two 180 ppm male rats.

Conclusion

In conclusion the high dose tested of 180 ppm (equivalent to maternal intake of 12.3 mg/kg bw/day) was the study NOAEL for both maternal and offspring.

IIA 5.7.1 Acute neurotoxicity - rat

An acute neurotoxicity study was submitted in the addendum to the monograph.

CA 5.7.2 Delayed polyneuropathy studies

No new studies have been carried out.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

A series of studies have been carried out with the main plant and animal metabolites of propineb and have included either in the Baseline Dossier or in the addendum to the monograph. In addition a few studies have been carried out and are presented in detail in this section. A list of these studies and their reference is presented in the table here below, while detailed summary are provided at the beginning of each section describing the results or the studies in detail.

Table 5.8.1-1 Summary of types of studies conducted with propineb metabolites. Studies not yet evaluated in EU are highlighted in bold.

Metabolite	Study	Reference	Submision status
Propylene Thiourea (AE F074263)	Acute oral toxicity	M-050174-01-1 M-104834-01-2	Baseline dossier
	In vitro Ames test	M-050164-01-1	Baseline dossier
	In vitro cytogenetic	M-050137-01-1	Baseline dossier
	In vitro gene mutation in mammalian cells	M-050139-01-1	Baseline dossier
	Carcinogenicity in rats	M-050230-02-1	Baseline dossier
	Carcinogenicity in mice	M-050457-01-1	Baseline dossier
	Studies on thyroid function	M-105120-02-2 M-050004-02-1 M-053435-01-1	Baseline dossier
	Study on hepatotoxicity in mice	M-359952-01-3	**New study**
	Two-generation study	M-182007-02-1*	Monograph addendum
	Developmental toxicity-rat	M-015527-02-1 M-389683-01-1 M-297866-01-1*	Baseline dossier/ Monograph addendum
Propylene Urea (AE 1379609)	Acute oral – rat	M-104828-01-2	Baseline dossier
	In vitro Ames test	M-116160-01-1	Baseline dossier
	In vitro cytogenetics	M-289106-01-1	**New study**
	In vitro gene mutation in mammalian cells	M-301079-01-1	**New study**
	Carcinogenicity – mice	M-050194-01-1	Baseline dossier
	Study on hepatotoxicity in mice	M-360438-01-1	**New study**
4-MMI (BCS-AB78877)	Acute toxicity	M-104849-01-1 M-105047-01-1	Baseline dossier
	In vitro Ames	M-491077-02-1	**New study**
	In vitro micronucleus	M-491079-02-1	**New study**
Propineb DIDT (BCS-CU99534)	**Acute oral rat**	M-491068-02-1	**New study**
	In vitro Ames test	M-481443-01-1	**New study**
	In vitro micronucleus	M-490043-01-1	**New study**
	In vivo rat 28-day toxicity	*M-491125-02-1*	**New *study***
Formyl PDA (BCS-CY52341)	**In vitro Ames test**	M-490977-01-1	**New study**
	In vitro micronucleus	M-491073-02-1	**New study**

* Study has been evaluated on EU level but is filed in the Supplemental Dossier due to technical reasons.

In addition a document (M-490628-02-1) has been prepared and submitted which summarizes consumer exposure and toxicological evaluation of propineb metabolites considering representative uses.

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Propylene Thiourea (AE F074263)

Propylene Thiourea (AE F074263) is often abbreviated as PTU which may create confusion with the drug Propylthiouracil a compound which partly shares the mode of action I the thyroid, i.e. both inhibit the enzyme Thyroid Peroxidase (although propylenethiourea inhibition is reversible upon withdrawn of the exposure) but propylenethiourea has no effects on the conversion of thyroxine (T4) and triiodothyronine (T3).

Only one study, which investigated possible the mode triggering liver tumors in the mouse, was not included in the Baseline Dossier and in the addendum to the monograph. This study is described in detail.

Here below a summary of the toxicological profile of PTU is presented, based on all the available studies.

PTU is moderately toxic following a single bolus administration with an oral LD_{50} value of 795 mg/kg in rats.
There is no evidence of mutagenic based on the results of three in vitro genotoxicity studies (bacterial reverse mutation test, chromosome aberration test and mammalian cell forward mutation test) carried out current standards and under GLP requirements.
Chronic rat and mouse oncogenicity studies have been conducted with PTU.
In the rat study, groups of 50 male and 50 female Wistar rat were administered diet containing PTU, at concentrations of 1, 10, 100 and 1000 ppm (equivalent to a mean daily intake of 0.055, 0.56, 5.71, 123 mg/kg bw/day and 0.073, 0.74, 7.26 , 128 mg/kg bw/day in males and females, respectively). The high dose levels of 1000 ppm exceeded the MTD: body weight in this group was 20% lower than that of the control group and after 3 month there was not more growth. Moreover, this dose level was lethal for the majority of animals as 62% of males and 70% of the females died during the first 6 months of the study. The thyroid was the target organ. Effects on iodine-bound concentration were observed from 100 ppm, associated with morphological changes like nodular hyperplasia. However, thyroid neoplastic findings, i.e. adenomas, were observed only in the 1000 ppm group. The dose level of 10 ppm (equivalent to 0.56 and 0.74 mg/kg bw/day) was the study NOAEL.

Based on the outcome of a mechanistic study to understand the mode of action behind the effects of PTU in the thyroid, propylenethiourea is a weak inhibitor of iodothyronine deiodinase.

In the mouse study, 50 male and 50 female CF1/W 74 mice per dose group were given diets containing PTU, at concentrations of 1, 10, 100 and 1000 ppm (equivalent to a mean daily intake of 0.16, 1.56, 17.1, 184 and 0.21, 2.1, 21.5 and 251 mg/kg bw/day in males and females, respectively). Additional 10 animals/sex/dose groups were subjected to an interim sacrifice after 12 months. The liver was the target organ and an increased incidence of hepatocellular adenomas and carcinomas was diagnosed in the livers of the treated male and female animals. The incidence was outside the historical control data from 10 ppm onward. There was no evidence of other type of tumours. Thyroid weight was increased at 1000 ppm in males. The enlarged thyroids correlated histologically with an increase in the number of follicle cells and slight follicle cell hypertrophy accompanied by reductions in follicle size. The study NOAEL was 1 ppm (equivalent to 0.16 and 0.21 mg/kg bw/day).

In the second mice oncogenicity study PTU was given in drinking water to groups of 50 B6C2F1 mice at concentrations of 0, 0.25, 1.25, 6, 30 and 150 ppm (equivalent to 0.04, 0.21, 0.89, 4.05 18.05 and 0.051, 0.25, 1.08, 4.61 and 21.9 mg/kg body weight in the females) over a period of up to 108 weeks. In this study body weight were affected at 30 and 1500 ppm in males (from 7% up to 22% lower) and at 150 ppm (-18%) in females. In this study there was no evidence of neoplastic finding and even no indication of treatment-related effects on the liver and thyroid. The study NOAEL was 6 ppm in male mice, equivalent to 0.89 mg/kg bw/day and 30 ppm in female mice, equivalent to 4.61 mg/kg bw/day.

A special study was conducted in 2009 to further investigate the mechanism behind liver tumor formation observed in the chronic study with CF1/W 74 mice. CFW male were given diets containing PTU at concentration of 1000 ppm (equivalent to approximately 139 mg/kg bw/day) for 15 days. The following parameters were assessed: liver cell proliferation, cytochrome P-450 content and enzymatic activity of specific P-450 isoforms and UDP-GT, phase I and phase II enzymes gene transcripts were analyzed by Q-PCR. Results showed increased absolute and relative liver weights (19 and 28% higher than the control group) associated with centrilobular hepatocellular hypertrophy, increased cell proliferation and mean total cytochrome P450 content (+ 30%). Quantitative PCR analyses of transcripts of genes of phase I and II revealed an up-regulation of Cyp2b9 and of epoxyhydrolase (Ephxl) gene transcripts. Overall this study provided some indication of a mode of action similar to that of phenobarbital (which was tested in parallel as positive control).

Table 5.8.1-2 Summary of toxicity study with PTU. New study in bold

Type of study	Species/test system	Result
Acute oral toxicity [redacted].; 1975 M-104834-01-2	Rat	LD_{50} =537 mg/kg
Acute oral toxicity [redacted].; 1977 M-050174-01-1	Rat	LD_{50} =795 mg/kg
Ames test [redacted].; 1995 M-050164-01-1	TA 98, TA100, TA1535 TA 1537	Negative
In vitro chromosome aberration. [redacted]; 1996 . M-050137-01-1	Chinese hamster V79 cells	Negative
In vitro forward mutations in the V79-HPRT assay. [redacted].; 1996 M-050139-01-1		Negative
Type of study	**NO(A)EL (mg/kg bw/day)**	**Effects at LOAEL and higher doses**
Chronic/ carcinogenicity Wistar rat 0, 1, 10, 100, 1000 ppm 0, 0.055, 0.56, 5.71, 123 mg/kg bw/day (♂) 0, 0.073, 0.74, 7.26 , 128 mg/kg/day (♀) [redacted].; [redacted]; 1980 M-050230-02-1	0.56(♂) – 0.74 (♀)	123 – 128 mg/kg bw/day: High mortality, anemia, renal calculi with blood in urine, thyroid adenomas 5.71 – 7.26 mg/kg bw/day Clinical signs (poor condition, rough coats, inactivity), ↑ thyroid wt, thyroid nodules and thyroid nodular hyperplasia.
Combined chronic carcinogenicity CF1/W74 mice 0, 1, 10, 100, 1000 ppm 0, 0.16, 1.56, 17.1, 184.1 mg/kg/day (♂) 0, 0.21, 2.1, 21.5, 251 mg/kg/day (♀) [redacted].; [redacted]; [redacted].; 1981 M-050457-01-1	0.16(♂) – 0.21 (♀)	From 1.56 mg/kg bw/day in both sexes: ↑hepatocellular adenomas and carcinomas
Oncogenicity study in B6C3F1-Mice Administration in drinking water over 2 years. 0, 0.25, 1.25, 6, 30 and 150 ppm 0, 0.04, 0.21, 0.89, 4.05 18.05 mg/kg bw/day (♂) 0, 0.051 0.25, 1.08, 4.61, 21.9 mg/kg bw/day (♀) [redacted]; 1998 M-050065-01-1	0.89 (♂)- 4.61 (♂)	18.05 mg/kg bw/day males: ↓ body weight (up to - 22%) 4.05 mg/kg bw/day: males ↓ body weight (- 7% 21.9 mg/kg bw/day: female ↓ body weight (up to - 18%) In this study there was no evidence of neoplastic

Studies on the mode of action		
Study	**Type of investigation**	**Results**
Effect of long-term administration of propylene thiourea (PTU) on the thyroid function of male and female rats 0, 1, 10, 100 and 1000 ppm [redacted]; 1979 M-105120-02-2	Thyroid tests function (accumulation of ^{125}I in the thyroid; level of protein-bound iodine in the blood plasma) 3, 7 and 14 days, and 1, 3 6, 12 and 24 months	Changes on thyroid function from 100 (~5 mg/kg bw/day) No significant effects at 1 - 10 ppm, equivalent to 0.05 – 0.5 mg/kg b per day.
Propylenethiourea (PTU): Effect of the compound on thyroid function of male Wistar-rats after uptake with the drinking-water in the low dose range up to a maximum of 10 ppm a.i. over a time interval up to 63 days [redacted].; 1991 M-050004-02-1	Measurement of thyroid: thyroid weight, ^{131}Iodine-accumulation, serum levels T4, T3 , TSH. Thyroids histopathology at days 21and 63	↓ of the iodine concentration in thyroid on day 63 in all treated animals; ↑ TSH serum concentration on day 7 and 63, but lower on day 21. Thyroid histopathology: no treatment related effects. Not conclusive
In vitro characterization of the goitrogenic properties of its metabolite 1,2-Propylenethiourea [redacted]; 1996. M-053435-01-1	In vitro test using hog thyroids to measure the effects of PTU on Thyroid Peroxidase activity and on the conversion of T4 to T3.	PTU suppressed TPO-catalyzed iodine formation temporarily and also suppresses the non-enzymatic and TPO-catalyzed iodination of L-tyrosine. PTU does not inhibit TPO-catalyzed oxidation of guaiacol and iodothyronine deiodinase
Propylene thiourea - Mechanistic 14-day toxicity study in the male mouse at 1000 ppm (139 mg/kg bw/day) [redacted].;2009; M-359952-01-3	Investigation of hepatotoxicity, induction of P-450 cell proliferation and gene transcriptinvestigation)	Increased liver weights associated with centrilobular hepatocellular hypertrophy, increased cell proliferation and mean total cytochrome P450 content. Quantitative PCR analyses up-regulation of Cyp2b9 and of epoxyhydrolase gene transcripts

A rat two-generation reproductive toxicity study with PTU has been performed according to the OECD 416 guideline (2001) and in compliance with GLP requirements. The compound was administered to groups of 25 Wistar rats/sex/dose group in the drinking water at concentrations of 0 (control), 1.5, 15, and 150 ppm (equivalent to 0.202, 2.03, 17.89 and 0.24, 2.20, 20.25 mg/kg bw/day in male and females, respectively). Administration via drinking water provoked severe toxicity in both parents and pups at the top dose: body weight was depressed by more than 20%, thyroid adenoma and hyperplasia were also observed in the parents and this was accompanied by increased number of pups with external and cranial malformations. The study NOAEL is 1.5 ppm (equivalent to 0.202-0.227 mg/kg bw/day).

Two rat developmental toxicity studies are available with PTU. Both studies are GLP and compliant with current regulatory requirement and have been included in a single report. In the first study Sprague-Dawley rats were administered 0, 1, 7, or 50 mg PTU/kg bw/day from gestation day 6 through 19.
Doses from 7 mg/kg bw/day provoked decreased maternal body weight and effects on thyroid hormone levels (increased TSH and decreased T4 and T3).

Developmental effects consisted of decreased body weight at the top dose and increased incidence of skeletal variation from 7 mg/kg bw/day.

As a few skeletal variations were also present at 1 mg/kg bw/day an additional study was performed to verify the potential relationship between the test compound administration and skeletal variations observed at 1 mg/kg bw/day. In the second study, gravid Sprague-Dawley rats were administered nominal doses of 0.3, and 1.2 mg PTU/kg body weight by oral gavage on days 6 through 19 of gestation. The incidence of the skeletal variations observed in the previous study at the dose level of 1 mg/kg bw/day was lower in the second study at the comparable dose of 1.2 mg/kg bw/day and was within the laboratory historical control data. Therefore, the overall NOAEL for developmental toxicity is considered to be 1.2 mg/kg bw/day.

Table 5.8.1-3 Summary of reproductive toxicity study with PTU

Multigeneration study	**NOAEL**	**LOAEL**		**Effects**
Two-generation rat 0, 1.5, 15 or 150 ppm in drinking water M-182007-02-1	0.202-0.227 (1.5 ppm)	2.20 –2.12 (15 ppm)	Adults	Decreased Body weight Reduced density of follicular colloid in the thyroid Thyroid adenomals at the top dose
	0.202-0.227 (1.5 ppm)	2.20 –2.12 (15 ppm)	Pups	Decreased Body weight Reduced density of follicular colloid in the thyroid Head malformation at the top dose
	2.20 –2.12 (15 ppm)	17.9 – 19.22 (150 ppm)	reproduction	↓number of implantation site
Developmental toxicity studies				
Embryotoxicity rat 0, 1, 7 and 50 mg/kg bw/day [redacted] 1993	1	7	dam	↑ plasma TSH and ↓ T3 and T4 ↓ Body weight at the top dose
	1	7	fetus	Slight delayed skeletal ossification ↑malformation at the top dose
Embryotoxicity rat 0, 0.3, 1.2 mg/kg bw/day [redacted] 1997 M-389683-01-1	1.2	-	dam	No adverse effects observed
	1.2	-	fetus	No adverse effects observed

Report:	[redacted] ;[redacted];2009;M-359952-01
Title:	Propylene thiourea - Mechanistic 14-day toxicity study in the male mouse (hepatotoxicity, cell proliferation and gene transcript investigations)
Report No:	SA 09069
Document No:	M-359952-01-3
Guidelines:	**US EPA OPPTS 870.SUPP**
GLP/GEP:	**yes**

Executive Summary

The objective of this study was to investigate the effects of propylene thiourea (PTU) on the liver following continuous dietary administration for 14 days in the male mouse. Liver cell proliferation, total cytochrome P-450 levels, liver enzymatic activities (P-450 and UDPGT) and gene transcript investigations (P-450 and phase II enzymes) were assessed as well as histopathology of the liver.

Propylene thiourea (AE B007299, batch number NLL 3790-7: a light beige powder, 98% w/w purity) was administered continuously via the diet to one group of 30 male Crl:CFW (SW) mice for at least 15 days at the concentration of 1000 ppm (equivalent to approximately 139 mg/kg/day). A similarly constituted group received untreated diet and acted as a control group. Finally, another group of 30 male mice received 80 mg/kg body weight/day of Phenobarbital (suspension w/v in 0.5% aqueous solution of methylcellulose) by oral gavage once per day (10ml/kg) and acted as a positive control group.

Animals were observed at least daily for clinical signs and twice daily for mortality. Detailed physical examinations were performed weekly. Body weight and food consumption were recorded once weekly. A subgroup of 15 mice from each main group received bromo-deoxy-uridine (BrDU) via their drinking water during the last week of the study in order to assess liver cell proliferation. Water consumption (BrdU administration period) was recorded on the day of scheduled sacrifice. Selected clinical chemistry parameters were determined at day 16. All animals were necropsied, selected organs weighed and portions of liver of the 15 animals from each group having received BrDU were taken and fixed for conventional histopathology examination as well as for cell proliferationmeasurement. The liver of the other 15 animals from each group were used for phase I and phase II enzymes gene transcripts analyses by Q-PCR and for microsomal preparations in order to determine total cytochrome P-450 content, cytochrome P-450 specific isoenzyme profiles and UDPGT activities.

One animal from group 2 (TT2M2612, Phenobarbital 80 mg/kg/day, sub-group 1) was sacrificed for humane reasons on Study Day 6 due to clinical signs (reduced motor activity, prostration, soiled fur and half-closed eyes). No clear cause of the animal status was established either after gross orlimited microscopic examination.

Mean body weight was reduced during the entire study period. On study Day 15, the mean body weight in the treated group was 4.9% lower than the control group. This effect resulted from a mean body weight loss of -0.13 g and of -0.03 g compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15. An overall cumulative body weight loss of 1.1 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls.

Mean food consumption was reduced by 8% and by 6% compared to the controls, respectively during the first and the second week of the study. Higher mean total cholesterol (+33%, p≤0.01), total protein (+7%, p≤0.01), albumin concentrations (+8%, p≤0.01) and mean alkaline phosphatase activity (+24%,

p≤0.05) were observed as compared to the control values. Additionally, mean urea concentration (-22%, p≤0.05) was lower relative to the control group.

At necropsy, mean terminal body weight was lower (-6%, p≤0.01) when compared to control animals. Mean absolute and relative liver weights were increased by between 19 and 28% (p≤0.01) when compared to control animals. Enlarged liver was found in 8/30 males. Microscopic examination revealed a centrilobular hepatocellular hypertrophy associated in some cases with a slightly exacerbated centrilobular hepatocellular vacuolation.

Assessment of cell proliferation in the liver revealed a 3 times higher mean BrdU labeling index in the centrilobular area in treated animals, when compared to the controls, whilst a similar mean BrdU labeling index was noted in the perilobular area. The overall BrdU labeling index (centrilobular + perilobular) was higher (approximately 2 times) than the controls.

The assessment of the P-450 isoenzyme and UDPGT activities revealed a slight increase in mean total cytochrome P450 content (+ 30%) without any change in EROD, PROD, BROD and UDPGT (bilirubin) activity when compared to the control groups. Quantitative PCR analyses of transcripts of genes of phase I enzymes revealed a down-regulation of Cyp1a1 and Cyp3a11 transcripts and an up-regulation of Cyp2b9 transcripts.

Concerning the phase II enzymes, sulfotransferase (Sult1a1) and UDP glucoronosyltrabsferase (Ugt2b1) gene transcripts were down-regulated whereas epoxyhydrolase (Ephx1) gene transcripts were up-regulated.

Animals receiving ***Phenobarbital (positive control)*** at 80 mg/kg/day by gavage, mean body weight was reduced during the entire study period. On study Day 15, the mean body weight in the treated group was 7.8% lower than the control group. This effect resulted from a mean body weight loss of -0.27 g and of -0.01 g compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15.

An overall cumulative body weight loss of 2.0 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls. Mean food consumption was reduced by 10% and by 8% compared to the controls, respectively during the first and the second week of the study.

Lower mean total bilirubin (-63%, p≤0.01) and total cholesterol concentrations (-21%, p≤0.01) were observed compared to the control values.

At necropsy, mean terminal body weight was lower (-7%, p≤0.01) when compared to control animals. Mean absolute and relative liver weights were increased by between 15 and 22% (p≤0.01) when compared to control animals. Enlarged liver was found in 9/29 males. Microscopic examination revealed a centrilobular hepatocellular hypertrophy associated with a decreased centrilobular hepatocellular vacuolation.

Assessment of cell proliferation in the liver revealed a 3 times higher mean BrdU labeling index in the centrilobular area in treated animals, when compared to the controls, whilst a similar mean BrdU labeling index was noted in the perilobular area. The overall BrdU labeling index (centrilobular + perilobular) was higher (approximately 2 times) than the controls.

The assessment of the P-450 isoenzyme and UDPGT activities revealed an increase in mean total cytochrome P450 content (+109%, p ≤0.01), a very slight increase in EROD activity (+60%, p≤0.01), a high increase in PROD activity (+3639%, p ≤0.01), a very high increase in BROD activity (+11236%, p ≤0.01) and a high increase in UDPGT (bilirubin) activity (+141%, p≤0.01). Quantitative PCR analyses of transcripts of genes of phase I enzymes revealed an up-regulation of Cyp2b9,

Cyp2b10 and Cyp 3a11 transcripts. Concerning the phase II enzymes, epoxyhydrolase (Ephx1), sulfotransferase (Sult1a1 andSult1d1), UDP glucoronosyltransferase (Ugt1a1 and Ugt2b1) gene transcripts were upregulated.
Comparison of the findings observed with PTU and Phenobarbital indicate that PTU hepatotoxicity mode of action share some commun characteristic with Phenobarbital, although a firm conclusion cannot be drawn from this study.

1. MATERIALS AND METHODS

A. MATERIALS

1. **Test Material:** AE F074263 (Propylene Thiourea)
   - **Description:** Light beige powder
   - **Lot/Batch #:** NLL 3790-7
   - **Purity:** 98 %
   - **CAS #:** 2122-19-2
   - **Stability of test compound:** The stability of the test substance at 1000 ppm in the diet was determined during the study for a time period which covers the period of storage and usage for the current study

2. **Vehicle and/or positive control:** Phenobarbital, a liver enzyme inducer in rodents (batch number 06100228; a white powder, 99.6% purity) was used as positive control.

   Other chemical: BrdU (5-Bromo-2'-deoxyuridine), an analogue of thymidine (batch number 097K0675; a white powder, 99.9% purity), was used to evaluate cell proliferation in the study

3. **Test Animals**
   - **Species:** Mouse
   - **Strain:** Crl:CPW (SW)
   - **Age:** 11 weeks old
   - **Weight:** Mean group weight of males: 30.4 g to 36.7 g;
   - **Source:** [redacted] Laboratories, USA.
   - **Acclimation period:** 6 days
   - **Diet:** Certified rodent powdered and irradiated diet A04CP1-10 from [redacted]., available *ad libitum,* except before blood sampling prior to sacrifice when animals were diet fasted overnight.
   - **Water:** Filtered and softened tap water from the municipal water supply, *ad libitum***.**
   - **Housing:** Individually in suspended, stainless steel, wire-mesh cages. The cage of each animal was identified by a card bearing a unique identification number.

Environmental conditions

Temperature:	20-24 °C
Humidity:	40-70 %
Air change:	10 to 15 changes/h
Photoperiod:	12 h dark/ 12 h light (7 am - 7 pm)

B. STUDY DESIGN

I. In life dates:

The study was conducted from 22 April to 7 July 2009 in the laboratory of the toxicology centre of Bayer CropScience in [redacted] (France).

II. Animal assignment and treatment

The dose levels were selected based on the evaluation of the results from a previous oncogenicity study conducted with PTU (M-).

The oral route was selected as it is an accepted route of exposure by regulatory authorities and since it is a possible route of human exposure.

Control animals were fed control diet for at least 14 days. Test animals were fed diet containing PTU at 1000 ppm for at least 14 days. Additionally, a positive control group was fed control diet and received phenobarbital at 80 mg/kg/day by oral gavage (10 ml/kg) for the same period. A solution of BrdU at 80 mg of BrdU/100 ml of drinking water was administered to all animals during the last week of the study.

Table 5.8.1-4 Study design

Test group	Dose level	Animals assigned (males)
Control	0	30
PTU	1000 (ppm)	30
Phenobarbital	80 mg/kg bw/day	30

III. Diet preparation and analysis

PTU was incorporated into the diet to provide the required dietary concentrations. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. There was one preparation at 1000 ppm for the study. When not in use, the diet formulation was stored at approximately -18° °C. The unused residue was discarded at the end of each administration period. Homogeneity of test substance in diet was verified to demonstrate adequate formulation procedures. Dietary levels of the test substance were verified. For PTU formulation the homogeneity and concentration results ranged between 96 and 97% of the nominal concentration.

The stability of the test substance at 1000 ppm in the diet was determined during the study for a time period which covers the period of storage and usage for the current study.

Phenobarbital was suspended (w/v) in an aqueous solution of methylcellulose 400 at 0.5%.
There was one preparation at 8 g/l for the study. When not in use, the suspensions were stored at +5° C (±3°C). The suspensions were mixed continuously before and during dosing with an electromagnetic stirrer. Analysis of the preparations showed that the homogeneity and concentration results ranged between 98 and 99% of the nominal concentration.

A solution of BrdU in filtered tap water from the municipal water supply was prepared at a concentration of 80 mg of BrdU/100 ml of drinking water. Bottles containing BrdU in drinking water were stored at room temperature and were protected from light. Analysis of the formulation indicated that the concentration of BrdU was 104%.

IV. Statistics

Mean and standard deviation were calculated for each group.
All statistical analyses were carried out separately for males and females. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). The following parameters were statistically analyzed:

- **A.** Body weight parameters
- **B.** Body weight change parameters calculated according to time intervals
- **C.** Average food consumption/day parameters calculated according to time intervals
- **D.** Clinical chemistry parameters
- **E.** Terminal body weight, absolute and relative organ weights parameters
- **F.** Total cytochrome P450 content and liver enzyme activities
- **G.** Cell proliferation
- **H.** Gene transcript data

Mean and standard deviation were calculated for each group.
The Phenobarbital (Group 2; gavage) and PTU (Group 3; diet) treated groups were compared to the control group (Group 1; diet) using the following procedures.

- Body weight change parameters, Terminal body weight, absolute and relative organ weight parameters, Clinical chemistry parameters,

Mean and standard deviation were calculated for each group and per time period for bod weight change parameters.

- Body weight and average food consumption/day parameters Total cytochrome P450 content

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.
The F test was performed to compare the homogeneity of group variances.
If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).
If the F test was significant ($p\leq0.05$), data were transformed using the log transformation.
If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.
If the F test was significant ($p\leq0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).
If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

The F test (5) was performed to compare the homogeneity of group variances. If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Enzymatic activities, cell proliferation parameter and Q-PCR data

Mean of the exposed group were compared to the mean of the control group using the exact Mann-Whitney test (2-sided). Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs (9) and for the Q-PCR data which were analyzed using Graph Pad Prism 4.

C. METHODS

A. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). All animals were observed for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose feces.

B. Body weight

Each animal was weighed once on the first day of test substance administration, then at leastweekly thereafter. Additionally, moribund and scheduled sacrifice animals were weighed before necropsy (terminal body weight).

C. Food consumption and compound intake

The weight of food supplied and that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 2 was calculated using the formula:

$$\text{Test item intake (mg/kg/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

D. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed at least once before scheduled sacrifice.

E. Clinical chemistry

On study Day 16, before necropsy, blood samples were taken from the retro-orbital venous plexus of each surviving 15 animals selected for P-450 determination. Animals were diet fasted overnight. Prior to blood sampling animals were anesthetized with Isoflurane ([redacted], France). Blood was collected on clot activator (for serum) for clinical chemistry. Any change in the general appearance of the serum was recorded.
Total bilirubin, urea, creatinine, total cholesterol, total protein and albumin, concentrations, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on serum samples using an Advia 1650 ([redacted], France).

F. Sacrifice and necropsy

On study Day 16, all surviving animals from all groups were sacrificed by exsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices, and all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded, but not sampled. Necropsy was performed at precise time interval in the morning.

G. Organ weight and tissue collection

Brain and liver were weighed fresh at scheduled sacrifice only.
Duodenum and two central sections of the liver taken in the left and median lobes of the liver of 15 animals per group selected randomly were collected and fixed by immersion in neutral buffered 10% formalin fixative for microscopic examination.
A liver sample was retained for potential additional cell proliferation analysis.
The entire liver of the other 15 animals per group was used for microsomal preparation. Moreover, a small piece of median of left liver lobes of these animals was collected and stored frozen below -70°C for phase I enzyme (cytochromes) and phase II enzyme (UDPGTs and Sulfotransferases) gene transcript analyses by Quantitative Polymerase Chain reaction (Q-PCR) analyses

a) For conventional histopathological examination and cell proliferation assessment:

Histological sections containing 2 liver samples and one piece of duodenum from 15 animals per group selected randomly were processed and embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and submitted to a conventional histopathological examination.

b) For cell proliferation assessment:

An immunohistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all surviving selected study animals. A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of

the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all surviving selected animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

H. Histopathology

Prepared liver slides were examined for all selected animals. Following the initial histopathological examination, a review of representative slides was performed by a second pathologist according to standard operating procedures.
For cell proliferation assessment, the zonal labeling index, expressed as the number of BrdU-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 perilobular cells using an automatic image analysis system. The mean labeling indexes (perilobular, centrilobular and combined) and standard deviations were calculated for each group.

I. Hepatotoxicity testing

P-450 activities

At final necropsy, the remaining portions of the liver also used for gene expression analysis was pooled by group of three and homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 and UDPGT-isoenzyme activities, to check the hepatotoxic potential of the test substance Phenobarbital.
Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. A single quantification was performed for each sample.
Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

and by HPLC with fluorimetric detection following derivatization by 4 -(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate).

Phase I: cytochromes P-450 and their induction:

Family	Enzymatic activity	Activity	Typical inducing agents
CYP 1A1 1A2	EROD	Activation of mutagens and carcinogens	β-naphtoflavone
CYP 2 2B1 2B2 2E	PROD	Detoxication of drugs and chemicals activation of nitrosamines	phenobarbital isoniazid

CYP 3A1 & 3A2	BROD	Detoxication of drugs and chemicals	pregnenolone 16 α - carbonitrile phenobarbital
CYP 4A	Lauric acid hydroxylation	Peroxisomal proliferation	clofibric acid

Enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) using a spectrophotometry method with 4-nitrophenol or bilirubin as substrate.

PCR ANALYSIS

Total cytoplasmic RNA was isolated from the liver of individual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies). Five µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, AmpliTaq Gold® PCR Master Mix on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

The list of Taqman assays used was as follows:

Gene family	Isoform	Refset ID	Taqman assay ID (Applied Biosystems)
Cytochrome P450	Cyp1a1	NM_009992.2	Mm00487218_m1
Cytochrome P450	Cyp2b9	NM_010000.2	Mm00657910_m1
Cytochrome P450	Cyp2b10	NM_009992.3	Mm00456588_mH
Cytochrome P450	Cyp2e1	NM_021282.2	Mm00491127_m1
Cytochrome P450	Cyp3a11	NM_007818.3	Mm00731567_m1
Cytochrome P450	Cyp4a10	NM_010011.2	Mm01188913_g1
Epoxyhydrolase	Ephx1	NM_010145.2	Mm00468752_m1
Epoxyhydrolase	Ephx2	NM_007940.3	Mm00514706_m1
Sulfotransferase	Sult1a1	NM_133670.1	Mm00467072_m1
Sulfotransferase	Sult1d1	NM_016771.3	Mm00502030_m1
UDP glucuronosyltransferase	Ugt1a1	NM_201645.2	Mm02603337_m1
UDP glucuronosyltransferase	Ugt2b1	NM_152811.1	Mm00514184_m1
UDP glucuronosyltransferase	Ugt2b5	NM_009467.1	Mm01623253_s1
Beta-2 microglobulin	B2m	NM_009735.2	Mm00437762_m1

Beta-2 microglobulin (B2m) was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct = (Cttest - CtB2m)$ treated - $(Cttest - CtB2m)$ control

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal TT1M2574.

2. RESULTS AND DISCUSSION

1. OBSERVATIONS

A. Clinical signs of toxicity

No treatment-related clinical signs were observed throughout the course of the study.

B. Mortality

One animal from group 2 (TT2M2612, Phenobarbital 80 mg/kg/day, sub-group 1) was sacrificed for humane reasons on Study Day 6 on the basis of clinical signs (reduced motor activity, prostration, tremors). No clear factor having contributed to the poor health condition of this animal was established either after gross or limited microscopic examination

2. BODY WEIGHT AND BODY WEIGHT GAIN

Propylene thiourea dietary administration at a nominal concentration of 1000 ppm induced a statistically significant reduction in mean body weight during the entire study period (reduced by between 3.2% p≤0.05 on Study day 8 and 4.9% p≤0.01 on Study Day 15). This effect resulted from a mean body weight loss of -0.13 g (p≤0.05) and of -0.03 g (p≤0.01) compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15. An overall cumulative body weight loss of 1.1 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls.

Phenobarbital administration by gavage at 80 mg/kg/day induced a statistically significant reduction in mean body weight during the entire study period (reduced by between 6.4% p≤0.01 on Study day 8 and 7.8% p≤0.01 on Study Day 15). This effect resulted from a mean body weight loss of -0.27 g (p≤0.01) and of -0.01 g (p≤0.05) compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15. An overall cumulative body weight loss of 2.0 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls.

3. FOOD CONSUMPTION AND COMPOUND INTAKE

Mean food consumption was reduced by 8% (p≤0.05) and by 6% (p≤0.01) in the PTU treated group compare to the controls, respectively during the first and the second week of the study.

Mean food consumption was reduced by 10% (p≤0.05) and by 8% (p≤0.01) in the PB treated group, respectively during the first and the second week of the study.

4. ACHIEVED DOSAGE

The mean achieved dosage intake of PTU throughout the study was 139 mg/kg/ day.

5. WATER CONSUMPTION

Mean water containing BrdU consumption in Group 3 (PTU) animals was increased by 14% (not statistically significant) whereas mean water containing BrdU consumption in Group 2 (PB) animals was decreased by 19% (p≤0.01) compared to the control values.

6. CLINICAL CHEMISTRY

When compared to the controls, higher mean total cholesterol (+33%, p≤0.01), total protein (+7%, p≤0.01) and albumin (+8%, p≤0.01) concentrations were noted in the treated group. Additionally, mean alkaline phosphatase activity was higher (+24%, p≤0.05) and mean urea concentration was lower (-22%, p≤0.05) relative to the control group.

When compared to the controls, lower mean total bilirubin (-63%, p≤0.01) and total cholesterol (-21%, p≤0.01) concentrations were noted in the Phenobarbital group.

7. SACRIFICE AND PATHOLOGY

1. Terminal body weight and organ weight

Mean terminal body weight was lower (-6%, p≤0.01) in group 3 (propylene thiourea) males and was also lower (-7%, p≤0.01) in group 2 (phenobarbital) males, when compared to control animals.

Mean absolute and relative liver weights were statistically significantly higher in group 3 (propylene thiourea) males and were also increased in group 2 (phenobarbital) males, when compared to control animals. These changes were considered to be treatment-related.

Table 5.8.1-5 Mean liver weight ±SD at scheduled sacrifice (% change when compared to controls)

Sex	Male		
Dose group	Control	Phenobarbital 80 mg/kg	Propylene thiourea 1000 ppm
Mean absolute liver weight	1.21±0.09	1.44±0.13** (+19%)	1.39±0.11** (+15%)
Mean liver to body weight ratio	3.975±0.254	5.086±0.369** (+28%)	4.856±0.284** (+22%)
Mean liver to brain weight ratio	280.729±23.998	341.603±32.788** (+22%)	327.747±26.825** (+17%)

*: p≤0.05; **: p≤0.01

2. Necropsy

Enlarged liver was found in 8/30 males in group 3 (propylene thiourea) and was also found in 9/29 males in group 2 (phenobarbital). Since this change was associated with microscopic findings and higher weights, it was considered to be treatment-related.

Table 5.8.1-6 Incidence of macroscopic changes in the liver- scheduled sacrifice

Sex	Male		
Dose group	Control	Phenobarbital 80 mg/kg	Propylene thiourea 1000 ppm
Enlarged	0/30	9/29	8/30

3. Microscopic pathology

Centrilobular hepatocellular hypertrophy was found in group 3 (propylene thiourea) males and was also found in group 2 (phenobarbital) males; this change was considered to be treatment-related. However, there were some slight differences in the morphological appearance of the hypertrophy. No change or a slight exacerbated centrilobular hepatocellular vacuolation was observed in group 3 (propylene thiourea) males, whereas in 14/14 group 2 (phenobarbital) males, hepatocellular hypertrophy was associated with a decreased centrilobular hepatocellular vacuolation.

Table 5.8.1-7 Incidence of microscopic changes in the liver- scheduled sacrifice

Sex	Male		
Dose group	Control	Phenobarbital 80 mg/kg	Propylene thiourea 1000 ppm
Number examined	15	14	15
Hepatocellular hypertrophy: centrilobular			
Minimal	0	0	0
Slight	0	2	0
Moderate	0	12	15
Total	**0**	**14**	**15**

4. Cell Proliferation

In the centrilobular area, the mean BrdU labeling index was found to be higher (approximately 3 times) in animals treated with propylene thiourea at 1000 ppm, when compared to the controls. This change in the centrilobular area was similar to what was observed in animals treated with phenobarbital at 80 mg/kg.

In the perilobular area, the mean BrdU labeling indexes in animals treated with propylene thiourea (1000 ppm) and phenobarbital (80 mg/kg) were similar to the controls.

Table 5.8.1-8 Cell Proliferation

Dose group		BrdU positive cells centrilobular zone	BrdU positive cells perilobular zone	Total BRDU positive cells
Control	N	15	15	15
	Mean	7.38	5.49	6.38
	STD	4.74	3.23	3.62
Phenobarbital 80 mg/kg	N	14	14	14
	Mean	23.87**	6.60	14.23**
	STD	9.70	3.59	4.25
Propylene thiourea 100 ppm	N	15	15	15
	Mean	22.06**	8.36	14.33*
	STD	16.78	7.27	9.88

**: p≤0.01

The total BrdU labeling index (centrilobular + perilobular) was found to be higher (approximately 2 times) in animals treated with propylene thiourea at 1000 ppm, when compared to the controls.

This change (centrilobular + perilobular areas) was similar to what was observed in animals treated with phenobarbital at 80 mg/kg.

5. Hepatotoxicity testing

Total cytochrome P-450:

Total cytochrome P-450 content was slightly increased (+30%; p≤0.01) by treatment with propylene thiourea 1000 ppm, when compared to the controls. The results obtained with phenobarbital 80 mg/kg displayed a significant increase (p≤0.01) of total cytochrome P-450 contents by 109%, when compared to the control group.

Enzymatic activities:

Propylene thiourea administration induced the following changes:

c) No change in EROD, PROD and BROD activity in male mice when compared to the control groups.

d) No change in UDPGT (bilirubin) activity when compared to the control group.

Phenobarbital administration induced the following changes:

- A very slight increase in EROD activity (+ 60%, p≤0.01) when compared to the control groups. A high increase in PROD activity (+ 3639%, p≤0.01).
- A very high increase in BROD activity (+11236%, p≤0.01) in the male mice treated with Phenobarbital when compared to the control group.
- A high increase (+141%, p≤0.01) in UDPGT (bilirubin) activity.

Table 5.8.1-9 Total Cytochrome P-450 content and specific isoenzyme P-450 and UDPGT activities (% change when compared to controls)

Dose group		Control	Propylene urea 2500 ppm (diet)	Phenobarbital 80 mg/kg/day (gavage)
Total P-450 (nmol/mg protein)	N	5	5	5
	Mean	0.76	0.98 **	1.59 **
	STD	0.06	0.06	0.11
			(+30%)	(+109%)
EROD (nmol/min/mg protein)	N	5	5	5
	Mean	39.82	42.47 NS	63.82 **
	STD	3.00	3.27	2.14
			(NC)	(+60%)
PROD (nmol/min/mg protein)	N	5	5	5
	Mean	3.81	3.28 NS	105.06 **
	STD	0.28	1.02	8.20
			(NC)	(+3639%)
BROD (nmol/min/mg protein)	N	5	5	5
	Mean	5.12	5.63 NS	580.40 **
	STD	0.78	0.75	75.44
			(NC)	(+11236%)
UDPGT Bilirubin (nmol/min/mg protein)	N	5	5	5
	Mean	0.387	0.355 NS	0.933 **
	STD	0.005	0.090	0.096
			(NC)	(+141%)

**: p≤0.01; NS: not statistically significant; N: represents a pool number of 3 livers; NC: no change.

Q-PCR ANALYSIS

Propylene thiourea administration induced the following changes:

- Cyp1a1 and Cyp3a11 gene transcripts were down regulated (-48%, p≤0.05 and -43%, p≤0.001; respectively) and Cyp2b9 gene transcripts were up regulated (+301%, p≤0.05).
- Sult1a1 and Ugt2b1 gene transcripts were down regulated (-15%, p≤0.05 and -23%, p≤0.01; respectively) and Ephx1 gene transcripts were up regulated (+43%, p≤0.001).

Phenobarbital administration induced the following changes:

- Cyp2b9, Cyp2b10 and Cyp3a11 gene transcripts were up regulated (+2116%, p≤0.001, +13307% p≤0.001 and +313%, p≤0.001; respectively).
- Ephx1, Sult1a1, Sult1d1, Ugt1a1 and Ugt2b1 gene transcripts were up regulated (+88%, p≤0.001, +59%, p≤0.001, +354%, p≤0.001, +239%, p≤0.001 and +46%, p≤0.001; respectively).

Table 5.8.1-10 Q-PCR Analysis: Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)

Gene transcripts	Control	Phenobarbital (80 mg/kg/day)	Propylene Thio Urea (1000 ppm)
Cyp1a1	1.034 ± 0.803	1.139 ± 0.538	0.541* ± 0.175 (-48)
Cyp2b9	39.080 ± 40.388	866.299*** ± 838.837 (+2116)	156.914* ± 157.949 (+301)
Cyp2b10	3.664 ± 3.590	491.245*** ± 225.745 (+13307)	4.864 ± 3.900
Cyp2e1	0.858 ± 0.163	2.071 ± 4.875	0.787 ± 0.185
Cyp3a11	0.821 ± 0.182	3.396*** ± 1.402 (+313)	0.465*** ± 0.142 (-43)
Cyp4a10	0.958 ± 0.383	2.503 ± 6.962	0.861 ± 0.313
Ephx1	0.950 ± 0.187	1.783*** ± 0.416 (+88)	1.356*** ± 0.352 (+43)
Ephx2	0.870 ± 0.136	0.757 ± 0.183	0.708 ± 0.167
Sult1a1	1.003 ± 0.223	1.600*** ± 0.305 (+59)	0.848* ± 0.161 (-15)
Sult1d1	0.735 ± 0.354	3.338*** ± 1.592 (+354)	0.714 ± 0.283
Ugt1a1	0.975 ± 0.217	3.302*** ± 0.964 (+239)	0.967 ± 0.136
Ugt2b1	0.980 ± 0.217	1.434*** ± 0.384 (+46)	0.755** ± 0.145 (-23)
Ugt2b5	0.891 ± 0.107	0.948 ± 0.166	0.948 ± 0.323

*: Statistically different from the control group (p≤0.05)
**: Statistically different from the control group (p≤0.01)
***: Statistically different from the control group (p≤0.001)

3. CONCLUSIONS

Comparison of the findings observed with PTU and Phenobarbital indicate that PTU hepatotoxicity mode of action share some commun characteristic with Phenobarbital, although a firm conclusion cannot be drawn from this study.

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Propylene urea (PU)

Three new studies are submitted that were not included in the Baseline Dossier and in the addendum to the monograph.
Here below a summary of the toxicological profile of PTU is presented, based on all the available studies
PU is of very low oral acute toxicity: the rat acute oral LD_{50} is higher than 5000 mg/kg bw.
In the mouse oncogenicity, PU was administered via the diet to groups of 50 male and 50 female CF_1 mice at concentrations of 0, 50, 500 and 2500 ppm (equivalent to 0, 8, 83, 502 and 0, 11, 115, 619 mg/kg bw/day in males and females, respectively) for 24 months. At 2500 ppm, males exhibited lower body weights throughout the entire study period, and these deviations from control were also statistically significant up to study week 18 (about 10% less). Similarly body weight of top dose females was statistically (about 10%) lower at the beginning of the study (up to study week 11). The liver was the target organ in both sexes: an increased incidence of enlarged and swollen livers and increased incidence of centrilobular hyperplasia of the liver was observed from 500 ppm groups. At 2500 ppm the incidence of hepatocellular adenoma and carcinoma in both sexes was higher than the control. The NOAEL was 50 ppm (equivalent to 8 and 11 mg/kg bw/day).
An epigenetic mechanism is presumed for the genesis of the liver tumour because there was no evidence of mutagenic potential in a series of in vitro test. The bacterial reverse mutation test was negative, as well as the new chromosome aberration tests and mammalian cell forward mutation tests performed in 2009.

A special study was conducted in 2009 to further investigate the mechanism behind liver tumor formation observed in the chronic study with CF_1 mice. CFW male were given diets containing PU at concentration of 2500 ppm (equivalent to approximately 360 mg/kg bw/day) for 15 days. The following parameters were assessed: liver cell proliferation, cytochrome P-450 content and enzymatic activity of specific P-450 isoforms and UDP-GT; phase I and phase II enzymes gene transcripts were analyzed by Q-PCR. Results did not show any effects on and liver weight.
Microscopic evaluation revealed focal hepatocellular single cell necrosis and an increased number of mitoses and Küpffer cell hyperplasia in the majority of the treated animals, increased cell proliferation (the overall BrdU labeling index was 30 times higher than the controls).
P-450 isoenzyme and UDPGT activities revealed a decrease in mean total cytochrome P-450 Quantitative PCR analyses of transcripts of genes of phase I enzymes revealed a down-regulation of Cyp2el and Cyp4al0 transcripts and an up-regulation of Cyp2b9 transcripts. Concerning the phase II enzymes, epoxyhydrolase 2, sulfotransferases and UDP glucoronosyltransferases gene transcripts were down-regulated (Ephx2 -50% $p<0.001$, Suit lal -15% $p<0.01$, Sultldl -48% $p<0.001$, Ugtlal -51%o $p<0.001$ and Ugt2bl -34% $p<0.001$) and Ephxl gene transcript was up-regulated (447% $p<0.001$).

Results suggest that PU provoked liver tumor due to cytotoxicity.

A summary of the toxicity effects of PU is given in the table here below.

Table 5.8.1-11 Summary of toxicity studies with propyleneurea (bold new studies)

Type of study	**Species/test system**	**Result**
Acute oral toxicity [redacted]. ; 1977 M-104834-01-2	Rat	LD50 > 5000 mg/kg bw
[redacted].; 1980 M-116160-01-1	TA 98, TA100, TA1535 TA 1537	Negative
In vitro chromosome aberration test with Chinese hamster V79 cells. [redacted].;2008 M-299106-01-1	Chinese hamster V79 cells	Negative
V79/HPRT-test in vitro for the detection of induced forward mutations [redacted]; 1996 M-301079-01-1	Chinese hamster V79 cells	Negative
Type of study	**NO(A)EL (mg/kg bw/day)**	**Effects at LOAEL and higher doses**
PropyleneUrea - Chronic toxicological study on mice (two year feeding study) 0, 50, 500 and 2500 ppm. 0, 8, 83 and 502 mg/kg body weight (♂) 0, 11, 115 and 619 mg/kg day (♀) [redacted] 1981 M-050194-01-1	8(♂) – 11(♀)	502 – 610 mg/kg bw/day: both sexes ↑incidence hepatocellular adenoma and carcinomas 83 – 115 mg/kg bw/day: both sexes ↑incidence of hepatic centrilobular hyperplasia
Studies on the mode of action		
Propylene Urea - Mechanistic 14-day toxicity in the male mouse at 2500 ppm (equivalent to approximately 360mg/kg bw/day) [redacted].;2009 M-360438-01-1	Investigation of hepatotoxicity, induction of P-450 cell proliferation and gene transcriptinvestigation)	Microscopic examination revealed focal hepatocellular single cell necrosis and an increased number of mitoses in the majority of the treated animals and Küpffer cell hyperplasia in 6/15 mice. Cell proliferation was 30-fold higher than the controls

Report:	[redacted]q; [redacted];2008;M-299106-01
Title:	AE 1379609 (propylene urea) - (project: AE 0172747) - In vitro chromosome aberration test with Chinese hamster V79 cells
Report No:	AT04452
Document No:	M-299106-01-1
Guidelines:	**OECD 473(1997); EEC 2000/32/EC Method B10; US EPA OPPTS 870 5375 (August 1998);not specified**
GLP/GEP:	**yes**

Executive summary

The clastogenic potential of AE 1379609 (Propylene Urea) was evaluated in a chromosome aberration test in vitro.
Initially Chinese hamster V79 cells were exposed in the absence and in the presence of S9 mix for 4 hours to concentrations of 275, 550 and 1100 µg/ml of AE 1379609 (Propylene Urea). Cultures of all concentrations were harvested 18 hours after the beginning of the treatment. In addition, cells treated with 1100 ug/ml were harvested 30 hours after the beginning of the treatment. Without S9 mix an additional experiment was performed using continuous treatment for 18 hours, harvest at the same time, and AE 1379609 (Propylene Urea) concentrations of 275, 550 and 1100 µg/ml.
There were no relevant cytotoxic effects were observed either without or with S9 mix.
None of the cultures treated with AE 1379609 (Propylene Urea) in the absence and in the presence of S9 mix showed biologically relevant increased numbers of aberrant metaphases.
The positive controls mitomycin C and cyclophosphamide induced clastogenic effects and demonstrated the sensitivity of the test system and the activity of the used S9 mix.
Based on this test, AE 1379609 (Propylene Urea) is considered not to be clastogenic for mammalian cells in vitro.

Material and methods

Test Material: AE 1379609 (Propylene Urea)

- Description: Fine white powder
- Lot/Batch: RDL 125-5-4
- Purity: 99.6 % (analytical result dated December 6, 2007)
- CAS: 6531-31-3
- Stability of test compound: The batch used was analytically examined prior to study initiation and was approved for use for the test period. A stability test in the solvent did not reveal significant degradation of the active ingredient

Solvent used: Deionized water

Control materials:

- Negative: Tissue Culture medium
- Solvent: Deionized water
- Positive: non- activation (-S9 mix): Mitomycin C in Hanks' balanced salt solution (Biochrom)., final concentration 0.1 µg/mL 0.1 for a treatment period of 4 hours and 0.03 µg/ml for a treatment period of 18 hours

Positive: with activation (+S9 mix): Cyclophosphamide in Hanks' balanced salt solution (Biochrom), final concentration 2 µg/mL

Activation : The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male Sprague Dawley rats. The used S9 fraction was derived from the preparation dated March 20, 2007. Prior to first use, each batch was checked for its metabolizing capacity by using 2 ug/ml cyclophosphamide; appropriate clastogenic activity was demonstrated. In addition, each batch was tested in parallel for possible contamination, possible cytotoxic effects and possible clastogeniceffects. Only batches without those effects were used
The S9 mix contained 40% S9 fraction. Cofactor solution per 25 ml S9 mix contained:
Sodium phosphate buffer (200 mM, pH 7.4) 15.0 ml, $MgCl_2$ x 6 H_2O 40.7 mg, KCl 61.5 mg, Glucose-6-phosphate (disodium salt) 38.0 mg and NADP (disodium salt) 78.8 mg.

Test cells :
V79 cells were obtained from [redacted] AG, [redacted]. The cells arrived at the Toxicology of Bayer HealthCare AG, [redacted] on November 8, 1993.

Culture medium:
Prior to the start of the study Chinese hamster V79 cells from a frozen permanent, which was stored in liquid nitrogen, were normally grown in 20 ml medium and 75 cm^2 flasks or under comparable conditions. Incubation of the cells was always performed at 37°C in a CO_2-incubator (5% CO_2). Unless reported otherwise, cells were grown in medium containing 10% fetal calf serum [PCS = fetal bovine serum (FBS)].
As medium, PAA Ready Mix was used. PAA Ready mix is commercially available by PAA, Paching, Austria and consists of Eagle's minimal essential medium (MEM, Earle) and consisted of1 % L-glutamine, 1% MEM-vitamins, 1 % MEM NEAA, 1 % Pen-Strep and either 10% FBS (=FCS) or 2% FBS (=FCS). A routine check for mycoplasma was performed on April 3, 2007 (when the used frozen permanent was prepared). There was no evidence of mycoplasma contamination. Therefore no check for mycoplasma contamination was needed for the actual culture.

Test compounds concentration used (µg/ml):
Based on the results of the pre-tests, the following concentrations of propylene urea, were selected in the main studies:

Harvest time in 18 hours	**4-hour treatment**
Non activated conditions	0, 275, 550 and 1100
Activated conditions	0, 275, 550 and 1100
Harvest time in 30-hours	**4-hour treatment**
Non activated conditions	0 and 1100
Activated conditions	0, 275, 550 and 1100
Harvest time in 18 hours	**18-hour treatment**
Non activated conditions	0, 275, 550 and 1100
Activated conditions	0, 275, 550 and 1100

Study Design and Methods:
Study performance
The study was conducted in the laboratory of Genetic Toxicology of Bayer Healthcare AG, 42096 [redacted] (Germany). The experimental start and completion dates of the study were 22 January 2008 and 4 February 2008, respectively. Chinese hamster V79 cells can be kept in culture as established cell lines (Kao and Puck, 1967). The mean generation time of the used cell line is approximately twelve hours.

Solvent selection, solubility, pH and osmolality
For AE 1379609 (Propylene Urea), deionized water was selected as solvent because in this solvent the tets material was soluble at least up to 110 mg/ml.
Concentrations of up to 1100 µg/ml AE 1379609 (Propylene Urea) did not change the pH in the medium in the pre-test.
The osmolality in the medium of the pre-test was not changed by concentrations of up to 1100 µg/ml AE 1379609 (Propylene Urea).

Preliminary cytotoxicity Assay
Cytotoxic effects of the test substance were assessed in the pre-test as well as in the main-study. Cell survival as well as mitotic index were determined in the presence and absence of S9 mix.
At the end of the respective incubation period cells of all cultures of the respective period were trypsinized, and an appropriate dilution was counted using a hemocytometer (improved Neubauer) to determine cell survival.
The mitotic index was determined for all cultures. The number of mitotic cells among a total of 1000 cells per culture was determined. All cells which were not in interphase were defined as mitotic.
In the main study, cultures with a total incubation period of 8 hours were additionally and exclusively used to determine the cytotoxicity of AE 1379609 (Propylene Urea).

Cytogenetic assay

Cell treatment:

For the treatment without S9 mix, 20 ml of fresh solutions of medium containing 2% PCS and 0.2 ml solution of AE 1379609 were added to each flask.

For the treatment with S9 mix, 19 ml of fresh solutions of medium containing 2% PCS, 1 ml of S9 and 0.2 ml solution of AE 1379609 were added to each flask.

The cells were incubated for the respective period at 37°C.

After 4 hours of treatment, the medium was removed; the cells were washed with PBS. 20 ml of fresh medium containing 10 % PCS was added to the flasks and the flasks were placed in a CO_2-incubator for the remaining incubation time.

In cultures treated for 18 hours medium was not removed.

Positive controls and solvent controls (0.2 ml solvent per culture), and, if indicated, untreated controls (no addition of solvent) were set up in parallel and handled as described for AE 1379609 (Propylene Urea)-treated cultures.

Untreated controls and solvent controls were used as negative controls.

Spindle inhibition:

0.2 ml Colcemid-solution (40 µg/ml water) were added to each flask two hours prior to the end of the incubation period to arrest the cells in a metaphase-like stage of mitosis (c-metaphase).

Cell harvest:

The medium was removed from each flask and cells were removed from the bottom of the flask by trypsinization and suspended in medium. This medium was transferred to a centrifuge tube and spun for approximately 5 minutes at 700 rpm. The supernatant was carefully removed and 1-2 ml of a hypotonic solution (0.4% KCl; 37°C) was added to the tube. Within 4 minutes, the volume was brought to 6 ml with additional hypotonic solution and cells were resuspended. The cells were sedimented in the centrifuge as before and the supernatant was removed..

Slide preparation:

A few drops of cold (4°C) fixative [ethanol/acetic acid (3:1)] were added and mixed carefully with the cells. The volume was adjusted to 6 ml with the fixative and mixed again with the cells. The mixture was incubated at room temperature for 20 minutes. Cells were pelleted as before and the supernatant was discarded. Cells were again resuspended in fixative as before and centrifuged. Pelleted cells were resuspended carefully in a small volume of fresh fixative. This suspension was dropped onto clean slides. The slides were allowed to dry for at least 2 hours. Thereafter, they were submerged in pure methanol for 3 minutes and stained for 15-20 minutes in 3% Giemsa solution. Slides were rinsed twice in water and once in acetone and were then kept in xylene for about 30 minutes. The slides were allowed to dry completely and covered.

At least two slides were generated per culture. All solutions used during this preparation were freshly prepared each time. The Giemsa solution was filtered before usage.

Metaphase analysis:
The selected slides were coded. Coded slides were evaluated using a light microscope at a magnification of about 1000. Chromosomes of approximately 200 metaphases per concentration, 100 metaphases from each of two parallel cultures, were examined. In most cases at least 100 assessable metaphases were present on one slide prepared from an individual culture. Therefore, the back-up slide which was generated routinely from every culture was normally not utilized for the evaluation.

Assessment criteria:
An increased incidence of gaps of both types without concomitant increase of other aberration types was not considered as indication of a clastogenic effect.
A test was considered positive, if there was a relevant and statistically significant increase in the aberration rate.
A test was considered negative, if there was no such increase at any time interval. A test was also considered negative, if there were statistical significant values, which were, however, within the range of historical negative controls.
In addition, a test was considered equivocal if there was an increase of aberrant metaphases above the range of the laboratory historical negative controls, provided the increase was not significant and the result of the negative control was not closely related to the data of the respective treatment group.
A test was also considered equivocal, if its result was implausible.

Assay Acceptance Criteria
An assay was acceptable, if there was a biologically relevant increase in chromosome aberrations induced by the positive controls and if the numbers of aberrations for the negative controls were in the expected range based on results from our laboratory and from published studies.

Statistics
The statistical analysis was performed by pair-wise comparison of AE 1379609 (Propylene Urea)-treated and positive control groups to the respective solvent control group.
The mitotic index was statistically analyzed (provided that it was reduced compared to the mean of the corresponding solvent control) using the one-sided chi²-test.
The numbers of metaphases with aberrations excluding gaps were compared (provided that these data superceded the respective solvent control). The statistical analysis followed the recommendations outlined by Richardson et al. (1989). The one-sided chi2-test was used for the statistical evaluation.
A difference was considered to be significant, if the probability of error was below 5 %.

Results and discussion
There was no evidence of substance precipitation in the medium under all the treatment conditions in all the pre-test studies.
Concentrations used in the surviving cell and in the mitotic index pre-studies were: 0, 5, 10, 25, 50, 100, 250, 500 and 1100 µg/ml.

Mitotix index

Both with and without S9 mix the mitotic indices in the treated cultures were not relevantly reduced compared with the negative controls. The cultures treated with mitomycin C (without S9) or cyclophosphamide (with S9) showed also no reduction in mitosis rate.

Surival index

Both with and without S9 mix the survival indices in the treated cultures were not relevantly reduced compared with the negative controls. The cultures treated with mitomycin C (without S9) or cyclophosphamide (with S9) showed also no reduction in mitosis rate.

Cytogenetic assay - Chromosome Aberrations

Without S9 mix:

No biologically relevant and statistically significant increases of numbers of metaphases with aberrations were detected after 4 hours treatment and total culture times of 18 or 30 hours.

No biologically relevant increases of numbers of metaphases with aberrations were detected for a treatment period and total culture time of 18 hours. In agreement with the laboratory assessment criteria the statistically significance observed at 1100 µg/ml in chromosomal aberrations after 18 hour treatment period without metabolic activation was considered to be of no biological relevance, since it was within the range of the historical controls

The treatment with the positive control mitomycin C resulted in a clear and statistically significant increase of metaphases with aberrations and demonstrated the sensitivity of the test system.

AE 1379609 (Propylene Urea) with S9 mix:

No biologically relevant and statistically significant increases of numbers of metaphases with aberrations were detected after 4 hours treatment and total culture times of 18 or 30 hours.

The positive control cyclophosphamide induced statistically significant and biologically relevant increases of metaphases with aberrations and demonstrated the sensitivity of the test system and the activity of the used S9 mix.

Table 5.8.1-11: Chromosome aberration test without metabolic activation (-S9) : 4 hour-treatment

Harvest Time (hours)	Concentrations (µg/ml)		Cells with aberrations Excluding gaps Total number (%)	Cells with aberrations Including gaps Total number (%)
18	Control	Water	4 (2.0)	4 (2.0)
	PU	275	0 (0.0)	0 (0.0)
		550	1 (0.5)	1 (0.5)
		1100	3 (1.5)	3 (1.5)
	Control	0.1 (MMC)	65 (37.5)**	65 (37.5)**

Table 5.8.1-12: Chromosome aberration test with metabolic activation (+S9): 4 hour-treatment

Harvest Time (hours)	Concentrations (µg/mL)		Cells with aberrations Excluding gaps Total number (%)	Cells with aberrations Including gaps Total number (%)
18	Control	Water	9 (4.5)	9 (4.5)
	PU	275	18 (9.0)	18 (9.0)
		550	10 (5.0)	10 (5.0)
		1100	7 (3.5)	6 (3.0)
	Control	2 (CPA)	94 (47.0)***	93 (46.5)**

** = p< 0.01

Table 5.8.1-13: Chromosme aberration test with and without metabolic activation (±S9) 4 hour- treatment and harvest time: 30 hours

Experimental group	Concentration (µg/mL)	Cells with aberrations Excluding gaps Total number (%)	Cells with aberrations Including gaps Total number (%)
Without metabolic activation			
Water	0	2 (1.0)	2 (1.0)
PU	1100	3 (1.5)	2 (1.0)
With metabolic activation			
Water	0	2 (1.0)	2 (1.0)
PU	1100	1 (0.5)	1 (0.5)

Conclusions
Based on the results of this test, AE 1379609 (Propylene Urea) is considered not to be clastogenic for mammalian cells in vitro.

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Report:	[redacted]g; [redacted];2008-M-301079-01
Title:	AE 1379609 (propylene urea) [project: Propineb (AE F 074263)] - V79/HPRT-test in vitro for the detection of induced forward mutations
Report No:	AT04546
Document No:	M-301079-01-1
Guidelines:	**OECD 476 (1997)** **EEC 2000/32/EC Method B17 (2000)** **UP EPA OPPTS 870.5300 (August 1998); deviation not specified**
GLP/GEP:	**yes**

Executive summary
AE 1379609 (Propylene Urea) was evaluated for point mutagenic effects at the hypoxanthine-guanine phosphoribosyl transferase locus (forward mutation assay) in V79 cell cultures after treatment with concentrations of up to and including 1088 ug/ml, both with and without S9 mix.

AE 1379609 induced no decreases in survival to treatment or in relative population growth both with and without S9 mix when tested up to the requested limit concentration of 10 mM, which is equal to 1001 µg/ml AE 1379609.
There was no biologically relevant increase in mutant frequency above that of the negative controls both with and without S9 mix.
Ethylmethanesulfonate and Dimethylbenzanthracene induced clear mutagenic effects and demonstrated the sensitivity of the test system and the activity of the S9 mix.
Based on these results, AE 1379609 is considered to be non-mutagenic in the V79/HPRT Forward Mutation Assay, both with and without metabolic activation.

I. MATERIALS AND METHODS

1. MATERIALS:

1. Test Material: AE 1379609 (Propylene Urea)

Description: White, crystalline powder

Lot/Batch #: RDL125-5-4

Purity: 99.6 % (analytical result dated December 6, 2007)

CAS #: 6531-31-3

Stability of test compound: The batch used was analytically examined prior to study initiation and was approved for use for the test period. A stability test in the solvent2 did not reveal significant degradation of the active ingredient

Solvent used: Deionized water was selected as solvent. In this solvent AE 1379609 was soluble up to 110 mg/mL.

2. Control Materials:

Negative: Cells were exposed to vehicle alone either with or without metabolic activation. The final concentration of the vehicle (solvent is used synonymously for vehicle) in the medium of this control did not exceed 1% (v/v). Cells of the untreated controls remained completely untreated.

Positive:

Non-activation (- S9): Ethyl methanesulfonate (EMS) at a final concentration of 900 µg/mL (EMS is a liquid, no solvent was needed).

Activation (+S9): Dimethylbenzanthracene (DMBA) in DMSO at a final concentration of 20 µg/mL.

3. Activation: S9 mix was used for the simulation of the mammalian metabolism. The S9 fraction was isolated in house from the livers of Aroclor 1254 induced male Sprague Dawley rats. Prior to first use, each batch was checked for its metabolizing capacity by using 20 µg/mL DMBA; appropriate mutagenic activity was demonstrated. In addition, each batch was tested in parallel for possible contamination, possible cytotoxic effects and possible mutagenic effects. Only batches without

those effects were used.
For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with a cofactor solution (2:3). The S9 mix was kept on ice until use and only used on the same day.

S9 mix composition:

Component	Concentration
Sodium phosphate buffer	60 % (v/v)
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2 \times 6\ H_2O$	8 mM
S9 fraction	40 % (v/v)

4. Test Cells: Male Chinese hamster V79 cells (lung) obtained from Prof. G. Speit, University of [redacted], Germany.

5. Culture medium: PAA Ready Mix which and consists of Eagle's minimal essential medium (MEM, Earle) and the following supplements:

PAA Ready Mix (10% FBS)	PAA Ready Mix (2% FBS)
1% L-glutamine	1% L-glutamine
1% MEM-vitamins	1% MEM-vitamins
1% MEM NEAA	1% MEM NEAA
1% Pen/Strep	1% Pen/Strep
10% FBS(=FCS)	2% FBS (=FCS)

PAA Ready Mix (10% FBS) is referred to as culture medium. During treatment with AE 1887196, PAA Ready Mix (2% FBS) was used.

6. Test Concentrations:

Preliminary cytotoxicity study: 35, 70, 140, 280, 560, 840 and 1120 µg/mL without and with metabolic activation.

Main study: 34, 68, 136, 272, 544, and 1088 µg/mL without and with metabolic activation.
Concentrations of up to 1100 µg/ml AE 1379609 did not change the pH in the medium of the pre-test. The osmolality in the medium of the pre-test was not changed
by concentrations of up to 1100 µg/ml AE 1379609.

2. TEST PERFORMANCE

The study was conducted form 25 January to 12 March 2008 in the Genetic Toxicology laboratory of Bayer HealthCare AG, [redacted] (Germany)

- **Determination of Cytotoxicity**

Exponentially growing V79 cells were plated in 20 ml culture medium in a 75 cm^2 flask with a total of volume 275 ml, (4x106cells per flask). For each concentration one culture was available. After attachment (16-24 hours later), cells were exposed without S9 mix to vehicle alone and to a range of concentrations of the test substance for 5 hours in 20 ml medium containing 2% FCS.
In experiments with metabolic activation 1 ml of medium was replaced by 1 ml S9 mix. Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 5 ml culture medium at a density of 200 cells into each of 3 Petri dishes (0 60 mm). These dishes were incubated for 6 to 8 days to allow colony development.
Thereafter, colonies were fixed with 95% methanol, stained with Giemsa (Merck; stock solution diluted 1:5 with deionized water) and counted. If not interfered e.g. by precipitation on the plates or coloration of the plates, colonies were counted automatically using Biologies Accu Count 1000. Data were transferred to a PC and processed with the released MS Windows XP based HPRT Study Manager of Bayer HeaithCare AG, which was prepared with MS Excel 2002 and includes also counting of mutant colonies. For the preparation of the report tables MS Word 2002 was used in addition.
Cytotoxicity was expressed by comparison of colonies in treated cultures versus negative control cultures (relative cloning efficiency).

- **Treatment Protocol without Metabolic Activation**

The method is based on the publication of Myhr and DiPaolo (1978). Exponentially growing V79 cells were plated in culture medium at a final volume of 20 ml in two 75 cm^2 flasks per concentration (4x106 per flask) including all control groups. After attachment (16-24 hours later), the cells were exposed for 5 hours in 20 ml culture medium with reduced serum content (2%). The corresponding controls were incubated under the same conditions. Thereafter, cell monolayers were washed with PBS, trypsinized and replated in 20ml culture medium using 1.5x106 cells per 75 cm2 flask and in 5 ml culture medium using 200 cells per Petri dish (Ø 60 mm). Per culture one flask and 3 Petri dishes were used.
The Petri dishes were incubated (normally 6 days) to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment ("Survival to Treatment").
Cells in 75 cm^2 flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured (= count 1, normally after 3 days) by reseeding $1.5x10^6$ cells into 20 ml medium in 75 cm^2 flasks. At the end of the expression period (= count 2, normally a total of 6 days), cultures were reseeded in Petri dishes (Ø 100 mm) at 3x10 cells per dish (8 dishes per culture) in 20 ml culture medium without hypoxanthine but containing 10 ug/ml 6-TG for selection of mutants. In addition, 200 cells per dish (Ø 60 mm, 3 dishes per culture) were seeded in 5 ml culture medium to determine the absolute cloning efficiency for each concentration. After incubation for 6 to 8 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes.
At least two trials will be performed. Mutant frequencies for at least four concentrations should be determined in each trial.

- **Treatment Protocol with Metabolic Activation**

The activation assay was performed independently. The procedure was identical to the nonactivation assay except for the addition of S9 mix. In these experiments 19 instead of 20 mL culture medium and

additionally 1 mL of S9 mix were added to the flasks for the treatment period, resulting in a concentration of 5% S9 mix in the cultures. The number of 6-TG resistant mutants and viability were determined as in the nonactivation assay.

- **Data Presentation**

All data are presented in tabular form; using descriptive statistical methods (mean and standard deviation). While calculations were performed with absolute numbers, rounded data are presented in the tables.

The parameter **"Survival to Treatment"** in % was determined on the basis of the following calculation:

$$\frac{\text{mean number of colonies (treated cultures)} \times 100}{\text{mean number of colonies (negative control cultures)}}$$

The **"Absolute Population Growth"** was calculated using the following formula:

$$\text{Absolute Population Growth} = \text{cell no. count 1} \times \text{cell no. count 2}$$

The parameter **"Relative Population Growth"** shows the cumulative growth of the treated cell populations, relative to the negative control.

$$\frac{\text{Absolute Population Growth treated culture} \times 100}{\text{Absolute Population Growth of corresponding negative control culture}}$$

The ability of cells to form colonies at the time of mutant selection is measured by the parameter **"Absolute Cloning Efficiency"**. It is expressed in %.

$$\frac{\text{Mean number of colonies per dish} \times 100}{200}$$

The **"Mutant Frequency"** is calculated for each group by dividing the total number of mutant colonies by the number of cells seeded (usually 8-10 plates at 3×10^5 cells per plate), corrected for the absolute cloning efficiency. The mutant frequency is expressed as 6-TG resistant mutants per 10^6 clonable cells.

$$\frac{\text{total number of mutant colonies} \times 100}{\text{number of evaluated dishes} \times 3{\times}10^5 \times \text{CE}}$$

- **Acceptance Criteria**
  - The average cloning efficiency of the negative controls should be at least 50%.
  - The average of mutant frequency of the negative controls should not exceed $25{\times}10^{-6}$ cells.
  - The mutant frequency of the two cultures of the negative and/or the untreated control should differ only to an acceptable extent. As a rule of thumb, the difference of mutant frequencies should not be greater than $5{\times}10^{-6}$.
  - The positive control should induce an average mutant frequency of at least three times that of the negative control.

- If not limited by the solubility of the test substance in the vehicle the highest concentration should induce cytotoxicity of about 80 to 90% or should be a concentration where precipitation occurs in the medium. The survival at the lowest concentration should be in the range of the untreated control.
- For the calculation of an acceptable mutant frequency at least 5 dishes per culture should be available and relative survival to treatment, relative population growth and absolute cloning efficiency of negative controls and AE 1887196 treated groups should be 10% or greater.

However, these criteria may be overruled by good scientific judgement.

- **Assessment criteria**

A. Mutant frequencies will only be used for assessment, if at least 5 dishes per culture were available and relative survival to treatment, relative population growth and absolute cloning efficiency were 10% or greater.

B. A trial will be considered positive if a concentration-related and in parallel cultures reproducible increase in mutant frequencies is observed. To be relevant, the increase in mutant frequencies should be at least two to three times that of the highest negative or negative control value observed in the respective trial. If this result can be reproduced in a second trial, the test substance is considered to be mutagenic.

C. Despite these criteria, a positive result will only be considered relevant, if no significant change in osmolality compared to the negative control can be observed. Otherwise, unphysiological culture conditions may be the reason for the positive result (Scott etal, 1991).

D. A test substance will be judged as equivocal if there is no strictly concentration related increase in mutation frequencies but if one or more concentrations induce a reproducible and biologically relevant increase in mutant frequencies in all trials.

E. An assay will be considered negative if no reproducible and relevant increases of mutant frequencies were observed.

However, these criteria may be overruled by good scientific judgement

- **Statistics**

The statistical analysis relies on the mutant frequencies which are submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights (Hsie et al., 1981; Arlett et al., 1989). According to the acceptance criteria mutant frequencies based on less than 5 plates and/or on a relative survival to treatment and/or a relative population growth and/or an absolute cloning efficiency below 10% are not included in the statistical analysis. The two mutant frequency values obtained per group are, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay requires at least two independent trials, the overall analysis without respectively with activation is the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses were run for each trial in order to examine the consistency of the results.

All acceptable groups are included in the weighted analysis of variance followed by pairwise comparisons to the negative control on a nominal significance level of α = 0.05 using the Dunnett

test (Dunnett, 1955). The regression analysis part is performed on the basis of the actual concentrations thereby omitting the positive, untreated and negative controls. If there is a significant concentration related increase of the mutant frequency (a = 0.05) in the main analysis the highest concentration will be dropped and the analysis will be repeated. This procedure will be repeated until p > 0.05. In that way eliminated concentrations are flagged correspondingly.

II. RESULTS AND DISCUSSION

1. GENERAL REMARKS

In the absence and in the presence of S9 mix Chinese hamster V79 cells were exposed to AE 1379609 at concentrations of up to and including 1088 µg/ml. No substance precipitation occurred in the medium both with and without S9 mix. The means of the absolute cloning efficiency for the negative controls in the mutation experiments were 75.1% and 94.1% in the experiments without activation. In experiments with metabolic activation 76.8% and 86.9% were observed. These results demonstrate good cloning conditions for the experiments.

2. MUTATION ASSAY

- **Mutation assay without metabolic activation**

Under nonactivation conditions two trials were performed (Tables 3 and 4). The mutant frequencies of the untreated controls and of the negative controls were all within the normal range. The positive control EMS induced clear mutagenic and statistically significant effects in all trials. AE 1379609, which was tested up to the requested limit concentration of 10 mM equal to 1091 µg/ml AE 1379609, did not induce increases in mutant frequencies. In addition, the overall statistical analysis reveals no statistically significant increases

Therefore, AE 1379609 was evaluated as non-mutagenic in the non-activation trial.

Table 5.8.1-14: HPRT-Test without metabolic activation. Date of treatment 25.01.2008

Compound Concentration (µg/mL)	Survival to treatment (colony)			Population growth		Mutant colonies in all dishes	Cloning efficiency		Mutant frequency
	mean	SD	in % of NC	Absolute $\times 10^{12}$	in % of NC		%	SD	$\times 10^{6}$
Untreated control	165.	15.3	101.6	657.9	108.0	14	90.7	6.6	6.4
	170.3	5.7	99.2	830.6	134.2	5	84.0	2.2	2.5
Deionised water	162.7	16.3	100,0	609.0	100.0	10	94.3	3.3	4.4
	171.7	11.1	100.0	632.9	100.0	11	93.8	2.8	4.9
Propylene Urea									
34	149.	4.6	91.8	703.1	115.5	13	75.5	1.5	7.2
	161.0	10.5	93.8	675.0	106.7	9	78.7	1.9	4.8
68	154.7	14.0	95.1	739.4	121.4	25	82.8	2.0	12.6
	143.0	10.8	83.3	808.0	127.7	14	73.3	3.3	8.0
136	146.0	4.0	89.8	682.8	112.1	7	78.2	2.0	3.7
	139.0	5.0	81.0	771.4	121.9	14	75.9	2.0	7.7
272	163.3	9.2	100.4	669.5	109.9	19	89.7	5.8	8.8
	152.7	5.1	88.9	641.3	101.3	6	77.8	1.0	3.2
544	146.7	4.5	90.2	575.4	94.5	9	83.3	5.6	4.5
	134.0	6.1	78.1	903.5	142.8	4	69.2	3.5	2.4
1088	154.7	3.1	95.1	855.5	140.5	11	73.7	4.4	6.2
	170.3	17.2	99.2	878.6	138.8	9	74.2	4.5	5.1
EMS	100.7	4.2	61.9	230.3	37.8	753	69.4	5.5	450.4
900	45.7	3.2	26.6	165.0	26.1	657	52.3	3.7	513.3

- **Mutation assay with metabolic activation**

Two assessable trials were performed with S9 mix (Tables 5 and 6). The mutant frequencies of the untreated controls and of the negative controls were all within the normal range. The positive control DMBA induced clear mutagenic and statistically significant effects in all trials.
For AE 1379609 treated cultures, no cytotoxic effects of 80% to 90% were induced. AE 1379609 was tested up to the requested limit concentration of 10 mM equal to 1001 µg/mlAE 1379609.
AE 1379609 induced no relevant increases in mutant frequencies. In addition, the overall statistical analysis reveals no statistically significant increase.
With metabolic activation AE 1379609 was therefore evaluated as non-mutagenic.

Table 5.8.1-15 : HPRT-Test with metabolic activation. Date of treatment 29.02.2008

Compound Concentration (µg/mL)	Survival to treatment (colony)			Population growth		Mutant colonies in all dishes	Cloning efficiency		Mutant frequency
	mean	SD	in % of NC	Absolute $\times 10^{12}$	in % of NC		%	SD	$\times 10^{6}$
Untreated control	190.7	2.5	102.1	522.5	62.2	6	93.0	5.8	2.7
	191.7	6.5	95.2	534.3	99.2	8	83.7	15.2	4.0
Deionised water	186.7	1.5	100.0	840.4	100.0	5	79.5	6.0	2.6
	201.3	8.5	100.0	538.4	100.0	5	94.2	11.0	2.2
Propylene Urea									
34	189.0	7.0	101.3	795.4	94.6	5	78.7	3.8	2.6
	186.0	12.2	92.4	695.3	129.2	5	87.3	6.5	2.4
68	185.3	8.1	99.3	4428	52.7	7	105.7	7.8	2.8
	206.0	5.6	102.3	684.8	127.2	5	88.2	8.1	2.4
136	199.0	6.1	106.6	796.2	94.7	10	82.7	6.0	5.0
	216.3	2.1	107.5	529.4	98.3	6	97.	7.6	2.6
272	168.0	10.6	90.0	678.9	80.4	4	89.3	13.3	1.9
	205.3	11.0	102.0	555.8	103.2	4	98.2	3.8	1.7
544	167.7	4.9	89.8	439.7	52.3	6	109.7	6.2	2.3
	205.3	21.4	102.0	664.1	123.3	2	89.5	7.8	0.9
1088	184.0	13.5	98.6	446.3	53.1	6	106.	3.3	2.4
	219.3	12.0	108.9	622.7	115.7	5	79.3	5.8	2.6
DMBA	160.7	11.4	86.1	298.1	35.5	80	81.7	2.6	40.8
20	120.0	2.6	59.6	112.0	20.8	119	84.5	5.6	58.7

III. CONCLUSIONS

Results of the study showed that AE 1379609 was non-mutagenic in the V79/HPRT Forward Mutation Assay, both with and without metabolic activation.

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Report:	KCA 5.8.1 /35; [redacted] ;2009;M-360438-01-1
Title:	Propylene Urea - Mechanistic 14-day toxicity study in the male mouse (hepatotoxicity-cell proliferation and gene transcript investigations)
Report No:	SA 09091
Document No:	M-360438-01-1
Guidelines:	**US EPA OPPTS 870.3PP**
GLP/GEP:	**no**

Executive Summary

The objective of this study was to investigate the effects of propylene urea (PU) on the liver following continuous dietary administration for 14 days in the male mouse. Liver cell proliferation, total cytochrome P-450 levels, liver enzymatic activities (P-450 and UDPGT) and gene transcript investigations (P-450 and phase II enzymes) were assessed as well as histopathology of the liver.

Propylene Urea (PU - AE 1379609, batch number RDL 125-21-1: a white powder, 96.3% w/w purity) was administered continuously via the diet to group of 30 male Crl:CFW (SW) mice for at least 15 days at concentration of 2500 ppm (equivalent to approximately 360 mg/kg/day). A similarly constituted group received untreated diet and acted as a control group. Finally, a third additional group of 30 male mice received 80 mg/kg body weight/day of Phenobarbital (suspension w/v in 0.5% aqueous solution of methylcellulose) by oral gavage once per day (10ml/kg) and acted as a positive control group. These two last groups (control and positive control groups) were part of another study (SA 09069) run in parallel to the current study in the
same animal room.

Animals were observed at least daily for clinical signs and twice daily for mortality. Detailed physical examinations were performed weekly. Body weight and food consumptions were recorded once weekly. Water consumptions (BrdU administration period) were recorded on the day of scheduled sacrifice. Selected clinical chemistry parameters were determined at day 16. All animals were necropsied, selected organs weighed and portions of liver of 15 animals out of 30 were taken and fixed for conventional histopathology examination as well as for cell proliferation measurement. Small pieces of liver of the remaining 15 animals were used for phase I and phase II enzymes gene transcripts analyses by Q-PCR, the remaining were used for microsomal preparations in order to determine total cytochrome P-450 content, cytochrome P-450 specific isoenzyme profiles and UDPGT activities.

In the group receiving PU, mean body weight was reduced during the entire study period. On study Day 15, the mean body weight in the treated group was 4.9% lower than the control group. This effect resulted from a mean body weight loss of -0.13 g and of -0.03 g compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15. An overall cumulative body weight loss of 1.1 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls.

Mean food consumption was reduced by 8% and by 6% compared to the controls, respectively during the first and the second week of the study. Higher mean total cholesterol (+33%, $p \leq 0.01$), total protein (+7%, $p \leq 0.01$), albumin concentrations (+8%, $p \leq 0.01$) and mean alkaline phosphatase activity (+24%, $p \leq 0.05$) were observed as compared to the control values. Additionally, mean urea concentration (-22%, $p \leq 0.05$) was lower relative to the control group.

At necropsy, mean terminal body weight was lower (-6%, $p \leq 0.01$) when compared to control
animals.

Microscopic examination revealed focal hepatocellular single cell necrosis and an increased number of mitoses in the majority of the treated animals and Küpffer cell hyperplasia in 6/15 mice. Assessment of cell proliferation in the liver revealed a 30 times higher mean BrdU labeling index in the centrilobular area in treated animals, when compared to the controls, as well as a 35 times higher mean BrdU labeling index in the perilobular area, when compared to the controls. The overall BrdU labeling index (centrilobular + perilobular) was higher (approximately 30 times) than the controls.

The assessment of the P-450 isoenzyme and UDPGT activities revealed a decrease in mean total cytochrome P450 content (-26%; $p \leq 0.01$) without any apparent change in EROD, PROD, BROD and UDPGT (bilirubin) activity when compared to the control groups.

Quantitative PCR analyses of transcripts of genes of phase I enzymes revealed a down-regulation of Cyp2e1 and Cyp4a10 transcripts and an up-regulation of Cyp2b9 transcripts. Concerning the phase II

enzymes, epoxyhydrolase 2, sulfotransferases and UDP glucoronosyltransferases gene transcripts were down-regulated (Ephx2 -50% p<0.001, Sult1a1 -15% p<0.01, Sult1d1 -48% p<0.001, Ugt1a1 -51% p<0.001 and Ugt2b1 -34% p<0.001) and Ephx1 gene transcript was up-regulated (+47% p<0.001).

Among animals receiving ***Phenobarbital (positive control)*** at 80 mg/kg/day by gavage, one animal was sacrificed for humane reasons on Study Day 6 due to clinical signs (reduced motor activity, prostration, soiled fur and half-closed eyes). No clear cause of the animal status was established either after gross orlimited microscopic examination.

Mean body weight was reduced during the entire study period. On study Day 15, the mean body weight in the treated group was 7.8% lower than the control group. This effect resulted from a mean body weight loss of -0.27 g and of -0.01 g compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15.

An overall cumulative body weight loss of 2.0 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls. Mean food consumption was reduced by 10% and by 8% compared to the controls, respectively during the first and the second week of the study.

Lower mean total bilirubin (-63%, p≤0.01) and total cholesterol concentrations (-21%, p≤0.01) were observed compared to the control values.

At necropsy, mean terminal body weight was lower (-7%, p≤0.01) when compared to control animals. Mean absolute and relative liver weights were increased by between 15 and 22% (p≤0.01) when compared to control animals. Enlarged liver was found in 9/29 males. Microscopic examination revealed a centrilobular hepatocellular hypertrophy associated with a decreased centrilobular hepatocellular vacuolation.

Assessment of cell proliferation in the liver revealed a 3 times higher mean BrdU labeling index in the centrilobular area in treated animals, when compared to the controls, whilst a similar mean BrdU labeling index was noted in the perilobular area. The overall BrdU labeling index (centrilobular + perilobular) was higher (approximately 2 times) than the controls.

The assessment of the P-450 isoenzyme and UDPGT activities revealed an increase in mean total cytochrome P450 content (+109%, p ≤0.01), a very slight increase in EROD activity (+60%, p≤0.01), a high increase in PROD activity (+3639%, p ≤0.01), a very high increase in BROD activity (+11236%, p ≤0.01) and a high increase in UDPGT (bilirubin) activity (+141%, p≤0.01). Quantitative PCR analyses of transcripts of genes of phase I enzymes revealed an up-regulation of Cyp2b9, Cyp2b10 and Cyp 3a11 transcripts. Concerning the phase II enzymes, epoxyhydrolase (Ephx1), sulfotransferase (Sult1a1 andSult1d1), UDP glucoronosyltransferase (Ugt1a1 and Ugt2b1) gene transcripts were upregulated.

Comparison of the findings observed with PU and Phenobarbital indicate that PU hepatotoxicity mode of action does not share the same characteristics of Phenobarbital.

I MATERIALS AND METHODS

A. MATERIALS

1. Test Material:	Propylene urea (AE F074263)
Description:	White powder
Lot/Batch #:	RDL 125-21-1
Purity:	96.3 %
CAS #:	6531-31-3
Stability of test compound:	The stability of the test substance at 2500 ppm in the diet was determined during the study for a time period which covers the period of storage and usage for the current study.
2. Vehicle and/or positive control:	Phenobarbital, a liver enzyme inducer in rodents (batch number 06100228; a white powder, 99.6% purity) was used as positive control.
Other chemical:	BrdU (5-Bromo-2'-deoxyuridine), an analogue of thymidine (batch number 097K0675; a white powder, 99.9% purity), was used to evaluate cell proliferation in the study.
3. Test Animals	
Species:	Mouse
Strain:	Crl:CFW (SW)
Age:	11 weeks old
Weight:	Mean group weight of males: 30.4 to 36.8 g;
Source:	[redacted] Laboratories, USA.
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from [redacted], available *ad libitum,* except before blood sampling prior to sacrifice when animals were diet fasted overnight.
Water:	Filtered and softened tap water from the municipal water supply, *ad libitum*.
Housing:	Individually in suspended, stainless steel, wire-mesh cages. The cage of each animal was identified by a card bearing a unique identification number.
Environmental conditions	
Temperature:	20-24 °C
Humidity:	40-70 %
Air change:	10 to 15 changes/h
Photoperiod:	12 h dark/ 12 h light (7 am – 7 pm)

B. STUDY DESIGN

1. In life dates:

The study was conducted from 22 April to 7 July 2009 in the laboratory of the toxicology centre of Bayer CropScience in [redacted] (France).

2. Animal assignment and treatment

The dose levels were selected based on the evaluation of the results from a previous oncogenicity study conducted with PU (M-).

The oral route was selected as it is an accepted route of exposure by regulatory authorities and since it is a possible route of human exposure.

Control animals were fed control diet for at least 14 days. Test animals were fed diet containing PU at 2500 ppm for at least 14 days. Additionally, a positive control group was fed control diet and received phenobarbital at 80 mg/kg/day by oral gavage (10 ml/kg) for the same period. A solution of BrdU at 80 mg of BrdU/100 ml of drinking water was administered to all animals during the last week of the study.

Table 5.8.1-16 Study design

Test group	Dose level	Animals assigned (males)
Control	0	30
PU	2500 (ppm)	30
Phenobarbital	80 mg/kg bw/day	30

3. Diet preparation and analysis

PU was incorporated into the diet to provide the required dietary concentrations. The test substance was ground to a fine powder before being incorporated into the diet by dry mixing. There was one preparation at 2500 ppm for the study with the exception of Day 1 where only the animals from the sub-group 1 (group 3) received a first non homogeneous preparation of PU at 2500 ppm. Thereafter, the new preparation was used throughout the study. When not in use, the diet formulations were stored at approximately -18° C. The unused residue was discarded at the end of each administration period. For first formulation of propylene urea (Fl), homogeneity and concentration results ranged between 74 and 117% of the nominal concentration. As regard to these results (low homogeneity and one result out of the target range) this formulation (Fl) was discarded during the study and replaced by a second formulation (see protocol amendment 1). Homogeneity and concentration results of second formulation (Flbis) ranged within 91 and 117% of the nominal concentration and were therefore within the target range. The stability of the test substance at 2500 ppm in the diet was determined during the study for a time period which covers the period of storage and usage for the current study.

Phenobarbital was suspended (w/v) in an aqueous solution of methylcellulose 400 at 0.5%. There was one preparation at 8 g/l for the study. When not in use, the suspensions were stored at +5° C (±3°C). The suspensions were mixed continuously before and during dosing with an electromagnetic stirrer. Analysis of the preparations showed that the homogeneity and concentration results ranged between 98 and 99% of the nominal concentration.

A solution of BrdU in filtered tap water from the municipal water supply was prepared at a concentration of 80 mg of BrdU/100 ml of drinking water. Bottles containing BrdU in drinking water were stored at room temperature and were protected from light.Analysis of the formulation indicated that the concentration of BrdU was 104%.

4. Statistics

Mean and standard deviation were calculated for each group.

All statistical analyses were carried out separately for males and females. Group means were compared at the 5% and 1% levels of significance. Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics). The following parameters were statistically analyzed:

- Body weight parameters
- Body weight change parameters calculated according to time intervals
- Average food consumption/day parameters calculated according to time intervals
- Clinical chemistry parameters
- Terminal body weight, absolute and relative organ weights parameters
- Total cytochrome P450 content and liver enzyme activities
- Cell proliferation
- Gene transcript data

Mean and standard deviation were calculated for each group.

The Phenobarbital (Group 2; gavage) and PU (Group 3; diet) treated groups were compared to the control group (Group 1; diet) using the following procedures.

- Body weight change parameters, Terminal body weight, absolute and relative organ weight parameters, Clinical chemistry parameters

Mean and standard deviation were calculated for each group and per time period for bod weight change parameters

- Body weight and average food consumption/day parameters Total cytochrome P450 content

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.

The F test was performed to compare the homogeneity of group variances.

If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided).

If the F test was significant ($p\leq0.05$), data were transformed using the log transformation.

If the F test on log transformed data was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided) on log transformed data.

If the F test was significant ($p\leq0.05$) even after log transformation, mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

If one or more group variance(s) equal 0, means were compared using non-parametric procedures.

The F test (5) was performed to compare the homogeneity of group variances. If the F test was not significant ($p>0.05$), mean of the exposed group was compared to the mean of the control group using the t-test (2-sided). If the F test was significant ($p\leq0.05$), mean of the exposed group was compared to the mean of the control group using the modified t-test (2-sided).

- Enzymatic activities, cell proliferation parameter and Q-PCR data

Mean of the exposed group were compared to the mean of the control group using the exact Mann-Whitney test (2-sided). Group means were compared at the 5% and 1% levels of significance.

Statistical analyses were carried out using Path/Tox System V4.2.2. (Module Enhanced Statistics) except for liver enzyme parameters and cell proliferation parameters which were analyzed using SAS programs (9) and for the Q-PCR data which were analyzed using Graph Pad Prism 4.

C. METHODS

1. Observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). All animals were observed for clinical signs at least once daily. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility, and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health, such as blood or loose feces.

2. Body weight

Each animal was weighed once on the first day of test substance administration, then at leastweekly thereafter. Additionally, moribund and scheduled sacrifice animals were weighed before necropsy (terminal body weight).

3. Food consumption and compound intake

The weight of food supplied and that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 2 was calculated using the formula:

$$\text{Test item intake (mg/kg/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

4. Water consumption

Drinking water bottles containing BrdU were weighed on the first day of BrdU administration. Empty water bottles were weighed at least once before scheduled sacrifice..

5. Clinical chemistry

On study Day 16, before necropsy, blood samples were taken from the retro-orbital venous plexus of each surviving 15 animals selected for P-450 determination. Animals were diet fasted overnight. Prior to blood sampling animals were anesthetized with Isoflurane ([redacted], France). Blood was collected on clot activator (for serum) for clinical chemistry. Any change in the general appearance of the serum was recorded.

Total bilirubin, urea, creatinine, total cholesterol, total protein and albumin, concentrations, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-

glutamyltransferase activities were assayed on serum samples using an Advia 1650 ([redacted], France).

6. Sacrifice and necropsy

On study Day 16, all surviving animals from all groups were sacrificed byexsanguination under deep anesthesia by inhalation of Isoflurane ([redacted], France). Animals were diet fasted overnight prior to sacrifice. All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Significant macroscopic abnormalities were recorded, but not sampled. Necropsy was performed at precise time interval in the morning.

7. Organ weight and tissue collection

Brain and liver were weighed fresh at scheduled sacrifice only.

Duodenum and two central sections of the liver taken in the left and median lobes of the liver of 15 animals per group selected randomly were collected and fixed by immersion in neutral buffered 10% formalin fixative for microscopic examination.

A liver sample was retained for potential additional cell proliferation analysis.

The entire liver of the other 15 animals per group was used for microsomal preparation. Moreover, a small piece of median of left liver lobes of these animals was collected and stored frozen below -70°C for phase I enzyme (cytochromes) and phase II enzyme (UDPGTs and Sulfotransferases) gene transcript analyses by Quantitative Polymerase Chain reaction (Q-PCR) analyses

a) For conventional histopathological examination and cell proliferation assessment:

Histological sections containing 2 liver samples and one piece of duodenum from 15 animals per group selected randomly were processed and embedded in paraffin wax. Histological sections, stained with hematoxylin and eosin, were prepared and submitted to a conventional histopathological examination.

b) For cell proliferation assessment:

An immunohistochemical staining demonstrating the incorporation of BrdU and the determination of the labeling index were performed to assess hepatocytic cell cycling on all surviving selected study animals. A section from a formalin-fixed paraffin-embedded block containing 2 liver samples and one duodenum sample was prepared (the duodenum has a high proliferation rate and serves as a positive staining control). The immunohistochemical reaction included incubation with a monoclonal antibody raised against BrdU, amplification with a secondary biotinylated antibody and a streptavidin-horseradish peroxidase complex, detection of the complex with the chromogen diamino-benzidine (DAB) and nuclear counterstaining with hematoxylin. The immunohistochemical staining for BrdU and determination of the labeling index were performed on all surviving selected animals showing sufficient BrdU incorporation (estimated by water consumption or duodenum BrdU labeling).

8. Histopathology

Prepared liver slides were examined for all selected animals. Following the initial histopathological examination, a review of representative slides was performed by a second pathologist according to standard operating procedures.
For cell proliferation assessment, the zonal labeling index, expressed as the number of BrdU-positive hepatocytes per thousand, was measured separately on random fields comprising at least 1000 centrilobular and 1000 perilobular cells using an automatic image analysis system. The mean labeling indexes (perilobular, centrilobular and combined) and standard deviations were calculated for each group.

9. Hepatotoxicity testing

P-450 activities

At final necropsy, the remaining portions of the liver also used for gene expression analysis was pooled by group of three and homogenized for microsomal preparations in order to determine total cytochrome P-450 content and specific cytochrome P-450 and UDPGT isoenzyme activities, to check the hepatotoxic potential of the test substance Phenobarbital.
Total cytochrome P-450 content in microsomal preparations was determined by spectrophotometry using a reduced CO differential spectrum. A single quantification was performed for each sample.
Specific cytochrome P-450 enzymatic activities were evaluated by spectrofluorimetry using the following substrates:

- ethoxyresorufin (EROD)
- pentoxyresorufin (PROD)
- benzoxyresorufin (BROD)

and by HPLC with fluorimetric detection following derivatization by 4-(bromomethyl)-7-methoxycoumarin of 12-hydroxy-lauric acid (lauric acid used as substrate).

Phase I: Cytochromes P-450 and their induction:

Family	Enzymatic activity	Activity	Typical inducing agents
CYP 1A1 1A2	EROD	Activation of mutagens and carcinogens	β-naphtoflavone
CYP 2 2B1 2B2 2E	PROD	Detoxication of drugs and chemicals activation of nitrosamines	phenobarbital isoniazid
CYP 3A1 & 3A2	BROD	Detoxication of drugs and chemicals	pregnenolone 16 α - carbonitrile phenobarbital
CYP 4A	Lauric acid hydroxylation	Peroxisomal proliferation	clofibric acid

Enzymatic activities were also determined by measuring UDP-glucuronosyltransferase (UDPGT) using a spectrophotometry method with 4-nitrophenol or bilirubin as substrate.

PCR ANALYSIS

Total cytoplasmic RNA was isolated from the liver of individual control and treated animals using RNeasy Mini kits (Qiagen). RNA quality controls were performed based on the ribosomal RNA electrophoretic profiles using a Bioanalyser (Agilent Technologies). Five µg of total RNA was used for Reverse transcription (RT) using a High Capacity cDNA Archive kit (Applied Biosystems). The assay was performed in duplicate using Taqman assays (Assay on demand, Applied Biosystems), 1/50 diluted first strand cDNA, AmpliTaq Gold® PCR Master Mix on an ABI prism 7900 HT machine (Applied Biosystems). For each gene transcript measured, a negative control condition was included in which H2O MQ was used as template instead of first strand cDNA.

The list of Taqman assays used was as follows:

Gene family	Isoform	Refset ID	Taqman assay ID (Applied Biosystems)
Cytochrome P450	Cyp1a1	NM_009992.2	Mm00487218_m1
Cytochrome P450	Cyp2b9	NM_010000.2	Mm00657910_m1
Cytochrome P450	Cyp2b10	NM_009992.3	Mm00456588_mH
Cytochrome P450	Cyp2e1	NM_021282.2	Mm00491127_m1
Cytochrome P450	Cyp3a11	NM_007818.3	Mm00731567_m1
Cytochrome P450	Cyp4a10	NM_010011.2	Mm01188913_g1
Epoxyhydrolase	Ephx1	NM_010145.2	Mn00468752_m1
Epoxyhydrolase	Ephx2	NM_007940.3	Mm00514706_m1
Sulfotransferase	Sult1a1	NM_133670.1	Mm00467072_m1
Sulfotransferase	Sult1d1	NM_016771.3	Mm00502030_m1
UDP glucuronosyltransferase	Ugt1a1	NM_201645.2	Mm02603337_m1
UDP glucuronosyltransferase	Ugt2b1	NM_152811.1	Mm00514184_m1
UDP glucuronosyltransferase	Ugt2b5	NM_009467.1	Mm01623253_s1
Beta-2 microglobulin	B2m	NM_009735.2	Mm00437762_m1

Beta-2 microglobulin (B2m) was selected as reference gene for the quantitative calculations of transcripts. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$\Delta\Delta Ct$ = (Cttest – CtB2m) treated - (Cttest - CtB2m) control

$RQ = 2^{-\Delta\Delta Ct}$

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for the control animal TT1M2574

II RESULTS AND DISCUSSION

- **OBSERVATIONS**
- **Clinical signs of toxicity**

No treatment-related clinical signs were observed throughout the course of the study.

- **Mortality**

One animal from group 2 (TT2M2612, Phenobarbital 80 mg/kg/day, sub-group 1) was sacrificed for humane reasons on Study Day 6 on the basis of clinical signs (reduced motor activity, prostration, tremors). No clear factor having contributed to the poor health condition of this animal was established either after gross or limited microscopic examination.

- **BODY WEIGHT AND BODY WEIGHT GAIN**

PU dietary administration at a nominal concentration of 2500 ppm induced a statistically significant reduction in mean body weight during the entire study period (reduced by between 4.4% $p \leq 0.01$ on Study day 8 and 8.1% $p \leq 0.01$ on Study Day 15). This effect resulted from a mean body weight loss of -0.17 g ($p \leq 0.01$) and of -0.12 g ($p \leq 0.01$) compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15. An overall cumulative body weight loss of 2.0 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls..

Phenobarbital administration by gavage at 80 mg/kg/day induced a statistically significant reduction in mean body weight during the entire study period (reduced by between 6.4% $p \leq 0.01$ on Study day 8 and 7.8% $p \leq 0.01$ on Study Day 15). This effect resulted from a mean body weight loss of -0.27 g ($p \leq 0.01$) and of -0.01 g ($p \leq 0.05$) compared to a mean body weight gain of 0.01 g and of 0.06 g in the controls, respectively between Study Days 1 and 8 and Study Days 8 and 15. An overall cumulative body weight loss of 2.0 g was observed over the entire study period compared to an overall cumulative body weight gain of 0.5 g in the controls.

- **FOOD CONSUMPTION AND COMPOUND INTAKE**

Mean food consumption was reduced by 8% (not statistically significant) compare to the controls during the first week of the study.

Mean food consumption was reduced by 10% ($p \leq 0.05$) and by 8% ($p \leq 0.01$) in the PB treated group, respectively during the first and the second week of the study.

- **ACHIEVED DOSAGE**

The mean achieved dosage intake of PU throughout the study was 360 mg/kg/ day.

- **WATER CONSUMPTION**

Mean water containing BrdU consumption in Group 3 (PU) animals was similar to control values whereas mean water containing BrdU consumption in Group 2 (PB) animals was decreased by 19% ($p \leq 0.01$) compared to the control values.

- **CLINICAL CHEMISTRY**

When compared to the controls, higher mean alanine aminotransferase (+137%, p≤0.01) and alkaline phosphatase (+38%, p≤0.01) activities as well as higher mean total bilirubin (+92%, p≤0.01) and creatinine (+34%, p≤0.05) concentrations were noted in the treated group. Additionally, mean total cholesterol concentration was lower (-19%, p≤0.01) and total protein concentration was slightly lower (-5%, p≤0.05) relative to the control group.

When compared to the controls, lower mean total bilirubin (-63%, p≤0.01) and total cholesterol (-21%, p≤0.01) concentrations were noted in the Phenobarbital group.

- **SACRIFICE AND PATHOLOGY**

6. Terminal body weight and organ weight

Mean terminal body weight was lower (-7%, p≤0.01) in group 3 (Propylene urea) males and was also lower (-7%, p≤0.01) in group 2 (Phenobarbital) males, when compared to control animals.
Mean liver to body weight ratio was statistically significantly higher in group 3 (Propylene urea) males when compared to control animals. This change was considered to be not relevant since it was the result of lower terminal body weight.
Mean absolute and relative liver weights were statistically significantly higher in group 2 (Phenobarbital) males when compared to control animals. These changes were considered to be treatment-related.

Table 5.8.1-17 Mean liver weight ±SD at scheduled sacrifice (% change when compared to controls)

Sex	Male		
Dose group	Control	Phenobarbital 80 mg/kg	Propylene urea 2500 ppm
Mean absolute liver weight	1.21±0.09	1.44±0.13** (+19%)	1.19±0.14 (-2%)
Mean liver to body weight ratio	3.975±0.254	5.086±0.369** (+28%)	4.206±0.476* (+6%)
Mean liver to brain weight ratio	280.729±23.998	341.603±32.788** (+22%)	276.320±32.426 (-2%)

*: p≤0.05; **: p≤0.01

7. Necropsy

Enlarged liver was found in 9/29 males in group 2 (phenobarbital), but not in the group of animals treated with PU.

Table 5.8.1-18 Incidence of macroscopic changes in the liver- scheduled sacrifice

Sex	Male		
Dose group	Control	Phenobarbital 80 mg/kg	Propylene urea 2500 ppm
Enlarged	0/30	9/29	0/30

8. Microscopic pathology

In group 3 (Propylene urea) males, focal hepatocellular single cell necrosis, increased number of mitoses and Küpffer cell hyperplasia were observed and were considered to be treatment-related.
In group 2 (Phenobarbital) males, centrilobular hepatocellular hypertrophy was found and was considered to be treatment-related. In 14/14 males, hepatocellular hypertrophy was associated with a decreased centrilobular hepatocellular vacuolation.

Table 5.8.1-19 Incidence of microscopic changes in the liver- scheduled sacrifice

Sex	Male		
Dose group	Control	Phenobarbital 80 mg/kg	Propylene urea 2500 ppm
Number examined	15	14	15
Hepatocellular single cell necrosis: focal			
Minimal	0	0	2
Slight	0	0	7
Moderate	0	0	1
Total	**0**	**0**	**13**
Increased number of mitoses			
Minimal	0	0	6
Slight	0	0	8
Moderate	0	0	1
Total	**0**	**0**	**15**
Küpffer cell hyperplasia			
Minimal	0	0	6
Total	**0**	**0**	**6**
Hepatocellular hypertrophy: centrilobular			
Minimal	0	0	0
Slight	0	2	0
Moderate	0	12	15
Total	**0**	**14**	**15**

9. Cell Proliferation

In the centrilobular area, the mean BrdU labeling index was found to be higher (approximately 30 times) in males treated with Propylene urea at 2500 ppm, and were also increased (approximately 3 times) in males treated with Phenobarbital at 80 mg/kg, when compared to the controls.
In the perilobular area, the mean BrdU labeling index in animals treated with Propylene Urea (2500 ppm) was higher (approximately 35 times) than the controls, whereas the BrdU index in animals treated with Phenobarbital (80 mg/kg) was similar.

The total BrdU labeling index (centrilobular + perilobular) was found to be increased (approximately 30 times) in males treated with Propylene urea, and was also higher (approximately 2 times) in animals treated with Phenobarbital, when compared to the controls.

Table 5.8.1-20 Cell proliferation

Dose group		BrdU positive cells centrilobular zone	BrdU positive cells perilobular zone	Total BRDU positive cells
Control	N	15	15	15
	Mean	7.38	5.49	6.38
	STD	4.74	3.23	3.62
Phenobarbital 80 mg/kg	N	14	14	14
	Mean	23.87**	6.60	14.23**
	STD	9.70	3.59	4.25
Propylene urea 2500 ppm	N	15	15	15
	Mean	223.60**	196.95**	206.01**
	STD	137.41	130.85	127.62

**: p≤0.01

The total BrdU labeling index (centrilobular + perilobular) was found to be higher (approximately 2 times) in animals treated with propylene thiourea at 1000 ppm when compared to the controls. This change (centrilobular + perilobular areas) was similar to what was observed in animals treated with phenobarbital at 80 mg/kg.

10. Hepatotoxicity testing

Total cytochrome P-450:

Total cytochrome P-450 content were significantly decreased (-26%, p≤0.01) by treatment with propylene urea 2500 ppm, when compared to the controls. The results obtained with Phenobarbital 80 mg/kg displayed a significant increase (+ 109%, p≤0.01) of total cytochrome P-450 content when compared to the control group.

Enzymatic activities:

Propylene urea administration induced the following changes:

- No apparent change in EROD, PROD and BROD activity in male mice when compared to the control groups. A high inter-individual variability was noted in PROD and BROD activities.
- A slight significant decrease (p≤0.01) in UDPGT (bilirubin) activity when compared to the control group.

Phenobarbital administration induced the following changes:

1. A very slight increase in EROD activity (p≤0.01) when compared to the control groups.
2. A high increase in PROD activity (p≤0.01).
3. A very high increase in BROD activity (p≤0.01) in the male mice treated with Phenobarbital when compared to the control group.
4. A significant increase (p≤0.01) in UDPGT (bilirubin) activity.

The magnitude of the changes compared to controls is summarized in the tables below:

Table 5.8.1-21 Total Cytochrome P-450 content and specific isoenzyme P-450 and UDPGT activities (% change when compared to controls)

Dose group		Control	Propylene urea 2500 ppm (diet)	Phenobarbital 80 mg/kg/day (gavage)
Total P-450 (nmol/mg protein)	N Mean STD	5 0.76 0.06	5 0.56 ** 0.10 (-26%)	5 1.59 ** 0.11 (+109%)
EROD (nmol/min/mg protein)	N Mean STD	5 39.82 3.00	5 37.36 NS 5.36	5 63.82 ** 2.14 (+60%)
PROD (nmol/min/mg protein)	N Mean STD	5 2.81 0.28	5 1.62 NS 0.87	5 105.06 ** 8.20 (+3639%)
BROD (nmol/min/mg protein)	N Mean STD	5 5.12 0.78	5 4.48 NS 2.47	5 580.40 ** 75.44 (+11236%)
UDPGT Bilirubin (nmol/min/mg protein)	N Mean STD	5 0.387 0.005	5 0.240 NS 0.068	5 0.933 ** 0.096 (+141%)

**: p≤0.01; NS: not statistically significant; N: represents a pool number of 3 livers.

These data indicate that Propylene urea at a nominal concentration of 2500 ppm during 14 days, did not induce PROD, BROD and UDPGT bilirubin activities and so that PU did not display a Phenobarbital-like enzymatic profile in the male SW mouse.

Q-PCR ANALYSIS

Propylene urea administration induced the following changes:

- Cyp2e1 and Cyp4a10 gene transcripts were down regulated (-61%, p≤0.001 and -46%, p≤0.001; respectively) and Cyp2b9 gene transcripts were up regulated (+280%, p≤0.01).
- Ephx2, Sult1a1, Sult1d1, Ugt1a1 and Ugt2b1 gene transcripts were down regulated (-50%, p≤0.001, -15%, p≤0.01, -48%, p≤0.001, -51%, p≤0.001 and -34%, p≤0.001; respectively) and Ephx1 gene transcripts were up regulated (+47%, p≤0.001).

Phenobarbital administration induced the following changes:

- Cyp2b9, Cyp2b10 and Cyp3a11 gene transcripts were up regulated (+2116%, p≤0.001, +13307%, p≤0.001 and +313%, p≤0.001; respectively).
- Ephx1, Sult1a1, Sult1d1, Ugt1a1 and Ugt2b1 gene transcripts were up regulated (+88%, p≤0.001, +59%, p≤0.001, +354%, p≤0.001, +239%, p≤0.001 and +46%, p≤0.001; respectively).

Table 5.8.1-22 Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)

Gene transcripts	Control	Phenobarbital (80 mg/kg/day)	Propylene Urea (2500 ppm)
Cyp1a1	1.034 ± 0.803	1.139 ± 0.538	0.849 ± 0.436
Cyp2b9	39.080 ± 40.388	866.299*** ± 838.837 (+2116)	148.484** ± 141.251 (+280)
Cyp2b10	3.664 ± 3.590	491.245*** ± 225.745 (+13307)	3.604 ± 3.833
Cyp2e1	0.858 ± 0.163	2.071 ± 4.875	0.334*** ± 0.199 (-61)
Cyp3a11	0.821 ± 0.182	3.396*** ± 1.402 (+313)	0.976 ± 0.885
Cyp4a10	0.958 ± 0.383	2.503 ± 6.962	0.515*** ± 0.309 (-46)
Ephx1	0.950 ± 0.187	1.783*** ± 0.416 (+88)	1.398*** ± 0.268 (+47)
Ephx2	0.870 ± 0.136	0.757 ± 0.183	0.434*** ± 0.163 (-50)
Sult1a1	1.003 ± 0.223	1.600*** ± 0.305 (+59)	0.854** ± 0.413 (-15)
Sult1d1	0.735 ± 0.354	3.338*** ± 1.592 (+354)	0.383*** ± 0.095 (-48)
Ugt1a1	0.975 ± 0.217	3.302*** ± 0.964 (+239)	0.475*** ± 0.091 (-51)
Ugt2b1	0.980 ± 0.217	1.434** ± 0.384 (+46)	0.650*** ± 0.192 (-34)
Ugt2b5	0.891 ± 0.107	0.948 ± 0.166	0.831 ± 0.192

**: Statistically different from the control group ($p \leq 0.01$)

***: Statistically different from the control group ($p \leq 0.001$)

4. CONCLUSIONS

In conclusion the mode of action triggering liver tumours in the mouse appear to be secondary to induced hepatotoxicity.

***

4-Methyl-2-imidazoline

4-Methyl-2-imidazoline (BCS-AB78877) is a plant metabolite of propineb that has been found in the rat metabolism study at about 6% of the amount administered. However it is found in the residues at levels above the threshold for genotoxicity. Therefore, an in vitro genotoxicity package is planned and results showed no concern for genotoxicity.

An acute oral toxicity study in the rat is available for this metabolite and has been submitted in the Baseline Dossier.

Table 5.8.1-23 Studies available with 4-Methylimidazoline

Type of study	Species/test system	Result
Acute oral toxicity [redacted]. ; 1979 M-104849-01-1	Rat	LD_{50} = 1330
[redacted].; 2015 M-491077-02-1	Salmonella typhimurium TA 1535, TA 1537, TA 98, TA 100, and TA 102	Negative
In vitro micronucleus M-491079-02-1	Human lymphocytes	Negative

Report:	KCA 5.8.1 /38; [redacted].;2014;M-491077-02-1
Title:	BCS-AB78877: Salmonella typhimurium reverse mutation assay
Report No:	1645201
Document No:	M-491077-02-1
Guidelines:	**Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471: Bacterial Reverse Mutation Test, adopted July 21, 1997; Commission Regulation (EC) No. 440/2008 B13/14, dated May 30, 2008; EPA Health Effects Test Guidelines, OPPTS 870.5100, Bacterial Reverse Mutation Test; EPA 712-C-98-247, August, 1998**
GLP/GEP:	**yes**

Executive Summary

This study was performed to investigate the potential of BCS-AB78877 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.
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 dissolved in DMSO and tested at the following concentrations:
- Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
- Experiment II : 33; 100; 333; 1000; 2500; and 5000 µg/plate

No precipitation of the test item occurred up to the highest investigated dose.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-AB78877 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.

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

Therefore, BCS-AB78877 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay

I. Materials and Methods

A. Material

1. Test Material:	BCS-AB78877
Description:	colorless liquid
Lot/Batch:	BCOO 6454-11-4
Purity:	98.8%
Stability of test compound:	Stable for at least 22 hours in aqueous solution at pH 7 and at 20°C
2. Control materials:	
Negative:	Culture medium
Solvent:	DMSO
Positive:	Sodium azide (SERVA) for TA 1535, TA 100 at 10 µg/plate in deionized water without S9 mix
	4-nitro-o-phenylene-diamine, 4-NOPD ([redacted]) for TA 1537 at 50 µg/plate in DMSO, for TA 98 at 10 µg/plate in DMSO without S9 mix
	Methyl methane sulfonate, MMS ([redacted]) for TA102 at 2 µL/plate in deionised water without S9 mix
	2-aminoanthracene, 2-AA (SERVA) for TA 1535, TA 1537, TA 98, TA 100, at 2.5 µg/plate in deionized water and for TA 102 at 10 µg/plate in deionized water with S9 mix
3. Test organisms:	
Species:	Salmonella typhimurium LT2 mutants
Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, and TA 98.
Source:	Strains obtained from [redacted] GmbH ([redacted], Germany)
4. Test compound concentrations:	
Range-finding or Experiment I:	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate
Experiment II:	For all strains with or without S9 mix: 33, 100, 333, 1000, 2500, and 5000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed from August 21st to September 22nd, 2014 at [redacted] GmbH - [redacted] CCR ([redacted], Germany).

1. Experimental performance

To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates.
For each strain and dose level, including the controls, three plates were used.
The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL Test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL Bacteria suspension,
- 2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution (solvent control), or reference mutagen solution (positive control), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacteria suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After preincubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.
After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark
The colonies were counted using the Petri Viewer Mk2 (Perceptive Instruments Ltd, Suffolk CB9 7BN, UK) with the software program Ames Study Manager (v.1.21). Due to extensive bacterial colony growth the colonies were partly counted manually.

2. Acceptability of the Assay

The Salmonella typhimurium reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of the laboratory historical data
- the positive control substances should produce a significant increase in mutant colony frequencies
- a minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed (3). A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (2). An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.
A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

4. Statistics

According to the OECD guideline 471, a statistical analysis of the data is not mandatory.

II. Results and discussion

No precipitation of the test item occurred up to the highest investigated dose.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-AB78877 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

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

Table 5.8.1-23 Summary of Experiment I

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	DMSO	0	28 ± 2 B M	9 ± 1	21 ± 4	146 ± 3	390 ± 18
	Untreated	0	35 ± 6 B M	9 ± 1	29 ± 1	150 ± 8	414 ± 47
	BCS-AB78877	3 µg	31 ± 1 B M	11 ± 2	17 ± 6	149 ± 2	413 ± 36
		10 µg	30 ± 2 B M	9 ± 3	22 ± 7	139 ± 3	430 ± 11
		33 µg	30 ± 6 B M	9 ± 2	23 ± 4	150 ± 21	425 ± 14
		100 µg	30 ± 5 B M	9 ± 3	23 ± 7	139 ± 4	432 ± 23
		333 µg	30 ± 5 B M	7 ± 2	24 ± 6	137 ± 5	436 ± 31
		1000 µg	31 ± 4 B M	8 ± 2	25 ± 5	134 ± 18	414 ± 15
		2500 µg	31 ± 5 B M	6 ± 2	24 ± 6	133 ± 1	375 ± 10
		5000 µg	40 ± 5 B M	7 ± 1	20 ± 3	128 ± 2	425 ± 40
	NaN3	10 µg	2805 ± 148			1650 ± 246	
	4-NOPD	10 µg			391 ± 20		
	4-NOPD	50 µg		79 ± 15			
	MMS	2.0 µL					4398 ± 53
With Activation	DMSO	0	19 ± 3	15 ± 6	32 ± 7	138 ± 11	484 ± 7
	Untreated		26 ± 2	15 ± 5	40 ± 5	158 ± 16	541 ± 54
	BCS-AB78877	3 µg	21 ± 4	12 ± 3	39 ± 3	131 ± 7	497 ± 35
		10 µg	20 ± 3	11 ± 4	40 ± 3	136 ± 7	489 ± 54
		33 µg	17 ± 5	14 ± 2	38 ± 7	130 ± 12	515 ± 46
		100 µg	15 ± 6	15 ± 1	35 ± 2	145 ± 3	521 ± 39
		333 µg	16 ± 4	12 ± 2	39 ± 10	131 ± 16	545 ± 65
		1000 µg	19 ± 3	14 ± 5	34 ± 2	131 ± 6	577 ± 16
		2500 µg	31 ± 2	11 ± 2	28 ± 1	126 ± 10	519 ± 95
		5000 µg	24 ± 4	10 ± 1	29 ± 4	136 ± 6	544 ± 12
	2-AA	2.5 µg	455 ± 19	104 ± 9	2982 ± 77	3436 ± 242	
	2-AA	10.0 µg					1307 ± 107

Key to Positive Controls		Key to Plate Postfix Codes	
NaN3	sodium azide	B	Extensive bacterial growth

2-AA	2-aminoanthracene	M	Manual count
MMS	methyl methane sulfonate		
4-NOPD	4-nitro-o-phenylene-diamine		

5.8.1-24 Summary of Experiment II

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	DMSO		11 ± 2	10 ± 1	18 ± 3	133 ± 11	493 ± 22
	Untreated		10 ± 3	10 ± 3	31 ± 8	169 ± 17	487 ± 24
	BCS-AB78877	33 µg	10 ± 2	9 ± 1	20 ± 7	143 ± 13	475 ± 44
		100 µg	11 ± 2	12 ± 2	29 ± 5	127 ± 14	492 ± 30
		333 µg	10 ± 2	9 ± 2	20 ± 4	140 ± 25	509 ± 37
		1000 µg	12 ± 4	9 ± 2	20 ± 4	134 ± 5	508 ± 34
		2500 µg	9 ± 1	9 ± 1	17 ± 4	145 ± 6	509 ± 46
		5000 µg	11 ± 3	6 ± 0	17 ± 2	129 ± 15	522 ± 33
	NaN3	10 µg	1635 ± 48			2073 ± 128	
	4-NOPD	10 µg			322 ± 14		
	4-NOPD	50 µg		88 ± 3			
	MMS	2.0 µL					5571 ± 112
Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
With Activation	DMSO		10 ± 3	13 ± 4	29 ± 4	139 ± 14	604 ± 25
	Untreated		12 ± 2	16 ± 3	33 ± 5	187 ± 4	636 ± 17
	BCS-AB78877	33 µg	11 ± 4	15 ± 3	30 ± 4	132 ± 6	595 ± 22
		100 µg	10 ± 2	14 ± 3	31 ± 2	128 ± 23	600 ± 11
		333 µg	12 ± 2	12 ± 4	33 ± 5	126 ± 9	609 ± 19
		1000 µg	9 ± 1	14 ± 2	34 ± 3	122 ± 19	587 ± 55
		2500 µg	10 ± 2	13 ± 3	30 ± 6	138 ± 16	581 ± 24
		5000 µg	11 ± 2	13 ± 3	32 ± 1	127 ± 2	524 ± 82
	2-AA	2.5 µg	384 ± 24	192 ± 23	3556 ± 172	3611 ± 70	
	2-AA	10.0 µg					1430 ± 152

Key to Positive Controls

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

III. CONCLUSION

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the BCS-AB78877 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

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Report:	KCA 5.8.1 /38; [redacted] .;2014; M-491079-02-1
Title:	BCS-AB78877: Micronucleus Test in Human Lymphocytes In vitro
Report No:	1645202
Document No:	M-491079-02-1
Guidelines:	**OECD Guidelines for Testing of Chemicals No. 487** **Commission Regulation (EU) No 640/2012 B49**
GLP/GEP:	**yes**

Executive Summary

BCS-AB78877, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes in vitro in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix
	Exp. I	Exp. II	Exp. I & II
Stimulation period	48 hrs	48 hrs	48 hrs
Exposure period	4 hrs	20 hrs	4 hrs
Recovery	16 hrs	—	16 hrs
Cytochalasin B exposure	20 hrs	20 hrs	20 hrs
Total culture period*	88 hrs	88 hrs	88 hrs

In each experimental group two parallel cultures were analysed and per culture at least 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (852.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the current OECD Guideline 487.
Dose selection of the cytogenetic experiment was performed considering the toxicity data and the occurrence of test item precipitation in accordance with OECD Guideline 487. The chosen treatment concentrations ranged between 5.5 and 852.0 µg/mL in both experiments with and without S9 mix.

In experiment I in the absence and in presence of S9 mix and in experiment II in presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration, whereas in experiment II in presence of S9 mix cytotoxicity was observed at the highest concentration tested.

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed after treatment with BCS-AB78877.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes.

Therefore, BCS-AB78877 is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to cytotoxic or the highest required concentrations.

I. Materials and Methods

A. Material

1. Test Material:	BCS-AB78877
Description:	Colourless liquid

Lot/Batch:	BCOO 6454-11-4
Purity:	98.8% w/w (correction for purity was made)
Stability of test compound:	Stable in the refrigerator at +2°C to +8°C for the study duration

2. Control materials:

Negative:	Culture medium with 1.0 % DMSO
Solvent:	DMSO
Positive controls	
a) Without metabolic activation	MMC; mitomycin C (pulse treatment), 2.0 µg/mL dissolved in deionized water
	Demecolcin (continuous treatment), 150 ng/mL dissolved in deionized water
b) With metabolic activation	CPA; cyclophosphamide, 15 µg/mL dissolved in Saline (0.9 % NaCl [w/v])

Microsomal fraction S9 mix

Phenobarbital/β-napht[redacted]lavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the [redacted]CCR SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.
An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ 8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).
The protein concentration of the S9 preparation used for this study was 35.6 mg/mL (Lot no. 150514).

3. Test system:

Human lymphocytes

Blood samples were drawn from healthy non-smoking donors not receiving medication. For this study, blood was collected from a female donor (25 years old) for Experiment I and from a male donor (24 years old) for Experiment II.
The lymphocytes of the respective donors have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. All donors had a previously established low incidence of micronuclei in their peripheral blood lymphocytes.

B. Study Design and methods

The experimental phase of the study was performed from 27 August 2014 to 22 October 2014 at [redacted] GmbH - [redacted]CCR ([redacted], Germany).

1. Experimental performance

Test system preparation

Human lymphocytes were stimulated for proliferation by the addition of the mitogen PHA to the culture medium for a period of 48 hours. The cell harvest time point was approximately 2 –2.5 x AGT (average generation time). Any specific cell cycle time delay induced by the test item was not accounted for directly.
Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 hrs after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3

µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).
All incubations were done at 37 °C with 5.5 % CO_2 in humidified air.

Test item preparation
Stock formulations of BCS-AB78877 and serial dilutions were made in DMSO. The final concentration of DMSO in the culture medium was 1.0 %. The solvent was chosen due to its solubility properties and its relative non-toxicity to the cell cultures. All formulations were prepared freshly before treatment and used within two hours of preparation.
There were no effects of the test item osmolarity in the medium as measured in the solvent control and the maximum concentration without metabolic activation. The pH was adjusted to physiological values using small amounts of 1M HCl (Experiment I without S9 mix) or 2M HCl (Experiment I with S9 mix and experiment II with and without S9 mix).

Dose selection
Dose selection was performed according to the current OECD Guideline for the in vitro micronucleus test. The highest test item concentration should be 5000 µg/mL, 5 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

With regard to the molecular weight and prutity (98.8%) of BCS-AE78877, 852.0 µg/mL of the test item (approx. 10 mM) were applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations between 5.51 and 852.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test for toxicity, no precipitation of the test item was observed. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity no cytotoxic effects were observed in experiment I after 4 hours treatment in absence and presence of S9 mix. Therefore, 852.0 µg/mL was chosen as top treatment concentration for experiment II

Pre-experiment
A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay.
The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9 mix). The preparation interval was 40 hrs after start of the exposure.

Cytogentic experiment
Pulse exposure
About 48 hrs after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)

About 48 hrs after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Preparation of slides

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Evaluation of cytotoxicity and cytogenetic damage

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

$$\mathrm{CBPI} = \frac{(\mathrm{MONC} \times 1) + (\mathrm{BINC} \times 2) + (\mathrm{MUNC} \times 3)}{n}$$

CBPI Cytokinesis-block proliferation index
n Total number of cells
MONC Mononucleate cells
BINC Binucleate cells
MUNC Multinucleate cells

Cytostasis % = 100 – 100 [($CBPI_T$ – 1) / ($CBPI_C$ – 1)]
T Test item
C Solvent control

3. Assessment criteria
The micronucleus assay will be considered acceptable if it meets the following criteria:

The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
The rate of micronuclei in the positive controls is statistically significant increased.
The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.

A test item can be classified as non-clastogenic and non-aneugenic if:
- the number of micronucleated cells in all evaluated dose groups is in the range of the historical laboratory control data and
- no statistically significant or concentration-related increase of the number of micronucleated cells is observed in comparison to the respective solvent control.

A test item can be classified as clastogenic and aneugenic if:
- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.

An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. Results and discussion
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 20 hours without S9 mix.
In each experimental group two parallel cultures were analysed. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. To determine a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is described as % cytostasis.

The highest treatment concentration in this study, 852.0 Lg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the purity (98.8%) of the test item and with respect to the OECD Guideline 487 for the in vitro mammalian cell micronucleus test.

No visible precipitation of the test item in the culture medium was observed.

No relevant influence on osmolarity was observed. The pH was adjusted to physiological values.

In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying micronuclei was observed. The micronucleus rates of the cells after treatment with the test item (0.10 – 0.80 % micronucleated cells) did not exceed the range of the solvent control values (0.15 – 0.90 % micronucleated cells) and were within the range of the laboratory historical control data.

In both experiments, either Demecolcin (150.0 ng/mL), MMC (2.0 µg/mL) or CPA (15.0 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

Table 5.8.1-25 Summary of the results

Test item	Concentration (µg/mL)	Proliferation index CBPI	Cytostasis in %	Micronucleated cells in %	HCD range
Experiment I : Exposure period 4 hours without S9 mix					
Solvent control: DMSO	0.5 % (v/v)	1.82	/	0.90	0.15 - 1.40
Positive control: MMC	2.0	1.44	46.7	**10.60**	**3.60 – 25.10**
BCS-AB78877	278.2	1.91	n.c.	0.80	
	486.9	1.99	n.c.	0.70	
	852.0	1.84	n.c.	0.65	
Experiment I : Exposure period 4 hours with S9 mix					
Solvent control: DMSO	0.5 % (v/v)	1.99	/	0.30	0.20 - 1.65
Positive control: CPA	15.0	1.59	40.8	**3.25**	**2.20 -11.05**
BCS-AB78877	278.2	1.88	11.2	0.20	
	486.9	1.99	0.3	0.10	
	852.0	1.89	10.4	0.45	
Experiment II : Exposure period 20 hours without S9 mix					
Solvent control: DMSO	0.5 % (v/v)	1.64	/	0.45	0.10 - 1.35
Positive control: Demecolcin	0.15	1.28	55.7	**5.55**	**1.40 – 6.10**
BCS-AB78877	278.2	1.59	7.5	0.75	
	486.9	1.40	37.7	0.70	
	852.0	1.25	60.4	0.55	
Experiment IIA : Exposure period 4 hours with S9 mix					
Solvent control: DMSO	1.0 % (v/v)	1.84	/	0.15	0.20- 1.65
Positive control: CPA	15.0	1.60	27.9	**6.10**	**2.20 -11.05**
BCS-AB78877	278.2	1.89	n.c.	0.15	
	486.9	1.81	3.1	0.25	
	852.0	1.79	5.3	0.20	

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

III. CONCLUSION

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes.
Therefore, BCS-AB78877 is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to cytotoxic or the highest required concentration.

PROPINEB-DIDT (BCS-CU99534)

Propineb-DIDT (BCS-CU99534) is a plant metabolite of propineb that has not been found in the animals and therefore it is necessary to characterise its toxicological profile.
In vitro genotoxicity studies showed that Propineb-DIDT is devoid of genotoxic potential.

The acute oral toxicity of this metabolite is less than 2000 mg/kg bw, but higher than 300 mg/kg bw.

A 28-day dietary toxicity study has been conducted in the rat. BCS-CU99534, was administered continuously via the diet to groups of Wistar rats (10/sex/group) at concentrations of 5, 10 and 20 ppm (equating approximately to 0.188, 0.409, 0.861 mg/kg body weight/day in males and 0.229, 0.466, 1.037 mg/kg body weight/day in females). Results showed no effects on thyroid hormones and on the thyroid. This metabolite provoked neurological effects like decreased spontaneous motor activity,

weak responses in the flexor reflex and in the tail pinch test and low grip strengths for both fore- and hind-limb. At histology examination, minimal focal/multifocal interstitial mixed cell infiltration and minimal focal/multifocal myofiber degeneration were observed. The effects were more pronounced in the females and occurred at doses equivalent and higher than 1 mg/kg bw/day. The NOAEL in the female rats was equivalent to 0.466 mg/kg bw/day and to 0.861 mg/kg bw/day in males..

5.8.1-26 Summary of new studies with the metabolite Propineb-DIDT

Type of study	Species/test system	Result
Acute oral toxicity [redacted];2014 M-491068-02-1	Rat	LD_{50} > 300 mg/kg bw and < 2000 mg/kg bw
	Repeated toxicity	
Type of study	**NO(A)EL (mg/kg bw/day)**	**Effects at LOAEL and higher doses**
28-day toxicity study, 0, 5, 10 and 20 ppm. 0, 0.188, 0.409, 0.861 mg/kg body weight (♂) 0.229, 0.466, 1.037 mg/kg day (♀) [redacted], 2015 M-491125-02-1	0.861(♂) – 0.466(♀)	20 ppm 1.037 mg/kg bw/day in females ↓ bodyweight, neurological effects (↓ motor activity, weak response in the flexor reflex and in the tail pinch test, ↓ mean grip strengths for both fore- and hind-limb minimal myofiber degeneration in the skeletal muscle
	Genotoxicity	
[redacted]; 2014. M-481443-01-1	Salmonella typhimurium TA 1535, TA 1537, TA 98, TA 100, and TA 102	Negative
In vitro micronucleus [redacted].; 2014 . M-490043-01-1	Human lymphocytes	Negative

Report:	KCA 5.8.1 /36; [redacted].;2014; M-491068-02-1
Title:	BCS-CU99534 Acute oral toxicity study in rats
Report No:	14/222-001P
Document No:	M-491068-02-1
Guidelines:	OECD 423 and Commission Regulation (EC) NO 440/2008 of 30 May 2008, B.1.Tris
GLP/GEP:	**yes**

Executive summary

An acute oral toxicity with BCS-CU99534 (Propineb-DIDT) was performed according to the acute toxic class method (OECD 423 and Commission Regulation (EC) NO 440/2008 of 30 May 2008, B.1.Tris) in RccHan:WIST rats.

A single oral treatment was carried out by gavage for each animal after an overnight food withdrawal. Food was made available again 3 hours after the treatment. BCS-CU99534 was administered as a solution prepared in PEG 400 at a concentration of 200 and 30 mg/mL at a dosing volume of 10 mL/kg bw.

Clinical observations were performed at 30 minutes, 1, 2, 3, 4 and 6 hours after dosing and daily for 14 days thereafter. Body weight was measured on Days -1, 0 and 7 and Day 14 before necropsy. All animals were subjected to a necropsy and a macroscopic examination

Initially, three males were treated at a dose level of 2000 mg/kg bw. The test item caused mortality in this group (1/3). Therefore a second group of three males was treated at the same dose level. Two of three rats died at this dose group. A lower dose group of three males was treated at a dose level of 300 mg/kg bw. As no mortality was observed, a confirmatory group of three males was treated at the same dose level. The test item did not cause mortality in the confirmatory group, so no further testing was required according to OECD 423 and Commission Regulation (EC) NO 440/2008 of 30 May 2008, B.1.Tris.

Initially, three females assigned to were treated at a dose level of 2000 mg/kg bw. The test item caused mortality in this group (2/3). Therefore a second group was treated at a dose level of 300 mg/kg bw. The test item did not cause mortality in this group; therefore a confirmatory group of three females was treated at the same dose level. The test item did not cause mortality in the confirmatory group, so no further testing was required according to OECD 423 and Commission Regulation (EC) NO 440/2008 of 30 May 2008, B.1.Tris.

Under the conditions of this study, the acute oral LD50 value of the test item BCS-CU99534 (Propineb-DIDT) was found to be between 300 mg/kg bw and 2000 mg/kg bw in female and male RccHan:WIST rats.

I. Materials and methods

A. Materials

1. Test material:	BCS-CU99534
Synonyms(s)	Propineb-DIDT
Description:	Yellow solid
Lot/Batch no:	SES 11956-8-1
Purity:	99.5 % (w/w)
Stability of test compound:	The test item preparation was made freshly on the morning of administration by the laboratory Pharmacy
2. Vehicle:	PEG 400
3. Test animals:	
Species:	Rat
Strain:	RccHan:WIST rats
Age:	8-12 weeks old
Weight at dosing:	150- 300 g
Source:	[redacted] Laboratories S.r.l. [redacted] (UD), (Italy)
Acclimatisation period:	at least 5 days
Diet:	[redacted] SM R/H complete diet for rats and mice ([redacted], Germany)
Water:	tap water, ad libitum

Housing:	The animals were housed in polypropylene/polycarbonate cages. Each cage contained three rats of the same sex and group during the treatment period. Each cage contained deep wood sawdust bedding (Lignocel, Hygienic Animal Bedding).
Environmental conditions:	Temperature: 22 ±3 °C Humidity: 30 to70% Air changes: Approximately 15-20 changes per hour Photoperiod: Alternating 12-hour light and dark cycles

B. Study Design and methods

1. In life dates

The study was conducted at [redacted] Ltd from June 11 to July 1st, 2004

2. Animal assignment and treatment

The starting dose level (2000 mg/kg bw) was selected because a limit dose is normally used unless there is information evidence that this dose is not appropriate. If two or more animals die sex at 2000 mg/kg bw dose, a full study will be conducted according to OECD 423. If no or one animal die, the dose of 2000 mg/kg bw will be repeated with 3 additional animals each sex.

3. Statistics

II. Results and discussion

B. Study Design and methods

1. In life dates

The study was conducted at [redacted] Ltd from June 11 to July 1st , 2014

2. Animal assignment and treatment

The initial dose level was selected by the study director. In the lack of any preliminary toxicological information, 2000 mg/kg bw was selected to be the starting dose.

A single oral gavage administration was followed by a fourteen-day observation period. Before treatment the animals were fasted. The food, but not water, was withheld during an overnight period. Animals were weighed just before treatment. The test item was administered by oral gavage in the morning. The food was returned 3 hours after the treatment

Clinical observations were performed on at the all animals at 30 minutes and at 1, 2, 3, 4 and 6 hours after dosing and daily for 14 days thereafter.

Macroscopic examination was performed on all animals.

3. Statistics

The method used was not intended to allow the calculation of a precise LD50 value.
The test item was ranked into categories of Globally Harmonized Classification System (GHS) described in the OECD Guideline No. 423 (Annex 2d), EU Directive 1999/45/EC (as amended) and Regulation (EC) No 1272/2008 (CLP).

II. Results and discussion

A. Mortality

BCS-CU99534 caused mortality at a dose level of 2000 mg/kg bw (7/9) and 300 mg/kg bw (1/12). Details are provided in Table B6.8.1-25.

Table 5.8.1-27: Doses, mortality /clinical signs/ animals treated

Dose (mg/kg bw)	Toxicological results*		Occurrence of mortality	Mortality (%)
2 000	Males 4/6/6	Females 3/3/3	Day 1(both sexes)	78
300	Males 1/6/6	Females 0/6/6	Day 2 (males only)	8.3

* : number of animals which died spontaneously and/or were sacrificed in moribund state/number of animals with signs of toxicity/total number of animals used per group

B. Clinical observations

Clinical signs were observed in animals treated at the dose level of 2000 mg/kg bw with BCS-CU99534. The onset of signs occurred within 24 hours from administration.
These included decreased activity (9/9), hunched back (6/9), faeces liquid (7/9), piloerection (4 /9) and death (7/9).
Treatment with 300 mg/kg bw caused decreased activity (12/12), hunched back (8/12), piloerection (7/12) and death (1/12).

C. Body weight

Body weight and body weight gain of BCS-CU99534 treated animals showed no indication of a treatment-related effect.

D. Necropsy

At the found death animals test item-related changes were observed in the stomach, digestive content of the duodenum and/or jejunum , including brown/non-glandular stomach mucosa, red discoloured glandular stomach mucosa, yellow-green liquid material in the digestive content of the duodenum and/or jejunum. In addition, yellow green perianal fur was also occasionally seen. In affected rats dosed at 2000 mg/kg bw, liquid material clinically observed in the faeces could indicated diarrhea in these animals.
Dark/red discoloration of the non-collapsed/collapsed lungs was regarded as typical agonal/post mortem change.

At the terminal test item-related findings were noted in the non-glandular mucosa of the stomach. Diffuse thickness was present in 3/6 females dosed at 300 mg/kg bw. Similar change was not recorded in the males at a dose level of 300 mg/kg bw.

Thickness and brown focal discoloration of non-glandular mucosa was manifested in two surviving males dosed at 2000 mg/kg bw.

III. Conclusions

Under the conditions of this study, the acute oral LD_{50} value of the test item BCS-CU99534 (Propineb-DIDT) was found to be between 300 mg/kg bw and 2000 mg/kg bw in female and male RccHan:WIST rats.

The study result triggers the following classification/labelling:

- EU directive 1999/45/EC: XnR22 (Harmful if swallowed)
- Regulation (EC) No 1272/2008 (CLP): Cat. 4 H302 (Harmful if swallowed)

.

***

Report:	KCA 5.8.1 /37; [redacted].; 2014;M-491125-02-1
Title:	Propineb-DIDT(BCS-CU99534): Placeholder for the In vivo rat 28-day comparative toxicity study planned till end of 2014
Report No:	M-491125-02-1
Document No:	M-491125-02-1
Guidelines:	**OECD guideline 407 (July, 1995)** **EEC Directive 96/54/EC, Method B.7 (July, 1996)**
GLP/GEP:	**No (the study was performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance Unit, but it was not subjected to specific QAU inspections)**

Executive Summary

This study was conducted according to the Organization for Economic Cooperation and Development (OECD) guideline 407 (July, 1995) and European Economic Community (EEC) Directive 96/54/EC, Method B.7 (July, 1996) guideline. The objectives of this study were to determine the potential toxic effects of BCS-CU99534 in rats following continuous dietary administration for at least 28 days and to provide information for selection of dose levels for future toxicity studies in this species. For this reason the study was carried out in a GLP laboratory and performed according to standard operating procedures which were previously accepted and periodically inspected by the Quality Assurance Unit, but it was not subjected to specific QAU inspections.

BCS-CU99534, (batch number: BCS-T&C-2012-0475-1, a Yellow powder, 98.0% w/w purity) was administered continuously via the diet to groups of Wistar rats (10/sex/group) for at least 28 days at concentrations of 5, 10 and 20 ppm (equating approximately to 0.188, 0.409, 0.861 mg/kg body weight/day in males and 0.229, 0.466, 1.037 mg/kg body weight/day in females). A similarly constituted group received untreated diet and acted as a control group. Animals were observed daily for mortality and clinical signs. Physical examinations were performed weekly. The first 10 surviving animals were subjected to a neurotoxicity assessment during Week 3 of the study. Body weight and food consumption were recorded once weekly. During the acclimatization phase all animals were subjected to an ophthalmic examination. All animals were re-examined during Week 4. During Week 1 and Week 4, a blood sample was collected from the retro-orbital venous plexus of each animal for hormone level determinations (TSH, T3 and T4). Hematology, clinical chemistry and urine parameters were determined at the end of the study. All animals were necropsied, selected organs weighed and a range of tissues were taken, fixed and examined microscopically.

The dietary administration of BCS-CU99534 to rats up to 20 ppm did not cause any mortality or clinical signs or any relevant effects in ophthalmology, hematology, urinalysis or at macroscopic examination.

At 20 ppm:

In females, mean body weight and body weight gain parameters were affected compared to controls. Mean absolute and relative body weight gains were significantly lower than controls throughout the study for most intervals. At the end of the study, there was a 43% decrease of the absolute body weight gain compared to controls. From day 15 onwards, mean body weight was significantly lower (-

10% to -12%, compared to controls). The marginal reduction of food consumption in females at this dose level (-4% overall) was attributed to lower bodyweights.
The neurotoxicity assessment showed effects in females compared to controls: a weaker response in the flexor reflex test and in the tail pinch test and significant lower mean grip strengths for both fore- and hind-limb. In addition, although not statistically significant, there was a slight decrease in the total spontaneous motor activity.
In clinical chemistry, the few changes noted were considered as not relevant and not adverse since they were within normal range of variation and/or do not represent a functional impairment of the test organism and were not associated with any relevant histopathological change.
At necropsy, mean terminal body weight was significantly lower in females compared to controls. Mean liver weight was minimally higher in both sexes compared to controls, however, in the absence of any corroborative findings in histology or blood biochemistry, this minimal change was considered not to be adverse.
At histology examination, the only histopathological change considered to be test item-related was observed in the skeletal muscle in a few females and comprised minimal focal/multifocal interstitial mixed cell infiltration and minimal focal/multifocal myofiber degeneration.

In conclusion, the dose level of 20 ppm (equivalent to 0.861 mg/kg bw/day) was considered to be the NOAEL in the males. The NOAEL in the females was 10 ppm (equivalent to 0.466 mg/kg bw/day) based on the neurological and histopathological effects observed in the skeletal muscle at 20 ppm.
At 10 ppm and 5 ppm, there was no treatment related effect in either sex.

As a conclusion, in females, based on the effects observed at 20 ppm on body weight parameters, in the neurotoxicity assessment and in the skeletal muscle at histopathological examination, the NOAEL was considered to be 10 ppm (equivalent to 0.466 mg/kg bw/day).
In males, the NOAEL was considered to be 20 ppm (equivalent to 0.861 mg/kg bw/day).

I. MATERIALS AND METHODS

A. Materials:

1. Test Material: BCS-CU09534 (Propineb-DDT)

Description:	yellow powder
Lot/Batch:	BCS-T&C-2012-0475-1
Purity:	98% w/w
Stability of test compound:	Stable when stored air tight, light resistant container at approximately 4°C

3. Test animals:

Species:	Male/Female/Rat
Strain:	Wistar Rj WI (IOPS HAN)
Age:	7 weeks
Weight at dosing:	males: 240 to 268; females: 167 to 194 g
Source:	R. [redacted], Le Genest St Isle, France
Acclimation period:	6 days
Diet:	Certified rodent powdered and irradiated diet A04CP1-10 from [redacted]. ([redacted] France)
Water:	Tap water ad libitum in polycarbonate bottles, ad libitum
Housing:	Rats were housed individually in suspended, stainless steel, wire mesh cages.
Environmental conditions:	Temperature: 20-24 °C Humidity: 40-70 % Air changes: Approximately 10 changes per hour

Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study design:
1. In life dates

The in-life phase was carried out from October 24th to November 28th at the Research Centre of Toxicology, Bayer CropScience, [redacted] (France).

Animal assignment and treatment

Groups of 15 male and 15 female rats were given the vehicle control diet or the appropriate diet mixture

Table 5.8.1-28 Animal assignment

Group	Test Item	Dose level (ppm)	Number of animals Per group
		males	
1	Control	0	15
2	BCS-CU99534	5	15
3		10	15
4		20	15
		females	
1	Control	0	15
2	BCS-CU99534	5	15
3		10	15
4		20	15

Dietary levels were selected based on the results of a preliminary tolerability study (SA 14217) in which BCS-CU99534 was initially administered to male and female rats (5 animals/sex/group) at 200, 400 and 600 ppm in the diet for 14 days. Exposure to 400 and 600 ppm provoked marked clinical signs (including piloerection, hunched posture, wasted appearance, reduced motor activity or prostration and uncoordinated movements) and animals of these groups were sacrificed prematurely for humane reason after 3 days of treatment. In a complementary phase, dose levels of 25, 50 and 100 ppm in the diet were administered to groups of female rats since females appeared to be more sensitive than male. Similar types of effects were observed at 100 and 50 ppm. At 100 ppm, the group was sacrificed prematurely on Day 5 for humane reason as well as 2 females at 50 ppm (on day 5 and 13, respectively). In addition at 50 ppm, bodyweight parameters were clearly affected. At 25 ppm, no clinical signs were observed however, the body weight gain throughout the study was below the expected value for animals of the age and strain.

3. Diet preparation and analysis of the test substance

The test item was incorporated into the diet to provide the required dietary concentrations. The test item was ground to a fine powder before being incorporated into the diet by dry mixing. There were five preparations for each concentration. When not in use, the diet formulations were stored at approximately 18°C. They were issued daily to the animal unit in polyethylene containers for everyday use.

The homogeneity of test item in diet was verified before the study for the lowest and highest concentrations to demonstrate adequate formulation procedures.

Dietary levels of the test item were verified for each concentration on dietary preparation F1, F3 and F5. For the first formulation (F1), the mean values obtained from the homogeneity checks were taken as measured concentrations.

The stability of the frozen dietary formulation was determined during the study for all concentrations after 1 day at room temperature and after 11 or 13 days frozen followed by 1 day at room temperature.

- After 1 day at room temperature, the stability was 52%, 58% and 71% at 5, 10 and 20 ppm respectively.
- After 11 days or 13 days frozen and 1 day at room temperature, the stability was 52%, 54% and 57%, respectively.

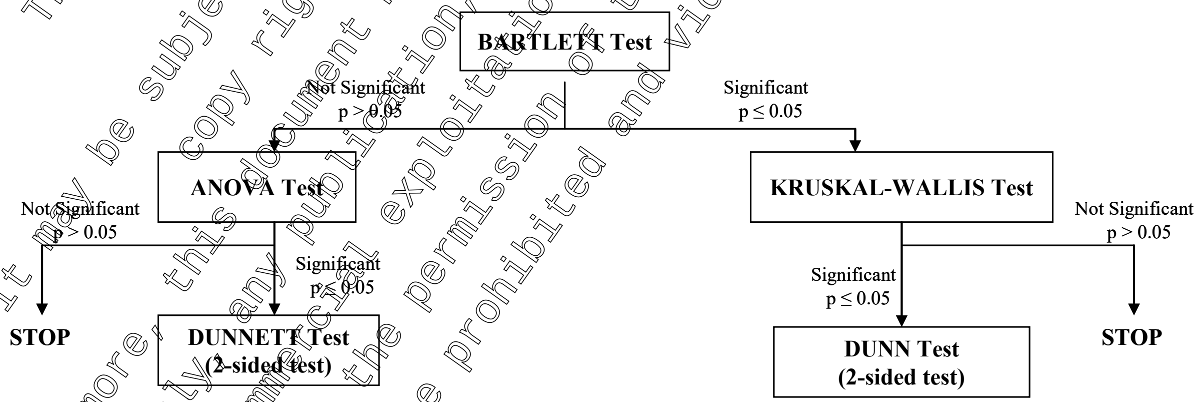
For the achieved intake calculation, the worst case was considered and correction factors of 52%, 54% and 57% were applied for the dose levels of 5, 10 and 20 ppm, respectively

4. Statistics

Mean and standard deviation were calculated for each group. All statistical analyses were carried out separately for males and females. Group means were compared at least at the 5% level of significance. Statistical analyses were carried out using Pristima, version 6.3.2 build 17, Xybion Corp.

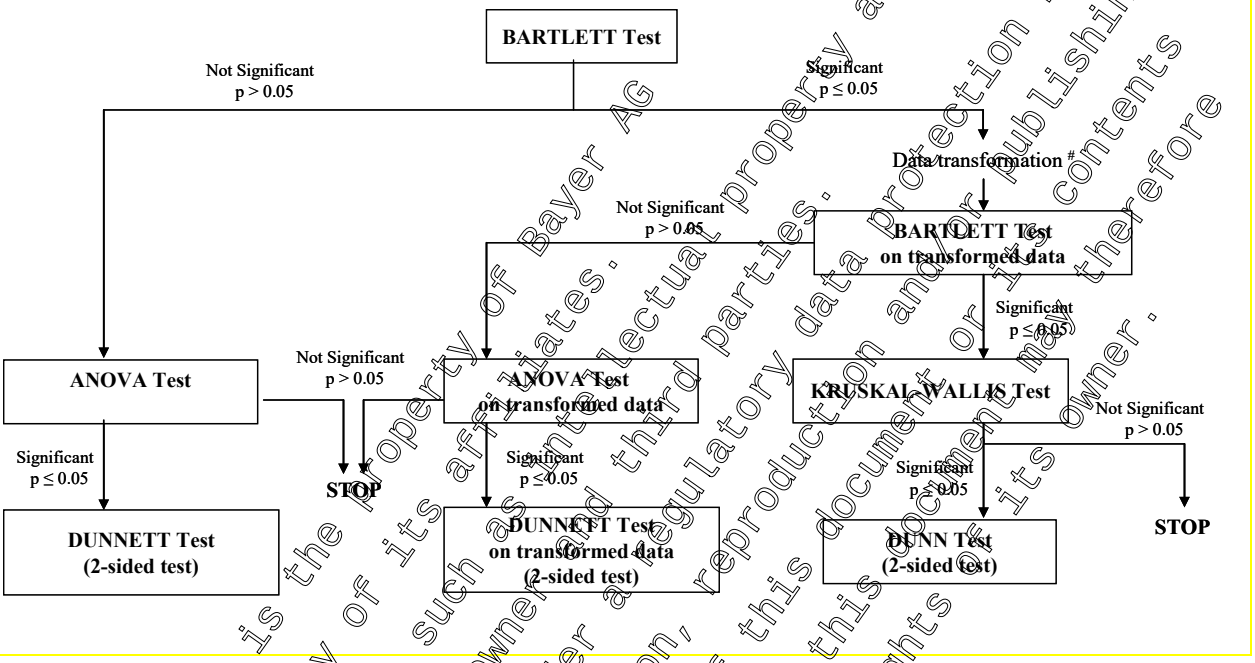
- Body weight change parameters,
  - Terminal body weight, absolute and relative organ weight parameters,
  - Hematology parameters (prothrombin time, hemoglobin concentration, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, %neutrophils, %lymphocytes, %reticulocytes),
  - Clinical chemistry parameters,
  - Quantitative urinalysis parameters (volume and refractive index),
  - Spontaneous motor activity, grip strength, landing foot splay and rectal temperature parameters.

Mean and standard deviation were calculated for each group and per time period for body weight change parameters



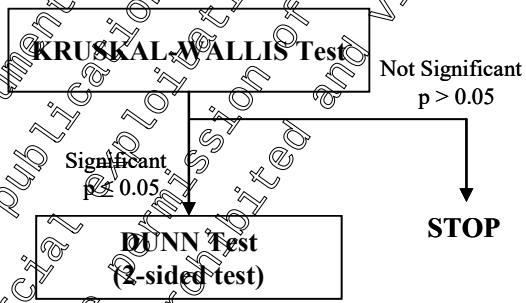
- Body weight and average food consumption/day parameters
  - Hematology parameters (red blood cell count, platelet count, white blood cell count, neutrophil count, lymphocyte count, reticulocyte count)
  - Hormonal parameters

Mean and standard deviation were calculated for each group and per time period for average food consumption/day parameters.



Data were transformed using the log transformation for body weight and food consumption parameters and for hormonal parameters or using the square root transformation for hematology parameters.

- Quantitative urinalysis parameter (pH)



If one or more group variance(s) equaled 0, means were compared using non-parametric procedures.

C. Methods

1. Daily observations

All animals were checked for moribundity and mortality twice daily (once daily on weekends or public holidays). Animals were observed for clinical signs at least once daily for all animals. Detailed physical examinations were performed at least weekly during the treatment period. The nature, onset, severity, reversibility and duration of clinical signs were recorded. Cages and cage-trays were inspected daily for evidence of ill-health such as blood or loose feces.

2. Body weight

Each animal was weighed once during the acclimatization period, on the first day of test item administration, then at weekly intervals throughout the treatment period. Additionally, diet fasted animals were weighed before scheduled necropsy (Terminal body weight).

3. Food consumption

The weight of food supplied and of that remaining at the end of the food consumption period was recorded weekly for all animals during the treatment period. Any food spillage was noted. From these records, the weekly mean achieved dosage intake in mg/kg/day for each week and for Weeks 1 to 4 was calculated, for each sex using the formula:

$$\text{Test item intake (mg/kg/day)} = \frac{\text{Dose level (ppm)} \times \text{Group mean food consumption (g/day)}}{\text{Group mean body weight (g) at the end of the food consumption period}}$$

4. Neurotoxicity

During study Week 3, a neurotoxicity assessment was performed for all animals. Each animal was individually tested (the order of animal testing was randomly determined) by the observer who was blind regarding the animal's group assignment.

<u>Functional Observational Battery included:</u>

- Home cage observation: while the animal was in its home cage, observations were collected regarding posture, piloerection, involuntary motor movements, gait abnormalities, vocalizations or any abnormal behavior.
- Observation during handling including ease to remove from cage, reaction to being handled, muscle tone, eyelid, lacrimation, salivation, nasal discharge, staining or any other signs such as alopecia, emaciation, temperature upon touching ("cold to touch")
- Open-field observation: each animal was individually observed in an open field for 2 minutes for piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalizations and number of rearings, urine and feces spots.
- Reflex and physiologic observations/measurements included:
  - Pupil size
  - Pupillary reflex (by covering the eyes of the animal for a few seconds and then observing pupillary constriction by focusing a narrow beam of light in the eyes).
  - Surface righting reflex (by putting the animal on its back and evaluating its ability/rapidity to reassume a normal standing position).
  - Corneal reflex (by touching the medial canthus with a fine object and observing the quick and complete closure of the eyelids).
  - Flexor reflex (by pinching the toes and evaluating the presence/strength of the flexor response of each hindlimb).
  - Auditory startle response (by evaluating the animal response to an auditory stimulus

- Tail pinch response (by pinching the tail with a forceps and evaluating the animal reaction).
- Grip strength: the fore- and hindlimb grip strength of animals was measured quantitatively using a grip strength apparatus equipped with one pull and one push strain gauge ([redacted], France).
- Landing foot splay: the animal was dropped from approximately 30 cm above the surface and hindlimb foot splay was marked, measured and recorded.
- Body weight
- Rectal temperature

Spontaneous motor activity

Animals were tested individually using an automated photocell recording apparatus ([redacted], France) designed to measure quantitatively spontaneous exploratory motor activity in a novel environment. Spontaneous exploratory motor activity was recorded during the first 60 minutes with data being collected at regular intervals throughout the session (due to technical limitation of the data collecting system, activity was actually planned to be recorded for 65 minutes to be sure to have the first 60 minutes recorded and saved).

5. Ophthalmological examination

During the acclimatization period all animals were subjected to an ophthalmic examination. After instillation of an atropinic agent (Mydriaticum, [redacted]) each eye was examined by means of an indirect ophthalmoscope. During week 4, animals from control and high dose groups were re examined.

6. Clinical pathology and hematology

Blood sampling

On Study Days 30, 31, 32 or 33, blood samples were taken from all animals in all groups by puncture of the retro orbital venous plexus prior to sacrifice. An approximately equal number of animals randomly distributed amongst all groups were sampled on each day. Animals were diet fasted overnight prior to bleeding and anesthetized by inhalation of Isoflurane ([redacted], France). Blood was collected on EDTA for hematology (approximately 0.5 mL), on clot activator for serum clinical chemistry (approximately 1.1 mL), on sodium citrate for coagulation parameters (approximately 0.9 mL) and on lithium heparin for plasma hormone analysis (approximately 0.6 mL).
In addition, on days 1, 2, 3 or 4, blood was collected on lithium heparin for plasma hormone analysis (approximately 1.1 mL).

Hematology parameters:

Red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin , mean corpuscular hemoglobin concentration , reticulocyte count, white blood cell count and differential count evaluation and platelet count were assayed using an Advia 2120i ([redacted], France).
A blood smear was prepared and stained using May-Grünwald Giemsa method. It was examined when the results of Advia 2120i determinations were abnormal.
Prothrombin time was assayed on an ACL Elite Pro (Instrumentation Laboratory, Paris, France).

Clinical Chemistry parameters

Total bilirubin, glucose , urea, creatinine, total protein, albumin, total cholesterol, triglycerides, chloride, sodium , potassium, calcium and inorganic phosphorus concentrations, and aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and gamma-glutamyltransferase activities were assayed on serum samples using an Advia 1800 ([redacted], France).
Globulin concentrations and albumin/globulin ratio values were calculated.

Hormone measurement

Plasma samples were prepared and kept frozen at -74°C + 10°C until sending off to the principal investigator for hormone measurement. Samples were then sent frozen to CiToxLabs for the determination of TSH hormone level with the Luminex MAP technology and the determination of T3 and T4 hormone level with the analytical method solid phase extraction on Oasis HLB cartridges.

Urine Collection and analysis

On Study Day 24, 25 or 26, in the morning, overnight urine samples were collected from all animals in all groups. Food and water were not accessible during urine collection. Any significant change in the general appearance of the urine was recorded.

Urinary volume was measured. pH was assayed using a Clinitek 500 and Multistix dipsticks ([redacted], France). Urinary refractive index was measured using a RFM329 refractometer (Bioblock Scientific, Illkirch, France).

b/Semi quantitative parameters Glucose, bilirubin, ketone bodies, occult blood, protein and urobilinogen were assayed using a Clinitek 500 and Multistix dipsticks ([redacted], France).

c/Microscopic examination of the sediment Microscopic examination of the urinary sediment was performed after centrifugation of the urine. The presence of red blood cells, white blood cells, epithelial cells, bacteria, casts and crystals was graded.

6. Post-mortem examinations

Necropsy

On study Days 29, 30, 31 or 32, all animals from all groups were sacrificed by exsanguination under deep anesthesia (inhalation of Isoflurane). An approximately equal number of animals randomly distributed amongst all groups were sacrificed on each day. Animals were diet fasted overnight prior to sacrifice.

All animals were necropsied. The necropsy included the examination of the external surfaces, all orifices and all major organs, tissues and body cavities. Macroscopic abnormalities were recorded, sampled and examined microscopically.

Organ weights

Adrenal gland, brain, epididymis, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (with parathyroid gland) and uterus (including cervix) were weighed fresh at scheduled sacrifice. Paired organs were weighed together.

Tissue collection and histopathology

The following organs or tissues were sampled:

Adrenal gland, aorta, articular surface (femoro-tibial joint), bone (sternum), bone marrow (sternum), braine, epididymis, esophagus, ovary, pancreas, pituitary gland, prostate gland, sciatic nerve, seminal vesicle, skeletal muscle, skin, eye and optic nerve, spinal cord (cervical, thoracic, lumbar), exorbital (lachrymal) gland, spleen, harderian gland , stomach h eart, submaxillary (salivary) gland, intestine, (duodenum, jejunum, ileum, cecum, colon, rectum) testes, thymus , kidney, thyroid gland (with parathyroid gland), larynx/pharynx, tongue, liver, trachea, lung, urinary bladder, lymph nodes (submaxillary, mesenteric), uterus (including cervix) mammary gland, vagina, nasal cavities and macroscopic findings

For sacrificed animals, a bone marrow smear was prepared from one femur, stained with May Grünwald Giemsa, but not examined as no treatment related changes were observed in hematology or bone marrow histology. Samples were fixed by immersion in neutral buffered 10% formalin with the

exception of the eye, optic nerve, Harderian gland, epididymis and testis that were fixed in Davidson's fixative

Histopathological examinations were performed on all tissues specified above, in the first 10 animals in the control group and the highest dose group. Kidney, liver, lung, thyroid gland and macroscopic findings of all animals were examined in the intermediate dose groups.

Initial examinations were performed by the Principal Investigator in Histopathology ([redacted]).

The histopathological evaluation was subjected to a review of representative slides by Dr. [redacted], Bayer S.A.S., Bayer CropScience according to SOP's of the test facility. This procedure is documented in a peer review statement which will be archived with the study raw data. There were no relevant deviations between the principal investigator's and reviewing pathologist's diagnoses. The present report reflects the mutual agreement on the results.

II. RESULTS

A. Mortality

There was no mortality throughout the study at any dose in either sex.

B. Clinical Signs

There were no treatment-related clinical signs observed throughout the study at any dose levels compared to controls. At the weekly physical examination, one female of the 20 ppm group had one half-closed eye at the end of the study.

C. Body weight

At 20 ppm in females, mean body weight was significantly lower than controls from day 15 onwards (-10% to -12%; p≤0.01 compared to controls).

Mean absolute and relative body weight gain was significantly lower than controls throughout the study for most intervals. Overall, the mean absolute bodyweight gain was 43% (p≤0.01) lower compared to controls at the end of the study.

There was no adverse effect effect on body weight parameters at 10 or 5 ppm in females and at any dose level in males.

Table 5.8.1-29: Group mean body weight (BW) and body weight gain (BWG) in g

Groups	**5 ppm**	**10 ppm**	**20 ppm**
	Males		
Initial BW Day1 (%C)	**255**	**256 (100)**	**255 (100)**
BW Day 8	302	301 (100)	305 (101)
BW Day 15	348	349 (100)	354 (102)
Final BW Day 29	**400**	**408 (102)**	**411 (103)**
BWG Day 1-8	47	45 (97)	50 (107)
BWG Day 1-15	93	93 (100)	99 (107)
Overall BWG Day 1-29	**146**	**153 (105)**	**156 (107)**
	Females		
Initial BW day1	**181**	**180 (99)**	**180 (99)**
BW Day 8	202	200 (99)	197 (98)
BW Day 15	218	219 (100)	212 (97)
Final BW Day 29	**245**	**242 (99)**	**238 (97)**
BWG Day 1-8	20	21 (101)	16 (81) **
BWG Day 1-15	37	39 (107)	32 (87) **
Overall BWG Day 1-29	**64**	**62 (98)**	**58 (91) ****

** ; statistically different from the control (p< 0.001)

D. Food consumption

At 20 ppm in females, mean food consumption was very marginally affected (-4% overall compared to control, being statistically significant in a single occasion throughout the study). This marginal effect was attributed to lower bodyweight in this group.
Mean food consumption was not affected in any other group in females and males.
.
Test material intake is summarized in Table B 6.8.2-6

Table 5.8.1-30 Mean achieved dietary intake

Groups	**5 ppm**	**10 ppm**	**20 ppm**
Males	0.202	0.409	0.861
Females	0.225	0.466	1.037

E. Ophthalmological examination

There were no treatment-related effects observed in treated animals compared to controls

F. Neurotoxicity assessment

Functional Observation bactery (FOB)
At 20 ppm in females in the sensory reactivity tests, higher incidences of animals showing moderate flexor reflex response were observed (6/10 and 7/10 for left and right flexor, respectively compared to 0/10 in controls). In addition, no or week response in the tail pinch test was observed for 6/10 females compared to 0/10 in controls. There were no effects in males in the sensory reactivity tests at any dose level.

In addition, there were no treatment-related abnormal findings in treated animals compared to controls during the home cage, during handling and open field observations at any dose in either sex.

Table 5.8.1-30 Reflex observations (10 animals examined)

Dose level of BCS-CU99534 ppm	Control	5	10	20
Females				
Moderate flexor reflex response - left	0/10	0/10	1/10	6/10
Moderate flexor reflex response – right	0/10	0/10	0/10	7/10
Tail pinch response – Absence or weak	0/10	0/10	1/10	6/10

Grip strength, landing foot splay, rectal temperature and body weights

At 20 ppm in females, mean grip strengths were significantly lower compared to controls for both hind- and fore-limb (-37% and -43%, respectively; p≤0.01). In addition in this group, mean bodyweight was significantly lower compared to controls (-13%) which is aligned with the observation made on the body weight evolution.
There were no effects in other female treated groups or in any male treated groups compared to controls.

Table 5.8.1-31 Grip strength

Mean ±standard (% change when compared to control)				
Fore limb	853.2 ± 126.24	820.7 ± 97.06 (-4)	873.3 ± 120.49 (+2)	481.0* ± 126.24 (-43)
Hind limb	526.3± 58.99	555.6 ± 87.51 (+5)	588.2 ± 63.21 (+6)	330.9* ± 55.22 (-37)

* Dunnett LSD at 0.01 level

Spontaneous motor activity

There were no statistically significant effects on the mean spontaneous motor activity parameters in either sex at any dose level compared to controls. In addition, the general pattern of motor activity within the test session (i.e. habituation) was comparable between groups.

However at 20 ppm, the total mean motor activity was 30% and -31% lower in males and females, respectively compared to controls. In females, this slight decrease corroborates with other observations made during the FOB (i.e. decreased grip strength) or at histopathological examination (skeletal muscle) and was considered to be treatment-related. In males, it was not associated with any other corroborative findings and it was therefore concluded that they were not treatment-related.

F. Clinical pathology

There were no treatment-related changes in hematology parameters.

When compared to the controls, higher mean inorganic phosphorus concentrations were observed in females at each dose level. However these changes were considered to be incidental as they were not dose-related and as those values remained within our historical control database.

Table 5.8.1-33 Clinical chemistry changes

Mean ± standard deviation (% change when compared to controls)				
Dose level of BCS-CU99534 (ppm)	Control	5	10	20
	Females			
Inorganic phosphorus concentration (mmol/L)	2.085 ±0.1520 -	2.231 ±0.1410 * (+7%)	2.225 ±0.1881 (+7%)	2.332 ±0.1761 ** (+12%)

* p≤0.05 ** p≤0.01

G. Post-mortem examinations

1. Terminal body weight and organ weights

At 20 ppm, in females only, mean terminal body weight was statistically significantly minimally lower than that of the female control group (-11%, p≤0.01).

Minimal weight variations were noted for mean liver weights in the group treated at 20 ppm. In the males, mean absolute and relative liver weights were minimally higher than those of the controls, being statistically significant for the relative weights, only (see Text table 4-1). As there were no corroborative histological changes or effects in blood biochemical parameters, this minimal weight variation was not considered to be sufficient evidence of a test item effect.

In the females treated at 20 ppm, only the mean liver to body weight ratio was statistically significantly minimally higher than that of controls and was considered to be related to the lower mean terminal body weight in these animals. It was therefore not interpreted to be a direct effect of the test item

Table 5.8.1-33 Mean liver weight ±SD at scheduled sacrifice

(% change when compared to controls)								
Sex	**Males**				**Females**			
BCS-CU99534 dose level (ppm)	**0**	**5**	**10**	**20**	**0**	**5**	**10**	**20**
Mean absolute liver weight (g)	9.652 ±0.6335	9.935 ±0.9085 (+3%)	9.761 ±0.5810 (+1%)	10.405 ±0.9687 (+8%)	5.877 ±0.4145	5.860 ±0.5311 (0%)	5.568 ±0.3242 (-5%)	5.928 ±0.3791 (+1%)
Mean liver to body weight ratio (%)	2.558 ±0.1212	2.595 ±0.1525 (+1%)	2.533 ±0.1078 (-1%)	2.745** ±0.1875 (+7%)	2.572 ±0.1124	2.567 ±0.1336 (0%)	2.510 ±0.1028 (-2%)	2.934** ±0.1878 (+14%)
Mean liver to brain weight ratio (%)	463.583 ±30.6200	481.475 ±46.1422 (+4%)	466.643 ±26.8011 (+1%)	499.331* ±43.7876 (+8%)	299.311 ±20.9003	303.670 ±26.5427 (+1%)	288.555 ±15.9233 (-4%)	312.937 ±22.2536 (+5%)

*: p≤0.05, **: p≤0.01

Macroscopic pathology
No macroscopic findings related to administration of the test item were observed.

Microscopic pathology
The only histopathological change considered to be test item-related was observed in the skeletal muscle, in females treated at 20 ppm, and comprised minimal focal/multifocal interstitial mixed cell infiltration in 4/10 and minimal focal/multifocal myofiber degeneration in 4/10 females.

Table 5.8.1-34 Incidence and severity of microscopic changes in the skeletal muscle

Sex	**Males**				**Females**			
Group	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Dose (ppm)	**0**	**5**	**10**	**20**	**0**	**5**	**10**	**20**
Number of animals examined	10	10	10	10	10	10	10	10
Interstitial mixed cell infiltrate: focal								
Minimal	1	0	0	0	0	1	0	3
Myofiber degeneration: focal								

Minimal	0	0	0	0	0	0	0	4

A low number of other minor histopathological changes were seen in the organs and tissues of rats used in this study, without relevant inter-group difference between the test item-treated groups and the group receiving control diet.
The lesions were minimal in severity and considered to be adverse.

All of these changes were considered incidental and/or to correspond to the usual background pathology noted in rats of this strain and age used under experimental conditions.

III. CONCLUSION

In conclusion, the dose level of 20 ppm (equivalent to 0.861 mg/kg bw/day) was considered to be the NOAEL in the males. The NOAEL in the females was 10 ppm (equivalent to 0.466 mg/kg bw/day) based on the neurological and histopathological effects observed in the skeletal muscle at 20 ppm.

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Report:	[redacted]3;[redacted]2014;M-481443-01
Title:	BCS-CU99534: Salmonella typhimurium reverse mutation assay
Report No:	1589901
Document No:	M-481443-01-1
Guidelines:	**Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No, 471: Bacterial Reverse Mutation Test, adopted July 21, 1997; Commission Regulation (EC) No. 440/2008 B13/14, dated May 30, 2008; EPA Health Effects Test Guidelines, OPPTS 870.5100, Bacterial Reverse Mutation Test; EPA 712-C-98-247, August, 1998**
GLP/GEP:	**yes**

Executive Summary

This study was performed to investigate the potential of BCS-CU99534 to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.
The assay was performed with and without liver microsomal activation. Each concentration, including the controls, was tested in triplicate. The test item was dissolve din DMSO and tested at the following concentrations:

- Pre-Experiment/Experiment I: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
- Experiment Ia: TA 100 without S9 mix:0.1; 0.3; 1; 3; 10; 33; 100; and 333 µg/plate

- Experiment II without S9 mix: 0.1; 0.3; 1; 3; 10; 33; 100; and 333 µg/plate
- Experiment II with S9 mix: 0.3; 1; 3; 10; 33; 100; 333; and 1000 µg/plate

In the first experiment, the test item precipitated in the overlay agar in the test tubes at 2500 and 5000

µg/plate. Precipitation of the test item in the overlay agar of the incubated agar plates was observed at 5000 µg/plate with and without metabolic activation. The undissolved particles had no influence on the data recording.
In all of the experiments, reduced background growth was noted in all strains at higher concentrations with and without metabolic activation.
All of the experiments showed toxic effects, evident as a reduction in the number of revertants (below an indication factor of 0.5), in the test groups at higher concentrations with and without metabolic activation.

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-CU99534 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.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.
In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.
Therefore, BCS-CU99534 is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay

I. Materials and Methods

A. Material

1. Test Material: BCS-CU99534
Description: yellow solid
Lot/Batch: SES 11956-8-1
Purity: 99.5%
CAS:
Stability of test compound: Stable in the refrigerator at +2°C to +8°C for the study duration

2. Control materials:
Negative: Culture medium
Solvent: DMSO
Positive: Sodium azide (SERVA) for TA 1535, TA 100 at 10 µg/plate in deionized water without S9 mix
4-nitro-o-phenylene-diamine, 4-NOPD ([redacted]) for TA 1537 at 50 µg/plate in DMSO, TA 98 at 10 µg/plate in DMSO without S9 mix
methyl methane sulfonate, MMS ([redacted]) for TA102 at 2 µL/plate in deionised water without S9 mix

2-aminoanthracene, 2-AA (SERVA) for TA 1535, TA 1537, TA 98, TA 100, at 2.5 µg/plate in deionized water and for TA 102 at 10 µg/plate in deionized water with S9 mix

3. Test organisms:
Species: Salmonella typhimurium LT2 mutants

Strain:	Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, and TA 98
Source:	Strains obtained from [redacted] GmbH ([redacted], Germany)

4. Test compound concentrations:

Range-finding	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate
Range-finding for TA100	Assay repeated at lower concentration without metabolic activation: 0.1, 0.3, 1, 3, 10, 33, 100 and 333 µg/plate
Pre-incubation assay:	For all strains without S9 mix: 0.1, 0.3, 1, 3, 10, 33, 100 and 333 µg/plate For all strains with S9 mix: 0.3, 1, 3, 10, 33, 100, 333 and 1000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed between 6 to 16 December 2013 at [redacted] GmbH - [redacted] CCR ([redacted], Germany).

1. Experimental performance

To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates.

For each strain and dose level, including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL Test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL Bacteria suspension,
- 2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution (solvent control), or reference mutagen solution (positive control), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacteria suspension were mixed in a test tube and incubated at 37 °C for 60 minutes. After preincubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark

2. Acceptability of the Assay

The Salmonella typhimurium reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of the laboratory historical data
- the positive control substances should produce a significant increase in mutant colony frequencies
- a minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

3. Assessment criteria

A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed (3). A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (2). An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

II. Results and discussion

In the first experiment the test item precipitated in the overlay agar in the test tubes at 2500 and 5000 µg/plate. Precipitation of the test item in the overlay agar of the incubated agar plates was observed at 5000 µg/plate with and without metabolic activation. No precipitation was noted in experiment Ia and experiment II. The undissolved particles had no influence on the data recording.

The plates incubated with the test item showed reduced background growth at the following concentrations (µg/plate):

Strain	Experiment I		Experiment Ia	Experiment II	
	without S9 mix	with S9 mix	without S9 mix	without S9 mix	with S9 mix
TA 1535	100 - 5000	333 - 5000	n. p.	33 - 333	333 - 1000
TA 1537	100 - 5000	333 - 5000	n. p.	10 - 333	333 - 1000
TA 98	100 - 5000	333 - 5000	n. p.	10 - 333	333 - 1000
TA 100	33 - 5000	333 - 5000	10 - 333	10 - 333	333 - 1000
TA 102	333 - 5000	1000 - 5000	n. p.	100 - 333	1000

n. p. not performed

Toxic effects, evident as a reduction in the number of revertants (below an induction factor of 0.5), were observed at the following concentrations (µg/plate):

Strain	**Experiment I**		**Experiment Ia**	**Experiment II**	
	without S9 mix	with S9 mix	without S9 mix	without S9 mix	with S9 mix
TA 1535	100 - 5000	333 - 5000	n. p.	33 - 333	333 - 1000
TA 1537	100 - 5000	333 - 5000	n. p.	33 - 333	1000
TA 98	100 - 5000	333 - 5000	n. p.	10 - 333	333 - 1000
TA 100	33 - 5000	333 - 5000	33 - 333	10 - 333	333 - 1000
TA 102	333 - 5000	1000 - 5000	n. p.	100 - 333	1000

n. p. not performed

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-CU99534 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix).

There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

Table 5.8.1-35 Summary of Experiment I

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	DMSO	0	17 ± 5	11 ± 4	24 ± 8	101 ± 6	505 ± 9
	Untreated	0	15 ± 2	14 ± 1	31 ± 5	107 ± 8	469 ± 23
	BCS-CU99534	3 µg	19 ± 4	8 ± 4	29 ± 2	90 ± 15	467 ± 16
		10 µg	11 ± 3	9 ± 3	21 ± 5	83 ± 22	455 ± 45
		33 µg	14 ± 1	11 ± 4	21 ± 6	13 ± 4 MR	467 ± 12
		100 µg	4 ± 1 MR	4 ± 2 MR	4 ± 1 MR	2 ± 1 MR	419 ± 21
		333 µg	1 ± 1 MR	1 ± 1 MR	1 ± 1 MR	1 ± 1 MR	81 ± 4 MR
		1000 µg	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR
		2500 µg	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR
		5000 µg	0 ± 0 PMR	0 ± 0 PMR	0 ± 0 PMR	0 ± 0 PMR	0 ± 0 PMR
	NaN3	10 µg	2496 ± 67			2140 ± 76	
	4-NOPD	10 µg			296 ± 9		
	4-NOPD	50 µg		106 ± 1			
	MMS	2.0 µL					5202 ± 562
With Activation	DMSO	0	13 ± 3	12 ± 4	38 ± 13	108 ± 14	596 ± 9
	Untreated		17 ± 8	16 ± 7	38 ± 7	116 ± 12	614 ± 49
	BCS-CU99534	3 µg	13 ± 3	14 ± 5	41 ± 6	101 ± 12	618 ± 23
		10 µg	15 ± 5	14 ± 7	32 ± 5	110 ± 20	650 ± 11
		33 µg	14 ± 4	11 ± 1	33 ± 2	102 ± 8	634 ± 17
		100 µg	10 ± 3	7 ± 3	25 ± 4	94 ± 8	610 ± 41
		333 µg	2 ± 1 MR	4 ± 1 MR	1 ± 0 R	12 ± 3 RM	470 ± 20
		1000 µg	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	7 ± 2 MR
		2500 µg	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR	0 ± 0 MR
		5000 µg	0 ± 0 PMR	0 ± 0 PMR	0 ± 0 PMR	0 ± 0 PMR	0 ± 0 PMR
	2-AA	2.5 µg	397 ± 26	289 ± 8	2793 ± 121	3515 ± 78	
	2-AA	10.0 µg					1347 ± 50

Key to Positive Controls		Key to Plate Postfix Codes	
NaN3	sodium azide	M	Manual count
2-AA	2-aminoanthracene	R	Reduced background growth
MMS	methyl methane sulfonate	P	Precipitate
4-NOPD	4-nitro-o-phenylene-diamine		

5.8.1-36 Summary of Experiment Ia

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)
			TA 100
Without Activation	DMSO		87 ± 3
	Untreated		93 ± 6
	BCS-CU99534	0.1 µg	71 ± 6
		0.3 µg	76 ± 13
		1 µg	75 ± 6
		3 µg	84 ± 14
		10 µg	40 ± 3 MR
		33 µg	6 ± 2 MR
		100 µg	0 ± 1 MR
		333 µg	0 ± 0 MR
	NaN3	10 µg	2006 ± 213

Key to Positive Controls

NaN3 sodium azide

Key to Plate Postfix Codes

M Manual count

R Reduced background growth

5.8.1-27 Summary of Experiment II

Metabolic Activation	Test Group	Dose Level (per plate)	Revertant Colony Counts (Mean ±SD)				
			TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	DMSO		20 ± 5	9 ± 2	21 ± 2	92 ± 1	498 ± 12
	Untreated		20 ± 3	9 ± 3	28 ± 5	101 ± 9	471 ± 7
	BCS-CU99534	0.1 µg	18 ± 4	8 ± 2	19 ± 4	78 ± 9	484 ± 20
		0.3 µg	18 ± 8	8 ± 2	18 ± 3	92 ± 10	470 ± 6
		1 µg	17 ± 3	10 ± 4	21 ± 1	82 ± 2	472 ± 19
		3 µg	21 ± 7	9 ± 1	25 ± 4	77 ± 10	493 ± 37
		10 µg	15 ± 2	4 ± 1 M R	7 ± 3 M R	35 ± 7 M R	449 ± 27
		33 µg	9 ± 1 M R	3 ± 1 M R	5 ± 1 M R	2 ± 1 M R	365 ± 2
		100 µg	2 ± 1 M R	1 ± 1 M R	0 ± 0 M R	0 ± 0 M R	155 ± 41 M R
		333 µg	0 ± 0 M R	0 ± 1 M R	0 ± 0 M R	0 ± 0 M R	40 ± 8 M R
	NaN3	10 µg	2543 ± 149			1881 ± 82	
	4-NOPD	10 µg			334 ± 13		
	4-NOPD	50 µg		65 ± 12			
	MMS	2.0 µL					3795 ± 334
Metabolic Activation	**Test Group**	**Dose Level (per plate)**	**Revertant Colony Counts (Mean ±SD)**				
With Activation	DMSO		19 ± 3	20 ± 6	34 ± 3	100 ± 5	541 ± 38
	Untreated		15 ± 0	18 ± 7	45 ± 10	129 ± 40	612 ± 21
	BCS-CU99534	0.3 µg	17 ± 4	20 ± 6	34 ± 11	94 ± 7	587 ± 23
		1 µg	22 ± 2	18 ± 7	38 ± 7	94 ± 11	547 ± 16
		3 µg	19 ± 6	19 ± 5	39 ± 9	104 ± 5	542 ± 29
		10 µg	20 ± 2	21 ± 3	38 ± 10	99 ± 10	548 ± 76
		33 µg	17 ± 3	26 ± 3	32 ± 4	72 ± 5	565 ± 4
		100 µg	16 ± 0	17 ± 4	28 ± 7	81 ± 10	509 ± 69
		333 µg	6 ± 2 M R	14 ± 7 R M	0 ± 0 M R	0 ± 0 M R	404 ± 18
		1000 µg	0 ± 0 M R	0 ± 0 M R	0 ± 0 M R	0 ± 0 M R	5 ± 2 M R
	2-AA	2.5 µg	399 ± 19	304 ± 21	2447 ± 270	2858 ± 75	
	2-AA	10.0 µg					1203 ± 39

Key to Positive Controls		Key to Plate Postfix Codes	
NaN3	sodium azide	M	Manual count
2-AA	2-aminoanthracene	R	Reduced background growth
MMS	methyl methane sulfonate		
4-NOPD	4-nitro-o-phenylene-diamine		

III. CONCLUSION

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

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Report:	[redacted]x;[redacted];2014;M-490043-01
Title:	BCS-CU99534: In vitro micronucleus test in human lymphocytes
Report No:	1589902
Document No:	M-490043-01-1
Guidelines:	**OECD Guidelines for Testing of Chemicals No. 487, Commission Regulation (EU) No 640/2012 B49;none**
GLP/GEP:	**yes**

Executive Summary

BCS-CU99534, suspended (Exp. I and IIA) or dissolved (Exp. IIB) in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes in vitro in three independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix
	Exp. I	Exp. IIA & IIB	Exp. I & IIA
Stimulation period	48 hrs	48 hrs	48 hrs
Exposure period	4 hrs	20 hrs	4 hrs
Recovery	16 hrs	—	16 hrs
Cytochalasin B exposure	20 hrs	20 hrs	20 hrs
Total culture period*	88 hrs	88 hrs	88 hrs

In each experimental group two parallel cultures were analysed and per culture at least 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (1900.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the current OECD Guideline 487.

Dose selection of the cytogenetic experiment was performed considering the toxicity data and the occurrence of test item precipitation in accordance with OECD Guideline 487.

In Experiment I, in the absence of S9 mix, cytotoxicity of about 50 % cytostasis was observed at the highest evaluated concentration.

In Experiment I in the presence of S9 mix, in Experiment IIA in the absence and presence of S9 mix and in Experiment IIB in the absence of S9 mix, concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage.

Overall, in the absence and presence of S9 mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test item. However, in Experiment IIA after continuous treatment with 12.2 µg/mL in the absence of S9 mix one single increase in micronucleated cells (1.83 %) above the historical control range (0.05 – 1.45 %) was observed. Neither a dose-dependency nor statistical significance was observed. No relevant increase in the number of micronucleated cells was observed in Experiment IIB and thus the finding is regarded as biologically irrelevant.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes.

Therefore, BCS-CU99534 is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to cytotoxic or the highest evaluable concentrations.

I. Materials and Methods

A. Material

1. Test Material:	BCS-CU99534
Description:	Yellow solid
Lot/Batch:	SES 11956-8-1
Purity:	99.5%
CAS:	
Stability of test compound:	Stable in the refrigerator at +2°C to +8°C for the study duration
2. Control materials:	
Negative:	Culture medium with 1.0% DMSO
Solvent:	DMSO
Positive controls	
a) Without metabolic activation	MMC, mitomycin C (pulse treatment), 2.0 µg/mL dissolved in deionized water Demecolcin (continuous treatment), 100 ng/mL (Exp. IIA) or 75.0 ng/mL (Exp. IIB) dissolved in deionized water
b) With metabolic activation	CPA, cyclophosphamide (continuous treatment), 15 µg/mL dissolved in Saline (0.9% NaCl [w/v])

Microsomal fraction/S9 mix

Phenobarbital/β-naphth[redacted]flavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the [redacted]CCR SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ 8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 29.8 mg/mL (Lot no. 050913).

3. Test system:

Human lymphocytes

Blood samples were drawn from healthy non-smoking donors not receiving medication. For this study, blood was collected from a female donor (33 years old) for Experiment I and IIB and from a female donor (29 years old) for Experiment IIA.

The lymphocytes of the respective donors have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. All donors had a previously established low incidence of micronuclei in their peripheral blood lymphocytes.

4. Test compound concentrations:

Range-finding	First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate
Range-finding for TA100	Assay repeated at lower concentration without metabolic activation: 0.1, 0.3, 1, 3, 10, 33, 100 and 333 µg/plate
Pre-incubation assay:	For all strains without S9 mix: 0.1, 0.3, 1, 3, 10, 33, 100 and 333 µg/plate For all strains with S9 mix: 0.3, 1, 3, 10, 33, 100, 333 and 1000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed from 20 November 2013 to 17 February 2014 at [redacted] GmbH - [redacted] CCR ([redacted], Germany).

1. Experimental performance

Test system preparation

Human lymphocytes were stimulated for proliferation by the addition of the mitogen PHA to the culture medium for a period of 48 hours. The cell harvest time point was approximately 2 – 2.5 x AGT (average generation time). Any specific cell cycle time delay induced by the test item was not accounted for directly.

Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 hrs after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).

All incubations were done at 37 °C with 5.5 % CO_2 in humidified air.

Test system preparation

Stock formulations of BCS-CU99534 and serial dilutions were made in DMSO. The final concentration of DMSO in the culture medium was 1.0 %. The solvent was chosen due to its solubility properties and its relative non-toxicity to the cell cultures. All formulations were prepared freshly before treatment and used within two hours of preparation.

There were no effects of the test item on pH and osmolarity in the medium as measured in the solvent control and the maximum concentration without metabolic activation

Dose selection

Dose selection was performed according to the current OECD Guideline for the in vitro micronucleus test. The highest test item concentration should be 5000 µg/mL, 5 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

With regard to the molecular weight of the test item, 1900.0 µg/mL of BCS-CU99534 (approx. 10 mM) were applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations between 7.1 and 1900.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test for toxicity, precipitation of the test item was observed

microscopically at the end of treatment at 66.1 µg/mL and above in the absence of S9 mix and at 115.8 µg/mL and above in the presence of S9 mix. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity in Experiment I, toxic effects were observed after 4 hours treatment with 37.8 µg/mL and above in the absence of S9 mix and with 115.8 µg/mL and above in the presence of S9 mix. Therefore, 200.0 µg/mL (without S9 mix) and 400.0 µg/mL (with S9 mix) were chosen as top treatment concentration in Experiment IIA.
The experimental part without S9 mix was repeated with a top dose of 200.0 µg/mL and narrow concentration spacing (Exp. IIB) to obtain evaluable concentations in a cytotoxic range.

Pre-experiment
A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay.
The pre-test was performed with 4 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9 mix). The preparation interval was 40 hrs after start of the exposure.

Cytogentic experiment
Pulse exposure
About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose · H_2O, 192 mg/L $Na_2HPO_4 \cdot 2 H_2O$ and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)
About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated

once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Preparation of slides

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Evaluation of cytotoxicity and cytogenetic damage

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI Cytokinesis-block proliferation index
n Total number of cells
MONC Mononucleate cells
BINC Binucleate cells
MUNC Multinucleate cells

$$\text{Cytostasis } \% = 100 - 100\,[(CBPI_T - 1) / (CBPI_C - 1)]$$

T Test item
C Solvent control

3. Assessment criteria

The micronucleus assay will be considered acceptable if it meets the following criteria:

The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
The rate of micronuclei in the positive controls is statistically significant increased.
The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.

A test item can be classified as non-clastogenic and non-aneugenic if:

- the number of micronucleated cells in all evaluated dose groups is in the range of the historical laboratory control data and
- no statistically significant or concentration-related increase of the number of micronucleated cells is observed in comparison to the respective solvent control.

A test item can be classified as clastogenic and aneugenic if:

- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.

An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

II. Results and discussion

Three independent experiments were performed.
In Experiment I, the exposure period was 4 hours with and without S9 mix.
In Experiment IIA, the exposure periods were 4 hours with S9 mix and 20 hours without S9 mix.
In Experiment IIB, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test item.
In each experimental group two parallel cultures were analysed. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. To determine a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is described as % cytostasis.
The highest treatment concentration in this study, 1900.0 Lg/mL (approx. 10 mM) was chosen with regard to the molecular weight of the test item and with respect to the OECD Guideline 487 for the in vitro mammalian cell micronucleus test.

In Experiment I, visible precipitation of the test item in the culture medium was observed microscopically at 66.1 Lg/mL and above in the absence of S9 mix and at 115.8 Lg/mL and above in the presence of S9 mix at the end of treatment.
In addition, precipitation occurred microscopically in Experiment IIA and IIB in the absence of S9 mix at 200.0 Lg/mL at the end of treatment and in Experiment IIA in the presence of S9 mix at 70.0 Lg/mL and above.

Table 5.8.1-38 Summary of the results

Test item	Concentration (µg/mL)	Proliferation index CBPI	Cytostatsis in %*	Micronucleated cells in %**	HCD range
Experiment I : Exposure period 4 hours without S9 mix					
Solvent control: DMSO	1.0 % (v/v)	2.00	/	0.65	0.15 - 1.40
Positive control: MMC	2.0	1.37	63.3	**12.80**	***3.60 – 25.10***
BCS-CU99534	12.3	2.04	n.c.	0.50	
	21.6	1.90	9.9	0.55	
	37.8	1.52	48.4	1.25	
Experiment IIA : Exposure period 20 hours without S9 mix					
Solvent control: DMSO	1.0 % (v/v)	2.06	/	1.43***	0.10 - 1.35
Positive control: Demecolcin	0.1	1.90	15.4	**3.60**	1.40 – 6.10
BCS-CU99534	12.2	2.05	1.3	*1.83****	
	21.3	2.04	1.9	0.85	
	37.3	1.94	11.8	1.40	
Experiment IIB : Exposure period 20 hours without S9 mix					
Solvent control: DMSO	1.0 % (v/v)	1.57	/	0.50	0.10 – 1.35
Positive control: Demecolcin	0.075	1.53	7.5	**3.20**	**1.40 – 6.10**
BCS-CU99534	5.0	1.58	n.c.	0.50	
	10.0	1.65	n.c.	0.85	
	20.0	1.57	0.2	0.30	
Experiment I : Exposure period 4 hours with S9 mix					
Solvent control: DMSO	1.0 % (v/v)	2.12	/	0.75	0.20 – 1.65
Positive control: CPA	15.0	1.75	33.4	**3.25**	2.20 -11.05
BCS-CU99534	21.6	1.96	14.1	0.15	
	37.8	2.00	10.5	0.45	
	66.1	1.84	25.4	0.75	
Experiment IIA : Exposure period 4 hours with S9 mix					
Solvent control: DMSO	1.0 % (v/v)	2.09	/	0.95	0.20- 1.65
Positive control: CPA	15.0	1.81	25.9	**3.00**	2.20 -11.05
BCS-CU99534	40.0	2.10	n.c.	0.80	
	50.0	1.95	13.0	0.80	
	60.0	1.97	10.9	1.20	

For the positive control groups and the test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
*** The number of micronucleated cells was determined in a sample of 4000 binucleated cells
Bold The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value

In Experiment I in the absence of S9 mix, cytotoxicity (48.4 % cytostasis) was observed at the highest evaluated concentration.

In Experiment I in the presence of S9 mix, in Experiment IIA in the absence and presence of S9 mix and in Experiment IIB in the absence of S9 mix, concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage.

The micronucleus rates of the cells after treatment with the test item in Experiment I and IIB without S9 mix and in Experiment I and IIA with S9 mix (0.15 – 1.25 % micronucleated cells) were close to the range of the solvent control values (0.50 – 0.95 % micronucleated cells) and within the range of the laboratory historical control data.

In Experiment IIA after continuous treatment with 12.2 µg/mL in the absence of S9 mix one single increase in micronucleated cells (1.83 %) above the historical control range (0.05 – 1.45 %) was observed. However, there was no dose-dependency and the increase was not statistically significant.

As no relevant increase in the number of micronucleated cells was observed in Experiment IIB this the finding is regarded as biologically irrelevant.

All the positive controls, Demecolcin (75.0 or 100.0 ng/mL), MMC (2.0 Lg/mL) or CPA (15.0 Lg/mL) showed distinct increases in cells with micronuclei.

III. CONCLUSION

In conclusion, it can be stated that under the experimental conditions reported, BCS-CU99534 did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes.
Therefore, it is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to cytotoxic or the highest evaluable concentrations.

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Report:	[redacted]6; [redacted];2014;M-491085-01
Title:	BCS-CU99534; Statement on further toxicological assessment
Report No:	M-491085-01-1
Document No:	M-491085-01-1
Guidelines:	**-/-**
GLP/GEP:	**n.a.**

Position paper to summarize the toxicological information available for the Propineb metabolie Propineb-DIDT (BCS-CU99534). The position paper will be available after finalisation of the In vivo rat 28-day comparative toxicity study with Propineb-DIDT(BCS-CU99534).

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PROPINEB-FORMYL-PDA (BCS-CY52341)

Formyl-PDA (BCS-CY52341) is a metabolite of propineb that is not found in the animals and that exceeds the threshold level for genotoxicity. Therefore, an invitro package for genotoxicity has been started. The report of gthe Ames test is available and will be submitted with the Delta-dossier, whereas the in vitro micronucleus study is still ongoing

Table 5.8.1-39 Summary of the new studies with the metanolite Propineb-Formyl PDA

Type of study	Species/test system	Result
Ames test. [redacted].; 2014. M-490977-01-1	Salmonella typhimurium TA 1535, TA 1537, TA 98, TA 100, and TA 102	Negative
In vitro micronucleus [redacted].; 2014; M-491073-02-1	Human lymphocytes	Negative

Report:	[redacted];2014;M-490977-01
Title:	BCS-CY52341: Salmonella typhimurium reverse mutation assay
Report No:	1622001
Document No:	M-490977-01-1
Guidelines:	**Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471: Bacterial Reverse Mutation Test; adopted July 21, 1997; Commission Regulation (EC) No. 440/2008 B13/14, dated May 30, 2008; EPA Health Effects Test Guidelines, OPPTS 870.5100, Bacterial Reverse Mutation Test; EPA 712-C-98-247, August, 1998.**
GLP/GEP:	**yes**

Executive Summary

This study was performed to investigate the potential of the propineb metabolite propineb-formyl PDA (BCS-CY52341) to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.

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 dissolved in deionized water and 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, 250; and 5000 µg/plate

No precipitation of the test item occurred up to the highest investigated dose.

The plates incubated with the test item showed normal back¬ground growth up to 5000 µg/plate with and without S9 mix in all strains used. Only in experiment I reduced background growth was observed in the presence of metabolic activation in strain TA 1535 at 5000 µg/plate and in strain TA 1537 at 2500 and 5000 µg/plate.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5),

occurred in the test groups with and without metabolic activation.
No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-CY52341 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.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

In conclusion, BCS-CY52341 is considered to be non-mutagenic in this Salmonella typhimurium reverse mutation assay.

I. Materials and Methods

A. Material

1. Test Material: BCS-CY52341
Synonym: 1-formamidopropan-2-aminium chloride, Propineb-formyl PDA hydrochloride
Description: yellow solid
Lot/Batch: SES 12673-5-1
Purity: 94.1% w/w (BCS-CY52341) and 1.64% w/w water. Correction for purity was made
CAS:
Stability of test compound: Stable at room temperature for the study duration

2. Control materials:
Negative: Culture medium
Solvent: Deionized water
Positive: Sodium azide (SERVA) for TA 1535, TA 100 at 10 µg/plate in deionized water without S9 mix
4-nitro-o-phenylene-diamine, 4-NOPD ([redacted]) for TA 1537 at 50 µg/plate in DMSO, TA 98 at 10 µg/plate in DMSO without S9 mix
methyl methane sulfonate, MMS ([redacted]) for TA102 at 2 µL/plate in deionised water without S9 mix

2-aminoanthracene, 2-AA (SERVA) for TA 1535, TA 1537, TA 98, TA 100, at 2.5 µg/plate in deionized water and for TA 102 at 10 µg/plate in deionized water with S9 mix

Mammalian Microsomal Fraction S9 Mix
Phenobarbital/β-naphth[redacted]lavone induced rat liver S9 were used as the metabolic activation system.
The S9 was prepared and stored according to the currently valid version of the [redacted]CCR SOP for rat liver S9 preparation. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

The protein concentration of the S9 preparation was 33.2 mg/mL (Lot No.: 130314K) in both experiments.
An appropriate quantity of S9 supernatant is thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 10 % v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM	MgCl2
33 mM	KCl
5 mM	glucose-6-phosphate
4 mM	NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experiment, the S9 mix is stored in an ice bath

3. Test organisms:

Species: Salmonella typhimurium LT2 mutants
Strain: Histidine-auxotrophic strains TA 1535, TA 100, TA 1537, and TA 98
Source: Strains obtained from [redacted] GmbH ([redacted], Germany)

4. Test compound concentrations:

Pre-experiment/ Experiment 1: First assay for all strains with or without S9 mix: 3, 10, 33, 100, 333, 1000, 2500, and 5000 µg/plate
Pre-incubation assay: For all strains with or without S9 mix: 33, 100, 333, 1000, 2500, and 5000 µg/plate

B. Study Design and methods

The experimental phase of the study was performed from 28 May to 13 June 2014 at [redacted] GmbH - [redacted] CCR ([redacted], Germany).

1. Experimental performance

To evaluate the toxicity of the test item a pre-experiment was performed with all strains used. Eight concentrations were tested for toxicity and mutation induction with each 3 plates.
For each strain and dose level, including the controls, three plates were used.
The following materials were mixed in a test tube and poured onto the selective agar plates:

- 100 µL Test solution at each dose level (solvent or reference mutagen solution (positive control)),
- 500 µL S9 mix (for test with metabolic activation) or S9 mix substitution buffer (for test without metabolic activation),
- 100 µL Bacteria suspension,
- 2000 µL Overlay agar

In the pre-incubation assay 100 µL test solution (solvent control), or reference mutagen solution (positive control), 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacteria suspension were

mixed in a test tube and incubated at 37 °C for 60 minutes. After preincubation 2.0 mL overlay agar (45 °C) was added to each tube. The mixture was poured on minimal agar plates.
After solidification the plates were incubated upside down for at least 48 hours at 37 °C in the dark

2. Acceptability of the Assay
The Salmonella typhimurium reverse mutation assay is considered acceptable if it meets the following criteria:

- regular background growth in the negative and solvent control
- the spontaneous reversion rates in the negative and solvent control are in the range of the laboratory historical data
- the positive control substances should produce a significant increase in mutant colony frequencies
- a minimum of five analysable dose levels should be present with at least three dose levels showing no signs of toxic effects, evident as a reduction in the number of revertants below the indication factor of 0.5.

3. Assessment criteria
A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice (strains TA 98, TA 100, and TA 102) or thrice (strains TA 1535 and TA 1537) the colony count of the corresponding solvent control is observed (3).
A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration (2). An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment. A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remain within the historical range of negative and solvent controls such an increase is not considered biologically relevant.

H. Results and discussion

The test item BCS-CY52341 was assessed for its potential to induce gene mutations according to the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using Salmonella typhimurium strains TA 1535, TA 1537, TA 98, TA 100, and TA 102.
The assay was performed in two independent experiments both with and without liver microsomal activation. Each concentration and the controls, were 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

No precipitation of the test item occurred up to the highest investigated dose.
The plates incubated with the test item showed normal back¬ground growth up to 5000 µg/plate with and without S9 mix in all strains used. Only in experiment I reduced background growth was observed in the presence of metabolic activation in strain TA 1535 at 5000 µg/plate and in strain TA 1537 at 2500 and 5000 µg/plate.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5), occurred in the test groups with and without metabolic activation.

Table 5.8.1-40 Summary of Experiment I: Revertant Colony Counts (Mean ±SD)

Metabolic Activation	Test Group	Dose Level (per plate)	TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	Deionised water		17 ± 4	10 ± 3	28 ± 4	160 ± 16	397 ± 25
	Untreated		19 ± 3	11 ± 3	26 ± 6	157 ± 7	422 ± 21
	BCS-CY52341	3 µg	15 ± 3	10 ± 2	26 ± 5	156 ± 11	401 ± 43
		10 µg	13 ± 4	9 ± 3	30 ± 1	145 ± 5	392 ± 16
		33 µg	13 ± 2	13 ± 3	31 ± 7	165 ± 15	418 ± 43
		100 µg	14 ± 3	12 ± 2	24 ± 3	159 ± 19	408 ± 54
		333 µg	16 ± 3	10 ± 2	25 ± 5	157 ± 6	410 ± 38
		1000 µg	17 ± 9	10 ± 2	24 ± 8	164 ± 11	397 ± 14
		2500 µg	13 ± 2	12 ± 3	27 ± 11	160 ± 17	415 ± 9
		5000 µg	16 ± 7	6 ± 1	33 ± 5	178 ± 13	456 ± 67
	NaN3	10 µg	2695 ± 160			1726 ± 23	
	4-NOPD	10 µg			252 ± 28		
	4-NOPD	50 µg		57 ± 8			
	MMS	2.0 µL					3526 ± 331
With Activation	Deionised water		12 ± 5	12 ± 3	29 ± 5	136 ± 12	551 ± 22
	Untreated		10 ± 5	15 ± 2	36 ± 12	155 ± 11	537 ± 20
	BCS-CY52341	3 µg	11 ± 3	18 ± 8	35 ± 2	149 ± 21	568 ± 49
		10 µg	11 ± 3	17 ± 4	35 ± 2	144 ± 17	583 ± 44
		33 µg	13 ± 5	15 ± 5	40 ± 5	140 ± 11	513 ± 49
		100 µg	15 ± 6	17 ± 8	40 ± 10	159 ± 21	584 ± 25
		333 µg	12 ± 3	12 ± 6	40 ± 10	127 ± 7	509 ± 96
		1000 µg	12 ± 3	12 ± 5	38 ± 11	145 ± 5	463 ± 19
		2500 µg	13 ± 2	9 ± 3 R	44 ± 8	145 ± 16	570 ± 61
		5000 µg	18 ± 7 R	14 ± 4 R	34 ± 5	150 ± 6	502 ± 33
	2-AA	2.5 µg	606 ± 43	207 ± 42	2781 ± 447	3303 ± 372	
	2-AA	10.0 µg					1282 ± 31

Key to Positive Controls

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

Key to Plate Postfix Codes

R	Reduced background growth

Table 5.8.1-41 Summary of Experiment II: Revertant Colony Counts (Mean ±SD)

Metabolic Activation	Test Group	Dose Level (per plate)	TA 1535	TA 1537	TA 98	TA 100	TA 102
Without Activation	Deionised water		14 ± 1	11 ± 2	19 ± 2	178 ± 27	441 ± 24
	Untreated		13 ± 1	6 ± 2	22 ± 4	190 ± 9	405 ± 22
	BCS-CY52341	33 µg	11 ± 5	10 ± 3	22 ± 5	162 ± 11	423 ± 58
		100 µg	20 ± 4	10 ± 3	27 ± 4	193 ± 33	387 ± 30
		333 µg	14 ± 5	3 ± 0	23 ± 4	175 ± 39	450 ± 43
		1000 µg	10 ± 5	11 ± 3	23 ± 4	184 ± 11	342 ± 46
		2500 µg	14 ± 1	7 ± 5	18 ± 1	175 ± 10	427 ± 35
		5000 µg	13 ± 4	8 ± 1	22 ± 2	161 ± 29	394 ± 31
	NaN3	10 µg	2783 ± 115			1556 ± 152	
	4-NOPD	10 µg			303 ± 39		
	4-NOPD	50 µg		56 ± 5			
	MMS	2.0 µL					3822 ± 714
With Activation	Deionised water		14 ± 1	19 ± 5	40 ± 5	160 ± 14	600 ± 37
	Untreated		10 ± 4	18 ± 4	37 ± 2	171 ± 29	562 ± 57
	BCS-CY52341	33 µg	12 ± 4	18 ± 3	43 ± 1	159 ± 21	588 ± 30
		100 µg	11 ± 5	16 ± 7	40 ± 5	174 ± 10	603 ± 55
		333 µg	14 ± 2	18 ± 9	32 ± 2	230 ± 31	598 ± 53
		1000 µg	10 ± 4	17 ± 5	39 ± 5	184 ± 14	515 ± 56
		2500 µg	17 ± 2	22 ± 1	42 ± 13	162 ± 4	591 ± 38
		5000 µg	10 ± 4	21 ± 3	34 ± 0	176 ± 16	578 ± 64
	2-AA	2.5 µg	408 ± 39	167 ± 14	3778 ± 37	2562 ± 113	
	2-AA	10.0 µg					1614 ± 70

Key to Positive Controls

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

No substantial increase in revertant colony numbers of any of the five tester strains was observed following treatment with BCS-CY52341 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

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

III. CONCLUSION

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, BCS-CY52341 did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

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Report:	KCA 5.8.1 /42; [redacted].;2011;M-491073-02-1
Title:	BCS-CY52341: In vitro Micronucleus Test in Human Lymphocytes
Report No:	1622002
Document No:	M-491073-02-1
Guidelines:	**OECD Guidelines for Testing of Chemicals No. 487** **Commission Regulation (EU) No 640/2012 B49**
GLP/GEP:	yes

Executive Summary

The test item BCS-CY52341, dissolved in deionised water, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in two independent experiments. The following study design was performed:

	Without S9 mix		With S9 mix
	Exp. I	Exp. II	Exp. I & II
Stimulation period	48 hrs	48 hrs	48 hrs
Exposure period	4 hrs	20 hrs	4 hrs
Recovery	16 hrs	-	16 hrs
Cytochalasin B exposure	20 hrs	20 hrs	20 hrs
Total culture period	88 hrs	88 hrs	88 hrs

In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (1473.0 µg/mL of the test item, approx. 10 mM) was chosen with regard to the molecular weight and the content (94.1 %) of the test item and with respect to the current OECD Guideline 487.

Dose selection of the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487. The chosen treatment concentrations ranged between 9.6 and 1473.0 µg/mL in both experiments with and without S9 mix.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In the absence and the presence of S9 mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test item. However, in Experiment II in the absence of S9 mix one statistically significant increase in micronucleated cells (0.70 %) was observed at the highest dose tested (1473.0 µg/mL). Since the value is clearly within the range of the laboratory historical solvent control data (0.05 – 1.45 % micronucleated cells), this finding has to be regarded as biologically irrelevant.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes.

Therefore, BCS-CY52341 is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to the highest required concentration.

I. Materials and Methods

A. Material

	BCS-CY52341
Synonym:	1-formamidopropan-2-aminium chloride, Propineb-formyl PDA hydrochloride
Description:	yellow solid
Lot/Batch:	SES 12673-3-1
Purity:	94.1% w/w (BCS-CY52341) and 1.64% w/w water. Correction for purity was made
CAS:	
Stability of test compound:	Stable at room temperature for the study duration

2. Control materials:

Negative:	Culture medium
Solvent:	Deionized water
Positive controls	
a) Without metabolic activation	MMC; mitomycin C (pulse treatment), 2.0 µg/mL dissolved in deionized water (Experiment I)
	Demecolcin (continuous treatment), 75.0 ng/mL dissolved in deionized water (Experiment II)
b) With metabolic activation	CPA; cyclophosphamide (continuous treatment), dissolved in Saline (0.9 % NaCl [w/v]) at 15 µg/mL (Experiment I) and 12.5 µg/mL (Experiment II)

Microsomal fraction S9 mix

Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the [redacted] CCR SOP for rat liver S9 preparation. Each batch of S9 is routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ 8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

The protein concentration of the S9 preparation used for this study was 35.0 mg/mL (Lot no. 310114).

3. Test system:

Human lymphocytes

Blood samples were drawn from healthy non-smoking donors not receiving medication. For this study, blood was collected from a female donor (29 years old) for Experiment I and from a female donor (34 years old) for Experiment II. The lymphocytes of the respective donors have been shown to respond well to stimulation of proliferation with PHA and to positive control substances. All donors had a previously established low incidence of micronuclei in their peripheral blood lymphocytes.

4. Test compound concentrations:
Dose selection was performed according to the current OECD Guideline for the in vitro micronucleus test. The highest test item concentration should be 5000 µg/mL, 5 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

With regard to the molecular weight and the content (94.1 %) of the test item, 1473.0 µg/mL of BCS-CY52341 (approx. 10 mM) were applied as top concentration for treatment of the cultures in the pre-test. Test item concentrations ranging from 157.1 to 1473.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test for toxicity, no precipitation of the test item was observed. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated Experiment I.

Using a reduced Cytokinesis-block proliferation index (CBPI) as an indicator for toxicity, no cytotoxic effects were observed in Experiment I after 4 hours treatment in the absence and presence of S9 mix. Therefore, 1473.0 µg/mL was chosen as top treatment concentration for Experiment II.

B. Study Design and methods

The experimental phase of the study was performed from 4 June 2014 to 16 July 2014 at [redacted] GmbH - [redacted] CCR ([redacted], Germany).

1. Experimental performance
Stock formulations of the test item and serial dilutions were made in deionised water. The final concentration of deionised water in the culture medium was 10 %. The solvent was chosen due to its solubility properties and its relative non-toxicity to the cell cultures.
All formulations were prepared freshly before treatment and used within two hours of preparation. The osmolarity and pH-value were determined in the solvent control and the maximum concentration without metabolic activation and there was no effect of the test item on these parameters.

Pre-experiment
A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. Cytotoxicity is characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate. The experimental conditions in this pre-experimental phase were identical to those required and described below for the mutagenicity assay.
The pre-test was performed with 10 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 hrs (with and without S9 mix). The preparation interval was 40 hrs after start of the exposure.

Pulse exposure
About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation 50 µL S9 mix per mL culture medium was added. After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose •H2O, 192 mg/L Na2HPO4 • 2 H2O and 150 mg/L KH2PO4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)
About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation

for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Preparation of slides

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Evaluation of cytotoxicity and cytogenetic damage

Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The criteria for the evaluation of micronuclei are described in the publication of Countryman and Heddle (1976). The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI Cytokinesis-block proliferation index
n Total number of cells
MONC Mononucleate cells
BINC Binucleate cells
MUNC Multinucleate cells

$$\text{Cytostasis \%} = 100 - 100\,[(CBPI_T - 1) / (CBPI_C - 1)]$$

T Test item
C Solvent control

2. Assessment criteria

The micronucleus assay is considered acceptable if it meets the following criteria:
- The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
- The rate of micronuclei in the positive controls is statistically significant increased.
- The quality of the slides must allow the evaluation of a sufficient number of analyzable cells.

A test item can be classified as non-clastogenic and non-aneugenic if:
- the number of micronucleated cells in all evaluated dose groups is in the range of the historical laboratory control data and
- no statistically significant or concentration-related increase of the number of micronucleated cells is observed in comparison to the respective solvent control.

A test item can be classified as clastogenic and aneugenic if:

- the number of micronucleated cells is not in the range of the historical laboratory control data and
- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.
An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

III. RESULTS AND CONCLUSION

II. Results and discussion

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 20 hours without S9 mix.
The cells were prepared 40 hours after start of treatment with the test item.

In each experimental group two parallel cultures were analysed. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. To determine a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is described as % cytostasis.

The highest treatment concentration in this study, 1473.0 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the content (94.1 %) of the test item and with respect to the OECD Guideline 487 for the in vitro mammalian cell micronucleus test.

No visible precipitation of the test item in the culture medium was observed. No relevant influence on osmolarity or pH was observed.

No relevant cytotoxicity, indicated by reduced CBPI and described as cytostasis could be observed up to the highest applied concentration.

In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying micronuclei was observed.

The micronucleus rates of the cells after treatment with the test item (0.35 – 1.00% micronucleated cells) were close to the range of the solvent control values (0.25 – 0.60% micronucleated cells) and within the range of the laboratory historical control data (see Appendix 1).

However, in Experiment II in the absence of S9 mix one statistically significant increase in micronucleated cells was observed after treatment with 1473.0 µg/mL (0.70 % micronucleated cells). Since the value is clearly within the range of the laboratory historical solvent control data (0.05 – 1.45 % micronucleated cells), this finding has to be regarded as biologically irrelevant.

In both experiments, either Demecolcin (75.0 ng/mL), MMC (2.0 µg/mL) or CPA (12.5 or 15.0 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

III. CONCLUSION

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the in vitro micronucleus test in human lymphocytes.
Therefore, BCS-CY52341 is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to the highest required concentration.

Table 5.8.1-41 Summary of the results

Test item	Concentration (µg/mL)	Proliferation index CBPI	Cytostasis in %	Micronucleated cells in %	HCD range
Experiment I : Exposure period 4 hours without S9 mix					
Solvent control: DMSO	10.0 % (v/v)	1.95	/	0.45	0.15 - 1.40
Positive control: MMC	2.0	1.30	68.4	7.15	3.60 - 25.10
BCS-CY52341	481.0	1.89	5.8	0.75	
	841.7	1.93	1.5	0.70	
	1473.0	1.96	n.c.	0.60	
Experiment II : Exposure period 20 hours without S9 mix					
Solvent control: DMSO	10.0 % (v/v)	1.63	/	0.25	0.10 - 1.35
Positive control: Demecolcin	0.075	1.33	47.1	2.65	1.40 - 6.10
BCS-CY52341	481.0	1.62	0.5	0.35	
	841.7	1.56	10.4	0.50	
	1473.0	1.64	n.c.	0.70	
Experiment I : Exposure period 4 hours with S9 mix					
Solvent control: DMSO	10.0 % (v/v)	2.01	/	0.50	0.20 – 1.65
Positive control: CPA	15.0	1.86	15.1	5.05	2.20 -11.05
BCS-CY52341	481.0	1.91	10.5	0.75	
	841.7	1.93	7.9	0.60	
	1473.0	1.91	10.6	0.65	
Experiment IIA : Exposure period 4 hours with S9 mix					
Solvent control: DMSO	1.0 % (v/v)	1.77	/	0.60	0.20- 1.65
Positive control: CPA	12.5	1.87	n.c.	4.35	2.20 -11.05
BCS-CY52341	480.0	1.77	n.c.	0.85	
	841.7	1.89	n.c.	1.00	
	1473.0	1.75	11.6	0.50	

For the positive control groups and the test item treatment groups the values are related to the solvent controls
n.c. Not calculated as the CBPI is equal or higher than the solvent control value

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Report: KCA 5.8.1 /43[redacted];2014;M-490628-02-1
Title: Consumer exposure and toxicological evaluation of propineb metabolites considering representative uses
Report No: M-490628-02-1
Document No: M-490628-02-1
Guidelines: -/-
GLP/GEP: **n.a.**

A consumer exposure assessment was conducted for the plant and livestock metabolites of propineb which are not already included in the plant and animal residue definitions for risk assessment. Thus, the assessment does not include PTU (propylene thiourea).
The consumer exposure to these metabolites was assessed considering supported representative uses for the renewal of propineb in Europe (apple, grape and tomato).
The exposure calculations were mainly based on the results of propineb plant and livestock metabolism studies used in combination of the propineb results from the supervised field trials.

Residue levels for PU (M02), 4-Methyl-imidazoline (M03), Formyl-PDA (M07) and N-Formyl-PU (M12) were also determined in some residue trials on apple, cherry tomato and grapes conducted in 2014. The results show that in most of the cases residue levels of these metabolites are lower than the highest residue levels considered for the acute consumer exposure calculations, based on metabolism data. In these cases, the worst case, i.e. metabolism data are considered for the consumer exposure calculations. However, when higher residue levels were found in the field trials, these residue levels were considered and used to calculate the consumer exposure to these metabolites.

For the chronic consumer exposure, the toxicological evaluation of these metabolites was performed according to the Threshold of Toxicological Concern (TTC) approach. Thus, the chemical structure of each metabolite was first analysed using QSAR models to identify alerts for genotoxicity and neurotoxicity. As none of the metabolites resulted of concern for genotoxicity and neurotoxicity, they were then allocated to a Cramer class using the Toxtree software. Thereafter, the needs and types of toxicological testing for each metabolite were established by taking into account whether the estimated maximum level of dietary exposure exceeded the threshold of no concern for its Cramer structural class.

All metabolites exceed the threshold of 0.0025 µg/kg bw/day below which no genotoxicity testing is required. Therefore, genotoxicity studies have been conducted and/or information collected from structural similar compounds to show no concern for genotoxicity. For the metabolite “tricycle”, toxicity testing is not feasible because it cannot be synthetized in adequate amounts for toxicity testing. Nevertheless, a Derek evaluation did not pose toxic alerts for genotoxicity.

An assessment of the neurotoxic potential was also carried out for the metabolites that exceed the threshold of 0.3 µg/kg bw/day, either by assessing existing relevant information or by running repeated toxicity studies as for Propineb-DIDT.

As already mentioned, it is not possible to conduct toxicological tests for the metabolite “tricycle”, because it cannot be synthetized in adequate amounts. Nevertheless, a Derek evaluation did not provide any toxic and/or neurotoxic alerts.

All the metabolites belong to Cramer Class 3, with the only exception of PTU-S-trioxide (M06) which belongs to Cramer Class 1.

A comparison between the estimated maximum chronic consumer exposures and the Cramer Class exposure thresholds from toxicity data is presented in Table 1 for those plant metabolites of propineb which are not included in the residue definition for risk assessment.
As chronic oncogenicity data exist for PU (M2), the chronic dietary risk assessment for this metabolite was also carried out by comparing estimated maximum chronic consumer exposure with the chronic reference value (or ADI) derived from the actual chronic toxicity data.
No metabolite exceeded the threshold of concern of 1.5 µg/kg bw/day for chronic toxicity and/or the proposed ADI for PU (M02).

As acute or less than lifetime TTCs have not yet been set by EFSA, the acute consumer exposure of plant and livestock metabolites of propineb not included in the residue definition for risk assessment has been conducted by comparing the estimated maximum acute consumer exposures with the appropriate toxicological acute reference values (ARfD) derived from toxicity studies or appropriate published data, including those on structurally related compounds.
It is not possible to synthesise the metabolite Tricycle (M12), or to propose an ARfD by reading across from other structurally-related compound. This metabolite cannot be tested for its toxicological profile nor analysed in the field trials. However, SAR analysis using Dereck software did not highlight any concerns for acute toxicity and neurotoxicity.

For all the metabolites except propineb-DIDT (M05), the acute consumer exposure did not exceed the respective proposed ARfD. Therefore, no acute concern is expected for PU (Propylene urea, M02), 4-Methyl-imidazoline (M03), PDA (Propylene diamine, M04), PTU-S-trioxide (M06), Formyl-PDA (M07) and N-formyl-PU (M12).

For propineb-DIDT (M05), when metabolism data are considered, the acute consumer exposure exceeds the ARfD for the representative uses on apple and grape with post-flowering application.
However, since propineb-DIDT was not found in the metabolism studies on tomato and on grape with pre-flowering uses, the acute consumer exposure relative to these uses is not expected to exceed the proposed ARfD of propineb-DIDT. Considering that propineb and propineb-DIDT have similar toxicity effects, the cumulative acute consumer exposure to propineb-DIDT and propineb do not exceed 100% for tomato and grape with pre-flowering uses. Therefore, no acute concern is expected for the representative uses on tomato and grape with pre-flowering applications

The consumer exposure to this metabolite needs to be reassessed considering field data.
Propineb-DIDT is going to be measured in the 2014 field trials, provided that this metabolite is stable under storage. 8 grape residue trials (following the use in northern Europe with 2 pre-flowering applications) and 8 tomato residue trials (following the use in greenhouse) are also conducted in 2015. In these 2015 trials, propineb-DIDT will be analysed within 30 days after harvest. Final reports are expected end of March 2016.

CA 5.8.2 Supplementary studies on the active substance

Two supplementary new studies are submitted.
A mechanistic study which was carried out to understand the mode of action triggering the neuromuscular effects in the rat, but that was not submitted in the Baseline Dossier. The results of this in vitro study suggest that the muscular weakness observed in vivo after exposure to propineb might be related to zinc, rather than formation of CS_2.

The second study, a 28-day immunotoxicity study, was carried out to comply with new US-EPA requirement. The study was carrie out in the female Sprague-Dawley rats. Propineb was administered continuously via the diet to separate groups of 8 females per group at concentrations of 75, 150 or

300 ppm (equating approximately to 8.7, 17.2, 29.6 mg/kg bw/day) for at least 28 days. A similarly constituted group received untreated diet and acted as a control group. An additional group of 8 female rats was administered cyclophosphamide (immunosuppressive agent) daily by gavage for at least 28 days at a dose of 80 mg/kg body weight/day and acted as positive control group. Five days before necropsy, all animals were immunized with Sheep Red Blood Cell (SRBC) antigen by intravenous injection. At sacrifice, the spleens were removed and crushed to obtain single cell suspensions which were incubated over a slide with the appropriate amount of guinea pig complement. Assessment of the immunosuppressive potential was done by calculating the amount of Plaque-Forming Cells (PFC) per 10^6 spleen cells. No impairment of the immunological response following immunization with SRBC was observed in animals treated with propineb at dose levels up to 300 ppm for at least 28 days. Therefore, propineb was considered not to have any immunotoxic potential.

Table 5.8.2-1 Supplementary studies on propineb

Type of study	**Test system**	**Results**
Mechanistic investigations on Propineb in primary neuronal cell cultures and skeletal muscle cells of the rat [redacted].;1999;M-001024-01	Primary neuronal cell cultures and skeletal muscle (L6) cell line.	Analogy between propineb and zinc in affecting cytoskeletal structure in neuronal and non-neuronal tissues.
TYPE of study	**NOAEL (mg/kg bw/day)**	**Effects ta LOAEL**
Subacute oral immunotoxicity study in female Wistar rats (4 weeks administration by diet) 0, 75, 150 or 300 ppm 8.7, 17.2, 29. [redacted];2010; M-364424-01	17.2	<u>29.6 mg/kg bw:</u> Systemic toxicity: ↓ bodyweight

Report:	[redacted];[redacted];1999;M-001024-01
Title:	Mechanistic investigations on Propineb in primary neuronal cell cultures and skeletal muscle cells of the rat
Report No:	28789
Document No:	M-001024-01-1
Guidelines:	**-/-**
GLP/GEP:	**no**

Executive Summary

In the toxicity studies propineb induced muscle weakness in rats and dogs. Therefore, mechanistic investigations were carried out to clarify its mode of action. In principle, two different mechanisms have been discussed for propineb. One is the degradation to CS2 and propylenthiourea and the other are direct effects of zinc. Both, zinc and CS2 were shown to induce delayed neurotoxicity in animals.

Primary neuronal cell cultures of the rat are a well established model to identify delayed neurotoxic compounds like n-hexane or acrylamide. In this cell culture model endpoints like viability, energy supply, glucose consumption and cytoskeleton elements were determined. Additionally, skeletal muscle cells were used for comparison.

Beside propineb, the fungicide zineb and their metabolites propylene and ethylenthiourea were investigated respectively. Propylthiourea was tested in relation to propylenthiourea. CS_2 neurotoxicity

was demonstrated by CS_2. Disulfiram and its metabolite diethyldithiocarbamate were used as different thiocarbamates as well as potential CS_2 donors. The zinc toxicity was tested by ZnCl2.
It was shown clearly that propineb exerted strong effects on the cytoskeleton of neuronal und non-neuronal cell cultures (astrocytes, muscle cells). This was similar to $ZnCl_2$ but not to CS_2. With CS_2 effects on the energy supply were more prominent than cytoskeleton degradation.

I. MATERIALS AND METHODS

A. Materials:

1. Test Material
Propineb, Zineb and $ZnCl_2$ were purchased from [redacted] ([redacted]) in a purity of 99 %. Disulfiram, diethyldithiocarbamate, CS_2, propylthiourea, propylenthiourea and ethylenthiourea were from [redacted]/Aldrich ([redacted]) in a purity of 99 %.

2. Test system
Primary neuronal cell cultures from the rat cortex.

Non- neuronal cell lines: L 6 cell line (from rat skeletal muscle) was obtained by the American cell culture collection

B. Study design

1. Preparation of primary neuronal cell cultures
Pregnant Wistar rats were sacrificed by aphyxiation, the fetuses were removed from the uterus and decapitated. The embryonal brain was isolated with sterile forceps.
The preparation of the brain stem was performed under a stereo microscope. The neuronal cell cultures came from rat fetuses of the developmental stage E 18 -E 19 (day of preparation).
The cortex was dissected from the whole brain tissue under a stereo microscope with sterile forceps and subsequently ensheated from the cerebral membrane. The tissues were pooled in sterile cultivation medium (Opti-MEM (Gibco, Eggenstein)) containing 10 ml B27 (Gibco Eggenstein) and 625 ul of a protein solution (Seromed, Berlin (solved in 2.5 ml sterile destilled water)). The following isolation of individual cells from cortex tissues was performed by filtration of the neuronal cells through two Nylon meshes with different pore diameters (135 and 25 urn). The single cell suspension was centrifugated (500-700 g) and washed twice with culture medium. The cell pellet was then suspended in 10 ml of the culture medium and the cell number counted by a cell analyzer system (Scharfe System). The cells were transferred in a cell concentration of 5 x 10^ to 1 x 10^ cells per well in 24 well laminine coated cell culture plates (Biocoat Becton and Dickinson, Heidelberg). The medium was changed every 2-3 days.
Neuronal cell cultures generate a permanent neuronal network within 10 days. The test procedure started at day 10 and was finished at day 17

2. Preparation of the non-neuronal cell cultures
The L 6 cell lines were cultivated in 24 well plates in their growth medium.
The L6 cell lines were grown first in a proliferation medium (DMEM supplemented with 1 %

glutamine, 10 % horse serum and 2 % chicken extract (Gibco, Eggenstein) and during the test period with a differentiation medium (DMEM supplemented with 1% glutamine 10 % PCS, 0.3 % Insulin and 5 % horse serum).

3. Doses

Propineb, zineb, disulfiram, dithiocarbamate, ethylenthiourea, propylenthiourea, propylthiourea were dissolved in DMSO.

$ZnCl_2$ was dissolved in water and applied in doses between 1 and 100 µM in the cell culture medium. CS_2 was dissolved inDMSO and applied in doses between 10 and 1000 µM.

The test compounds were added to the cell cultures by medium change at day 1, 3 and 5. The treatment period was 7 days, afterwards a recovery period of 7 days were added.

Evaluations were made 7 and 14 days after first treatment.

4. Viability assay

Cells were eluated two times with PBS and subsequently incubated in a Calcein-AM/PBS solution (1: 2) (Molecular Probes) for 30 minutes in a cell incubator. Fluorescence was determined with a Fluostar spectrophotometer (SLT, Crailsheim, FRG) at 485/530 nm.

5. Tetramethylrhodamine

Tetramethylrhodamine was applicated at a concentration of 3.3 µM to the culture medium. The cells were incubated for 30 min. Afterwards the cells were washed with PBS and the fluorescence was determined with a Fluostar spectrophotometer (SLT, Crailsheim) at 355/538 nm.

6. ATP determination

The intracellular ATP concentration was determined with chemiluminescence using a kit from Molecular Probes (Eugene, U.S.A.). ATP produced chemiluminescence in a luceiferase reaction.

7. Glucose consumption

The glucose was determined in the cell culture medium. The glucose content ([redacted] „Trinter" kit: 315; Deisen[redacted]en) was determined colometrically by the quantification of H_2O_2, a product of the glucose oxidase reaction. H_2O_2 reacted with 4-aminoantipyridine, p-hydroxybenzene sulfonate, and a peroxidase to quinoneimine dye and water. The resulting red dye was measured in a photometer at 505 nm(Beckman, Munchen).

8. Cell ELISA's

The cell culture plates were fixed in cold methanol (-4 °C) for 10 minutes and subsequently incubated for one hour in a 0.1% human albumine/PBS solution. Then, the cells were treated with a detergent (0.3 % Triton X 100 in PBS) for 10 minutes and then eluated two times with PBS (+ 0.3 % gelatin). The first antibody (neurofilaments (mouse), actin (mouse) and GFAP (mouse) all [redacted] or NSE (rabbit) [redacted]) was added for 2 hours, the second antibody (anti-mouse, [redacted] or anti rabbit, [redacted], Deisen[redacted]en) for 1 hour at 4 °C. After removal of antibody 1 and 2, the plates were washed three times with PBS (+ 0.3 % gelatin). The attached antibodies were exposed to peroxidase as a ABTS-solution ([redacted]) for 30 minutes. The enzymatic activity was stopped by adding a 1 % SDS- solution. The quantification of the attached antibodies occurred at 405 nm in an ELISA reader.

9. Statistics

All experiments were repeated. In each experiment 4 replicates were used per concentration. The statistical analysis was performed by a student's t test (Exel, Microsoft).

II RESULTS

A. Cell viability

The cell viability was determined by the live/dead assay. The cytotoxic effects after 7 days treatment were generally very low with the exception of zineb, which had a NOEC level below 1 µM and an $EC_{50}$of 10 µM. However, after the recovery period some compounds were found to be more cytotoxic: propineb, disulfiram and $ZnCl_2$ (table 4; fig. 2-10).

Propineb reduced the NOEC level during the recovery period from 50 to 10 µM with an EC_{50} value of 40 µM. This was comparable to disulfiram.

$ZnCl_2$ was only moderately more cytotoxic, the EC_{50} value dropped from > 100 to 90 MM.

B. Mitochondrial functions

a) Mitochondrial membrane potential (tetramethylrhodamine):

The mitochondrial membrane potential (tetramethylrhodamine) was strongly reduced by disulfiram and ethylenthiourea and moderately by propineb. The strong reduction by zineb was in parallel to the cytotoxic effect. However, after the recovery period also propylenthiourea, CS, and $ZnCl_2$ showed marked effects on the mitochondrial membrane potential.

b) ATP

The decrease of the intracellular ATP level was in agreement with the reduction of the mitochondrial membrane potential.

C. Glucose consumption

The glucose consumption were strongly reduced than the viabilty by zineb, disulfiram, diethyldithiocarbamate, propineb and propylenthiourea. The glucose consumption was further decreased during the recovery period. All compounds except propylthiourea affected this endpoint.

D. Cytoskeleton

The cytoskeleton was represented in neuronal cells by neurofilaments and by NSE (neuron specific enolase). Both proteins are highly selective for neurons. Here, propineb, zineb and $ZnCl_2$ showed strong effects on neurofilaments with NOEC level below 1 µg/ml and EC_{50} level between < 1 and 2.5 µg/ml. Also effective on this endpoint was disulfiram (NOEC 1; EC_{50} 19 µg/rnl) and propylthiourea (NOEC 5; EC_{50} 50 µg/ml). After the recovery period, all compounds showed effects on the cytoskeleton.

The strong effects on neurofilaments by propineb, zineb and $ZnCl_2$ could be reproduced by other cytoskeleton proteins like NSE or GFAP (glial fibrillary acid protein), a specific marker for astrocytes.

Table 5.8.2-1 Specific effects of propineb, its metabolites and analogues on primary cortical neurons and a differentiated muscle cell line

Compound	Viability	Celluler energy	Cytoskeleton Day 7	Cytoskeleton Day 7
Primary cortical neurons				
Propineb	+	+	+++	+++
Propylenthiourea	-	+	+	++
CS_2	-	+	+	+
$ZnCl_2$	+	++	+++	+++
Disulfiram	-	+++	++	+++
Diethyldithiocarbamate	-	-	++	++
Muscle cells				
Propineb	-	++	+++	n.d.
$ZnCl_2$	+	++	+++	n.d.

Keys: + EC50 < 50 µM; ++ EC50 ≤ 50 µM ; +++ EC50 < 10 µM ; n.d. not determined; - no effect.

5. Effects on a skeletal muscle cell line L 6 by propineb and $ZnCl_2$

Propineb and $ZnCl_2$ were not cytotoxic (propineb) or only moderately cytototoxic ($ZnCl_2$) on L6 cells. Energy related parameters were more affected by both compounds than the cytotoxicity. However, the actin filaments were strongly affected. This indicates, that propineb and $ZnCl_2$ affected primarily the cytoskeleton. These results were in agreement with the findings in primary neuronal cell cultures and suggested that effects on cytoskeletal elements were not selective to the nervous system.

Table 5.8.2-2 Comparison of Propineb and $ZnCl_2$ in muscle cell line

Endpoint	EC50 (µM)	
	Propineb	**$ZnCl_2$**
Cytotoxicity (viability)	>100	> 100
Glucose consumption	39	38
Mitochondrial function (tetramethylrhodamine)	80	38
Intracellular ATP concentration	24	31
Cytoskeleton (Actin filaments)	2.5	3

III CONCLUSION

The *in vitro* data sjowed an analogy between propineb toxicity and zinc toxicity. Main targets for propineb and zinc are the cytoskeletal structures in neuronal (neurofilaments and NSE) and non-neuronal tissue (astrocytes: GFAP; skeletal muscle: actin). This was in in contrast to CS_2 and related compounds where cellular energy was more strongly affected than cytoskeleton. These in vitro results suggest that the neuromuscular effects of propineb *in vivo* may be mediated by zinc.

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Report:	[redacted]l; [redacted]; [redacted];2010;M-364424-01
Title:	Subacute oral immunotoxicity study in female Wistar rats (4 weeks administration by diet)
Report No:	AT05803
Document No:	M-364424-01-1
Guidelines:	**US-EPA OPPTS 870.7800, OPPTS 870.3050; OECD 407**
GLP/GEP:	**yes**

Executive Summary

The immunotoxic potential of Propineb was investigated by measuring the splenic cell counts and by a Plaque Forming Cell Assay (PFCA).
Propineb (batch No EDFU711100, purity 91.8% w/w) was administered in the diet to 8 female rats of the strain Wistar (Hsd Cpb: WU) per group, in doses of 0, 75, 150 or 300 ppm for a period of 4 weeks (groups 1 - 4). Another group of 8 females served as a positive control group, which was treated with cyclophosphamide (group 5).
Additionally 5 female rats per group of the strain Wistar (Hsd Cpb:WU), were administered Propineb via the diet in doses of 0, 75, 150 or 300 ppm for a period of 4 weeks (groups 6-9) for evaluation of possible toxic effects.
Test substance intake in the groups tested for immunotoxicity was equivalent to 8.7, 17.2 and 29.6 mg/kg bw/day and of 8.6,15.5 and35.3 mg/kg bw/day in the group tested for testing general toxicity.

Overall signs of toxicity were confined in the 300 ppm group. One animal had to be killed in moribund condition in the third week, but the cause of the death of could not be ascertained.
Body weights were decreased in the 300 ppm group only.

Results of immunotoxicological investigation (cell counts of spleen and PFCA) gave no evidence of treatment-related effects in the groups of animals treated with propineb.

A pronounced decrease in splenic cell counts and in plaque formation was noted in the group of animals treated with cyclophosphamide. In addition, spleen and thymus weight were significantly decreased in animals treated with cyclophosphamide.

In conclusion, Propineb was tolerated when administered via the diet to female rats without adverse effects up to and including 150 ppm corresponding to a mean daily dose of 15.5 mg/kg body weight, based on mortality and retardation of body weight development at 300 ppm.

The immunotoxicological investigation showed no immunosuppressive potential and the highest dose tested of 300 ppm corresponding to a mean daily dose of 29.6 mg/kg bw/day is the study NOAEL for spleen cell counts and plaque formation.

I. MATERIALS AND METHODS

A. Materials:

1. Test Material:	Propineb
Description:	white powder
Lot/Batch:	EDFU711100
Purity:	81.8%
CAS:	9016-72-2
Stability of test compound:	Stable during the study period
2. Vehicle and /or positive control:	Cyclophosphamide
Description:	white powder
Lot/Batch:	Purchased by [redacted]-Aldrich
Purity:	not given
CAS:	6055-19-2
Stability of test compound:	Stable at 80 mg/mL for a time period which covers the period of storage and usage for the current study
3. Test animals:	
Species:	Female Rat
Strain:	Wistar (HsdCpb:WU)
Age:	7 weeks
Weight at dosing:	132 to 167 g for females
Source:	[redacted] Nederland, [redacted], Netherlands
Acclimation period:	1 week
Diet:	[redacted] Maus/Ratte- Haltung-GLP, Article No.: 3883 G4 S25 (powder) by [redacted] SA, 4303-Kaiseraugst, Switzerland;
Water:	Tap water ad libitum in polycarbonate bottles, ad libitum
Housing:	2 or 3 animals each in Makrolon® cages Type IV, Bedding material: Low-dust wood granules (Lignocel BK 8-15)
Environmental conditions:	Temperature: 22 ± 2 °C Humidity: 55 % Air changes: Approximately 10 changes per hour Photoperiod: Alternating 12-hour light and dark cycles (7 am - 7 pm)

B. Study design:

1. In life dates

July 3, 2009 to August 18, 2009 performed at [redacted], in [redacted], Germany.

2. Animal assignment and treatment

The test item was administered to the animals from the first day of treatment until spontaneous death, moribund sacrifice or until scheduled death.

The dosing schedule and the distribution of the animals to the groups are given in Table 5.8.2-01.

In all groups, 8 female rats per dose were used for a subacute study with administration for a treatment period of 30 days and used for immunotoxicological investigation

Eight animals were used as positive control group (No. 5) for immuntoxicological investigation. These animals were treated like animals of group No. 1 (0 ppm) with the exception that 6 days before the end of the in-life phase of these animals they were treated with 80 mg cyclophosphamide/kg body weight. Cyclophosphamide was administered by intraperitoneal (i.p.) injection. The application volume was 1 mL/kg body weight.

In addition, 5 female rats per group were scheduled for toxicological investigation

Table 5.8.2-3: Study design

Group	Test Substance	Dose level (ppm)	Number of animals Per group
Immunotoxicity part			
1	Control	0	8
2	Propineb	75	8
3		150	8
4		300	8
5	Cyclophosphamide	80 (mg/kg bw/day)	8
Toxicology part			
6	Control	0	5
7	Propineb	75	5
8		150	5
9		300	5

The doses were selected based on the results of the existing dietary toxicity studies in the rat that show clinical signs and effects on body weight development at doses equivalent or higher than 300 ppm.

3. Diet preparation and analysis of the test substance

The test substance was mixed in the diet ([redacted] 3883 G4 S25) at the appropriate concentrations using a mixing granulator. They were stored at room temperature and maximally used over the stability period proven by analytical investigation. The test substance content (all doses including 0 ppm) and homogeneity (low and high dose only) were also checked twice during the study. The analytically determined concentrations were 95,147 and 255 ppm at the first check and 48, 121 and 237 ppm at the second check.

At the first determination the maximal deviation of the obtained value for the content from the nominal value of+/- 20 % required by the respective company SOP was exceeded in the diet formulation of 75 ppm and was within this limit for the 150 ppm and 300 ppm formulations. With respect to homogeneity the limits for the maximal deviation was exceeded at 75 ppm.

At the second determination the maximal deviation of the obtained value from the nominal value of+/- 20 % required by the respective company SOP was exceeded in the diet formulation of 75 ppm and 300 ppm and was within this limit for the 150 ppm formulation. The 75 ppm and 300 ppm formulations were homogenous according to the respective company SOP.
The determined content of the test item was taken into account for calculations.

4. Statistics
Statistical evaluations on body and organ weight data were done using the Dunnett-test in connection with a variance analysis. For all these tests SAS® routines were used.
All variables that were not dichotomous were described by sex, dose group and time point using appropriate measures of central tendency (mean, median) and general variability (standard deviation, minimum, maximum).
For the statistical evaluation of samples drawn from continuously distributed random variables three types of statistical tests were used, the choice of the test being a function of prior knowledge obtained in former studies. Provided that the variables in question were approximately normally distributed with equal variances across treatments, the Dunnett test was used, if heteroscedasticity appeared more likely, a p value adjusted Welch test was applied. If the evidence based on experience with historical data indicated that the assumptions for a parametric analysis of variance cannot be maintained, distribution-free tests in lieu of ANOVA were carried out, i.e. the Kruskal-Wallis test followed by adjusted Mann- Whitney-Wilcoxon tests (U tests) where appropriate.

In these types of statistical processing of measurement values a large number of comparisons were made, which may also lead to false-positive statements. On account of this problem for the evaluation not only the statistical significance but also the biological and toxicological relevance was considered**.**

C. Methods

1. Daily observations
On working days the experimental animals were inspected twice a day for morbidity and mortality (once on weekends and public holidays). Once before the start of treatment and once weekly thereafter any clinical signs (findings) and abnormalities were recorded. Body surfaces and orifices, posture, general behavior, breathing and excretory products were assessed. Findings and abnormalities were recorded either using a coding system or uncoded.

2. Body weight
The body weights of the individual experimental animals were determined before the beginning of the study and twice per week thereafter. Furthermore, body weights were recorded immediately before scheduled necropsies, for calculation of relative organ weights.

3. Food consumption
Food and water intake per group was determined weekly.
The weight of the food offered at the start of the measurement period minus the food at the end of the period is defined as the food consumption of the animal in g.

On the basis of these data the following parameters were calculated:

- for each interval: mean daily food intake per animal, mean daily food intake per kg body weight;
- for the total period: measurement of mean food intake per animal and day, mean food intake per kg body weight and day.
- Furthermore, cumulative food intake per animal and cumulative food intake per kg body weight were calculated.

Test substance intake was calculated from the food intake data.
Comparable calculations were done for the water intake.

4. Immunotoxicity

The following investigations were carried out:

- Determination of the cell counts in the spleen.
- Plaque Forming Cell Assay (PFCA): 5 days before necropsy all animals of groups 1-5 treated were immunized i.v. with sheep erythrocytes (SRBC) to make it possible to carry out the PFCA.

SRBC suspensions were adjusted to concentrations of 1 x 109 per ml BSS. Humoral immune reactions were induced by iv injection of 100 µl per animal of this suspension. Five days after this in vivo stimulation (day 29 after onset of treatment) the animals were sacrificed and the spleens were removed.

The spleens were crushed through a metal sieve resulting in single cell suspensions. These suspensions were adjusted to $1x10^7$ cells per ml for further analysis.
Spleen cells were counted manually by Trypan Blue exclusion
Four aliquots of these suspensions, two of each (100 µl and 10 µl) were used for the detection of PFC on glass slides (in duplicates) after incubation with the appropriate amount of guinea pig complement.
Evaluation was done by calculating the amount of PFC per 106 spleen cells.

5. Clinical pathology and hematology

<u>Blood sampling</u>

Clinical laboratory investigation on blood samples were performed on all animals of the animals allocated to the toxicity investigations (groups No. 6-9).

The blood samples for determination of glucose concentrations were taken in the morning from the caudal vein of non-fasted, non-anesthetized animals.
The blood samples used for determining the other parameters in peripheral blood were collected in the morning from the retro-orbital venous plexus of non-fasted animals anesthetized with CO_2/O_2 (80/20) (Nöller, H.G.; Die Blutentnahme aus dem retroorbitalen Venenplexus. Klin. Wschr. 33, 770-771, 1955).
The following blood clinical chemistry parameters were analysed: alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, glutamate dehydrogenase, gamma glutamyl transferase,

albumin, bilirubin, cholesterol, creatinine, total protein, triglycerides, urea, glucose, chloride, potassium, sodium.

The samples for the hematological determinations were collected in tubes coated with EDTA (anticoagulant).
The following hematological parameters were determined in peripheral blood: differential blood count, erythrocyte count, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, hemoglobin concentration, hematocrit, leucocyte count, reticulocyte count, thrombocyte count, thromboplastin time (Hepato-Quick).

6. Post-mortem examinations

Necropsy

All animals of groups 1-5 were sacrificed and subjected to gross necropsy examination. Possible changes of organs in the body cavity were noticed.
All animals of groups 6-9 were necropsied and their organs and tissues were subjected to thorough gross pathological examination.

Organ weights

At the end of the treatment of the animals of groups 1-5 the weights of spleen and thymus were determined. From all animals of groups 6-9 the weight of liver, kidneys and thyroids were determined.

Histopathology

No histopathological examination was performed.

II. RESULTS

A. Mortality

One animal of the 300 ppm group (toxicity investigations) had to be sacrificed due to the bad health conditions during the third week of the study. During the second treatment week this animal lost body weight and at clinical observation emaciation, bloody muzzle, abdominal position and decelerated breathing were observed. At necropsy the animal appeared skinny and the spleen was diminished in size. The cause of these poor health conditions could not be ascertained.

B. Clinical Signs

With the exception of the findings for animal of the 300 ppm group that had to be sacrificed, no further clinical findings were observed in any of animals of the study.

C. Body weight

Bodyweights were decreased in the 300 ppm groups.

Table 5.8.2-4 Body weights

Immunotoxicity groups	Propineb				Cyclophosphamide
Dose levels (ppm)	0	75	150	300	80 mg/kg bw/day
Day1 (g)	143	144	150	150	145
Day 29 (g)	200	205	213	190	205
Toxicity groups	**Propineb**				
Dose levels (ppm)	**0**	**75**	**150**	**300**	
Day1 (g)	148	143	154	149	
Day 29 (g)	219	207	218	175**	

** p≤ 0.01

D. Food and water consumption

Food intake was lower in the animals of the 300 ppm group allocated to immunotoxicity, but not in the animals of the 300 ppm group allocated to the additional toxicity assessment.
No effects were observed in the other groups.
There was no clear evidence of effects on water consumption.

Table 5.8.2-5 Food intake

Immunotoxicity groups	Propineb				Cyclophosphamide
Dose levels (ppm)	0	75	150	300	80 mg/kg bw/day
Intake (g/kg bw/day)	115.1	115.5	115.0	98.6	112.0
Toxicity study groups	**Propineb**				
Dose levels (ppm)	**0**	**75**	**150**	**300**	
Intake (g/kg bw/day)	110.9	115.1	103.0	117.6	

Test material intake is summarized in table 5.8.2-6.

Table 5.8.2-6 Mean achieved dietary intake of propineb (Weeks 1 - 4)

Groups	75 ppm	150 ppm	300 ppm
Immunotoxicity groups (mg/kg/day)	8.7	17.2	29.6
Toxicity groups (mg/kg/day)	8.6	15.5	35.3

E. Immunotoxicity assessment

Cell counts

The splenic cell counts did not show any test substance induced effect or a statistically significant difference between the test substance treated and the respective control group up to and including 300 ppm (group 4). A decrease in splenic cell counts was observed in animals of the positive control group treated with cyclophosphamide

Table 5.8.2-6 Mean Cell counts

Cell counts x10^6 per organ (Mean and standard deviation)					
Group	**0**	**75**	**150**	**300**	**80***
Mean	438.5 ±*103.0*	458.2 ± *52.3*	441.4 ± *106.2*	508.3± *92.8*	143.0 ± *46.4*

There was no statistically significant effect in the amount of plaque forming cells, no statistically significant effect was detected.
The vehicle treated animals exhibited a relatively low mean value (group 1), which is partly due to low plaques counts of one single animal. Although the actual reason for this low response is not known, it could be due to insufficient i.v. application of the antigen (SRBC) or to low responsiveness of that particular animal.
The cyclophosphamide pre-treated animals (positive control) revealed a pronounced suppression in plaque formation.

Similarly, the PFC per spleen did not show any statistically significant difference between the vehicle control and test item treated animals , while the PFC were significantly reduced in positive control animals.

Table 5.8.2-7 Plaque Forming Colonies

PFC x10^6 per spleen cells (Mean)					
Group	**0**	**75**	**150**	**300**	**80***
Mean	1168	1979	1608	1536	85
Mean number of PFC x10^6 per total spleen cells					
Group	**0**	**75**	**150**	**300**	**80***
Mean	489	944	738	803	11

F. Clinical pathology

There were no treatment-related effects in blood chemistry and hematology parameters.

G. Post-mortem examinations

1. Gross pathology

All the macroscopic changes were considered as incidental and not treatment-related.

2. Organ weights

A few variations were observed in the mean and absolute organ weights.
Mean absolute (10%) and relative (16%) weights of spleen were slightly increased at 300 ppm in main group animals (group 4). Due to missing dose dependence the statistically significantly decreased weights of thymus at 150 ppm are not regarded to be due to the treatment with the test substance.
Mean weights of spleen and thymus were significantly decreased in the positive control group.

Table 5.8.2-8 Mean spleen weights

Immunotoxicity groups	Propineb				Cyclophosphamide
Dose levels (ppm)	**0**	**75**	**150**	**300**	**80 mg/kg bw/day**
Terminal Body weight (g)	202	210	218	192	210
Spleen (absolute, mg)	483	470	474	530	301**
Spleen (% bw)	239	224	217	278*	144**
Thymus (absolute, mg)	527	474	439	484	248**
Thymus (% bw)	260	225	201**	251	119**

Table 5.8.2-9 Mean organ weights

Toxicity groups	Propineb			
Dose levels (ppm)	**0**	**75**	**150**	**300**
Terminal Body weight (g)	219	207	218	175**
Liver (absolute, mg)	9156	8477	8913	8303
Liver (% bw)	4.19	4.10	4.08	4.75
Thyroid (absolute, mg)	11	11	10	12
Thyroid (% bw)	5	5	5	7

In the 300 ppm group of the toxicity phase of the study, mean relative weight of the thyroids was increased even if the difference from the control was not statistically significant.

III. CONCLUSION

Under the conditions described the administration of Propineb in the diet to female rats was tolerated without adverse effects up to and including 150 ppm corresponding to a mean daily dose of 15.5 mg/kg body weight, based on mortality and retardation of body weight development at 300 ppm.
The immunotoxicological investigation resulted in a NOAEL of 300 ppm for plaque formation corresponding to a mean daily dose of 29.6 mg/kg body weight.

CA 5.8.3 Endocrine disrupting properties

Toxicoty studies have shown that propineb has an effect on the thyroid, with the rat being the most susceptible species, but is not classified for reproductive toxicity. New data from a rat developmental toxicity and a rat developmental neurotoxicity study showed no effects of developmental toxicity and/or on the development of the fetus brain and neurological development after birth. Therefore propineb does not meet the interim criteria as endocrine disrupter.

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Report:	[redacted]q; [redacted];2014;M-489312-01
Title:	Occupational medical experiences with propineb
Report No:	M-489312-01-1
Document No:	M-489312-01-1
Guidelines:	**US EPA FIFRA Guideline Requirement: N/A**
GLP/GEP:	**no**

Material and methods:

Staff members being involved in the production pf propineb formulations were examined by routine medical investigations. The production staff were working according to the usual precautions laid down for the production of Propineb TK83 and for the formulation procedure of Antracol.

No. of workers exposed: 22

Medical examinations: History and full physical examination

Commenced in: 1964

Examination intervals: annually

Laboratory examinations: FBC, liver enzymes, creatinine, olesterol, urine stick

Technical examinations: Lung function testing, ecg, vision testing and audiometry as needed for specific job tasks

Medical assessment:

Occupational medical surveillance of workers exposed to Propineb performed since 1964 annually on a routine basis, not directly related to exposures, did not reveal any unwanted effects in the workers. The examinations included the above laboratory parameters and clinical and technical examinations.

During the production period since 1964 no accidents with Propineb occurred in the workers. No further constitations of the Medical Department due to work or contact with Propineb were required.

CA 5.9.2 Data collected on humans

No cases of human poisoning have been reported up to now.

CA 5.9.3 Direct observations

See Baseline Dossier (= EU Dossier, which resulted in the Annex inclusion under Directive 91/414/EEC in 2003), KCA 5.9.2.

CA 5.9.4 Epidemiological studies

Up to now there are no epidemiological studies available.

CA 5.9.5 Diagnosis of poisoning (determ. AS, metabolites), spec. signs of poisoning, clinical tests

Nausea, Vomiting, Symptoms reinforced by alcohol (Antabuse effect).

See MSDS with document number M-078199-02-1 filed under KCA 3.8.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

Treat symptomatically. In case of ingestion gastric lavage should be considered in cases of significant ingestions only within the first 2 hours. However, the application of activated charcoal and sodium sulphate is always advisable. Follow-up measures: Strict abstinence from alcohol for 1 to 2 weeks, due to antabuse effect.

See MSDS with document number M-078199-02-1 filed under KCA 3.8.

CA 5.9.7 Expected effects of poisoning

See Baseline Dossier, (= EU Dossier, which resulted in the Annex inclusion under Directive 91/414/EEC in 2003), KCA 5.9.6 .