Propoxycarbazone-sodium

Herbicide

Dossier for Renewal of Approval according to Commission Regulation 844/2012

Document M-CA, Section 5

Toxicological and metabolism studies on the active substance

Bayer CropScience AG
[redacted]
Germany

OWNERSHIP STATEMENT

This document, the data contained in it and copyright therein are owned by Bayer CropScience. No part of the document or any information contained therein may be disclosed to any third party without the prior written authorisation of Bayer CropScience.

The summaries and evaluations contained in this document are based on unpublished proprietary data submitted for the purpose of the assessment undertaken by the regulatory authority. Other registration authorities should not grant, amend, or renew a registration on the basis of the summaries and evaluation of unpublished proprietary data contained in this document unless they have received the data on which the summaries and evaluation are based, either:

- **From Bayer CropScience; or**
- **From other applicants once the period of data protection has expired.**

Version history

Date	Data points containing amendments or additions [1]	Document identifier or version number

[1]Note how the amendments or additions are represented (italics/colour etc)

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

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

The EU dossier for Annex I inclusion of propoxycarbazone-sodium contained several ADME studies (see Table CA 5.1-1, p.8).
Since study authors have used different names or codes for the degradation products of propoxycarbazone-sodium (MKH 6561) in these studies, a single name and a single code number for each metabolite are always used in this summary. The different names, structures and code numbers used in the study reports are also listed in Table CA 5.1-2 on page 9 for cross reference purposes.

Furthermore, in order to fulfil the new data requirements of Regulation 283/2013, **a comparative *in-vitro* metabolism study in rat / human liver microsomes** has been included under Annex Point CA 5.1.2. However, as no study guideline currently exists the guidance document SANCO/10181/2013-rev 2.1 applies, which states that in cases where "test methods or guidance documents are not yet available for particular data requirements […] waiving of these particular data requirement points is considered acceptable as long as no test methods or guidance documents are published in form of an update of the Commission Communications 2013/C 95/01 and 2013/C 95/02.".

Absorption, distribution and elimination

The studies on rats using phenyl- as well as triazolinone-labelled active substance showed a low degree of absorption followed by fast elimination from the body. The low rate of absorption was confirmed by a bile fistulation experiment, where after intraduodenal administration only small amounts of radioactivity were detected in bile. After oral administration of propoxycarbazone-sodium, more than 88% of the administered radioactivity was excreted in all dose groups tested within 48 hours independent of the labelling position. The concentrations of radioactive residue in organs, tissues and GIT were very low indicating no tendency for accumulation of propoxycarbazone-sodium related residues. Due to the low degree of absorption the major portion of radioactivity was consequently excreted via faeces with more than 66% of the administered radioactivity after oral administration in all dose groups. Renal excretion accounted for approximately 21 to 31% of the administered dose. The excretion behaviour showed no significant differences with respect to dose rate and sex. Only a small amount of radioactivity was measured in the bile, corresponding to 3.4% of the dose administered to male rats. Less than 1% of the administered radioactivity was found in the expired air.

Metabolism

Propoxycarbazone-sodium was not intensively metabolised in rats. The major component in urine and faeces of all dose groups was unchanged parent compound accounting for 75 to 89% of the administered dose. Metabolism took place via cleavage of the amide bond, resulting in sulfonamide methyl ester (M05) and N-methyl propoxy triazolinone (M10). Sulfonamide methyl ester (M05) was further metabolised yielding sulfonamide acid (M06) and saccharin (M07). Sulfonamide methyl ester (M05) was observed in the high dose experiment in faeces in a maximum amount of 8.8% of the administered dose. Sulfonamide acid (M06) and saccharin (M07) did not exceed 4% of the given dose in any experiment. Identification rate ranged from 83 to approximately 100% of the administered dose.

These result are supported by the comparative *in-vitro* metabolism study of ^{14}C-propoxycarbazone-sodium in pooled rat liver microsomes (RLM; males), obtained from Wistar rats, and pooled human liver microsomes (HLM; males and females).
The results of the test with ^{14}C-propoxycarbazone-sodium demonstrated that the test item was moderately stable after incubation with RLM and HLM. Two small ^{14}C-containing products could be detected (namely Pr-1 and Pr-2). Pr-1 HPLC peak was considered as a degradation product of ^{14}C-propoxycarbazone-sodium since it was present either in microsome incubations at t=0 hours and in incubations at 37±1°C with buffer only, accounting for 4.5% to 6.2% of the total radioactivity in these

samples. Pr-2 compound was formed after incubation of ^{14}C-propoxycarbazone-sodium with liver microsomes from humans but not from rats. Therefore, it was considered as an actual human-specific metabolite of ^{14}C-propoxycarbazone-sodium. The MS spectra of metabolite Pr-2 containing its characteristic fragment ions demonstrated that this metabolite is most likely formed by O-demethylation of propoxycarbazone leading to the propoxycarbazone carboxylic acid metabolite (M04; see Chapter CA 5.8.1).

The proposed metabolic pathway for propoxycarbazone-sodium (MKH 6561) in rats is shown in Figure CA 5.1-1.

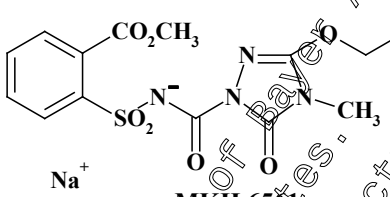


Figure CA 5.1-1: Propoxycarbazone-sodium (MKH 6561) - Proposed metabolic pathway in rats

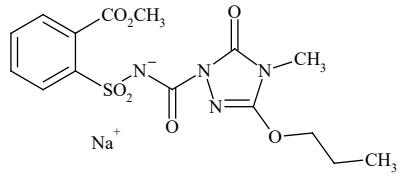
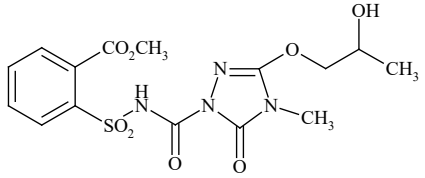
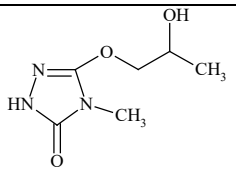
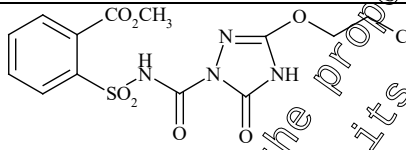
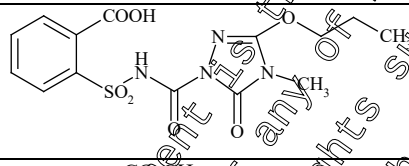
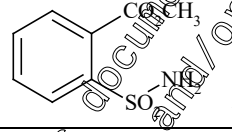
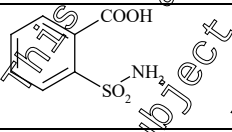
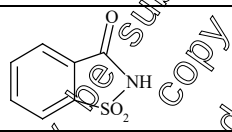
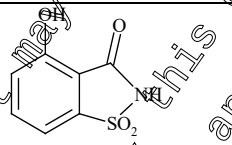
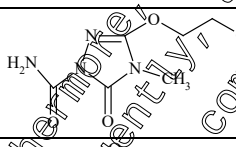
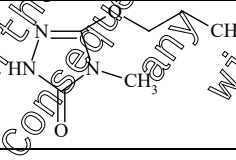
For chemical names and codes see list of metabolites at the end of Chapter 5.1.

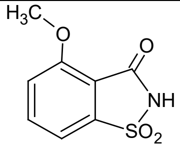
Table CA 5.1-1: Overview of ADME studies

Study Type (Ref. Point in dossier or DAR)	Test substance Dosing regime	Scope of study	Reference
ADME – rat CA 5.1.1 /01	[phenyl-UL-^{14}C]MKH 6561 Low dose: 2 mg/kg bw, single oral dose (♂+♀); Multiple dose: 14 x 2 mg/kg bw, oral dose once per day (non-radioactive compound) and 24 h after the last dose 2 mg/kg bw, single oral dose (radioactive compound) (♂ only); High dose: 200 mg/kg bw, single oral dose (♂ only); Expired air: 2 mg/kg bw, single oral dose (♂ only); Whole body autoradiography: 2 mg/kg bw, single oral dose and 2 mg/kg single i.v. (♂ only); Bile fistulation: 2 mg/kg bw single intraduodenal (♂ only)	Biokinetic behaviour and metabolism of propoxycarbazone-sodium.	[redacted] (1998) M-001693-01-1
ADME – rat CA 5.1.1 /02	[triazolinone-3-^{14}C]MKH 6561 Low dose: 2 mg/kg bw (one single oral dose, ♂ only); Expired air: 2 mg/kg bw, single oral dose (♂ only)	Biokinetic behaviour and metabolism of propoxycarbazone-sodium.	[redacted] (1997) M-005603-01-1
Metabolism/excretion – rat CA 5.1.1 /03	[phenyl-UL-^{14}C]MKH 6561 2 mg/kg bw (single oral dose, ♂ only)	Identify 2-hydroxypropoxy MKH 6561 (M01) as an intermediate metabolite in the liver of rats.	[redacted] (1999) M-015589-01-1
Metabolism *in*-vitro – rat/human CA 5.1.2 /01	[phenyl-UL-^{14}C]propoxycarbazone-sodium (MKH 6561) 15 µM	Relevance of the toxicological safety studies and investigation on possibility of formation of unique human metabolites	[redacted] (2014) M-488585-01-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

Table CA 5.1-2: List of the active substance and its metabolites

	Structure	Trivial Name / Chemical Name [CAS#]
a.s.		MKH 6561 Benzoic acid, 2-((((4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazol-1-yl)carbonyl)amino)sulfonyl)-methyl ester [145026-81-9]
M01		2-hydroxypropoxy MKH 6561 KTS 9001 Benzoic acid, 2-((((4,5-dihydro-5-oxo-3-(2-hydroxypropoxy)-1H-1,2,4-triazol-1-yl)carbonyl)amino)sulfonyl)-methyl ester
M02		2-hydroxy–N-methyl propoxy triazolinone
M03		N-des-methyl MKH 6561
M04		MKH 6561 carboxylic acid MKH 7018 MKH 8394 Benzoic acid, 2-[[(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1H-1,2,4-triazol-1-yl)carbonyl]amino)sulfonyl
M05		Sulfonamide methyl ester STI 4934 Benzoic acid, 2-(aminosulfonyl)-, methyl ester [157680-71-3]
M06		Sulfonamide acid MKH 7283 Benzoic acid, 2-(aminosulfonyl) [632-24-6]
M07		Saccharin MKH 7284 1,2-Benzisothiazol-3(2H)-one, 1,1,dioxide [81-07-2]
M08		4-hydroxy saccharin KTS 9357 1,2-Benzisothiazol-3(2H)-one, 4-hydroxy-1,1,dioxide [80563-77-5]
M09		N-methyl propoxy triazolinone amide KTS 9304 3H-1,2,4-Triazol-3-one-2-carboxamide-2,4-dihydro-4-methyl-5-propoxy
M10		N-methyl propoxy triazolinone MKH 7017 3H-1,2,4-Triazol-3-one, 2,4-dihydro-4-methyl-5-propoxy [145027-96-9]

	Structure	Trivial Name / Chemical Name [CAS#]
M11		MKH 6561-methoxy-saccharin BCS-AG71018 4-Methoxy-1,2-benzothiazol-3(2H)-one 1,1-dioxide

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

All studies for this endpoint have been previously submitted and evaluated.

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

Report:	[redacted];[redacted];2014;M-488585-01
Title:	[Phenyl-UL-14C]propoxycarbazone-sodium: Metabolic stability and profiling in liver microsomes from rats and humans for Inter-species comparison
Report No:	S46813
Document No:	M-488585-01-1
Guidelines:	**No guideline available**
Deviations:	**.** Not applicable
GLP/GEP:	**yes**

Executive Summary

The *in-vitro* metabolism of ^{14}C-propoxycarbazone-sodium was investigated in pooled rat liver microsomes (RLM), obtained from male Wistar rats, and pooled human liver microsomes (HLM, males and females). ^{14}C-propoxycarbazone-sodium was applied at a concentration 15 µM to rat and human liver microsomes in a NADPH regeneration system. Activity of microsomes was confirmed by incubation with testosterone. The metabolite pattern was investigated 0 and 1 hour after start of incubation. The test duration 1 hour for the test item was considered as reasonable because positive results were obtained from the enzymatic reaction of testosterone to hydroxy-testosterone already after 5 minutes. At the end of incubation the metabolic reaction was stopped by precipitation of the protein, the supernatant isolated by centrifugation and metabolite pattern investigated by reversed phase HPLC with radiochemical detection (HPLC-RAD).

The recovery of radioactivity in both tested test system was very high. i.e. > 99.5% for the 1 hour sample. Moreover, the results of the test with ^{14}C-propoxycarbazone-sodium demonstrated that the test item was moderately stable after incubation with RLM and HLM.

Two small ^{14}C-containing products could be detected (namely Pr-1 and Pr-2). Pr-1 HPLC peak was considered as a degradation product of ^{14}C-propoxycarbazone-sodium since it was present either in microsome incubations at t=0 hours and in incubations at 37±1°C with buffer only, accounting for 4.5% (RLM) to 6.2% (HLM) of the total radioactivity in these samples. Pr-2 compound was formed after incubation of ^{14}C-propoxycarbazone-sodium with liver microsomes from humans but not from rats, accounting for 2.3% of the relative percentage of peak area. Therefore, it might be an actual human-specific metabolite of ^{14}C-propoxycarbazone-sodium. The MS spectra of metabolite Pr-2 containing its characteristic fragment ions demonstrated that this metabolite is most likely formed by O-demethylation of propoxycarbazone leading to a propoxycarbazone carboxylic acid metabolite.

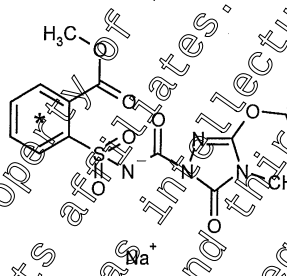
I. MATERIALS AND METHODS

A. MATERIALS

1. Test material (unlabelled):

Identification:	Propoxycarbazone-sodium BCS-AF93558
Description:	Off-white powder
Lot/Batch #:	AE 0298618 00 1B98 0001
Expiration:	2018-09-26
Purity:	98.0%
Stability of test compound:	Refrigerator at 2-8°C

2. Test material (radiolabelled):



Identification:	[Phenyl-UL-^{14}C]propoxycarbazone-sodium
Lot/Batch #:	KML-9702
Purity:	99.0%
Radiochemical purity:	99.0% by HPLC
Specific activity:	3.82 MBq/mg (103.3 µCi/mg)
Stability of test compound:	Deep freezer at -80±10°C

3. Control materials:

Metabolite standard:	6β-hydroxytestosterone
Description:	White powder
Lot/Batch #:	0446800-2
Expiration:	2014-07-05 (one year after aliquot preparation)
Purity:	99.7%
Stability of test compound:	Refrigerator at 2-8°C
Internal standard:	Dexamathasone Vetranal
Description:	White colourless powder
Lot/Batch #:	SZBB118XV
Expiration:	2016-04-28
Purity:	99.5%
Stability of test compound:	Refrigerator at 2-8°C

4. Test system:

Rat liver microsomes

Strain:	Wistar
Source:	[redacted], USA
Sex:	Male
No. of animals:	200
Lot No.:	1010126

Human liver microsomes

Source:	[redacted], USA
Sex:	Male/female
No. of humans:	50
Lot No.:	1210097

B. STUDY DESIGN AND METHODS

Treatment

[Phenyl-UL-^{14}C]propoxycarbazone-sodium was incubated with rat or human active microsomes and in the absence of microsomes and co-factors. In order to show the suitability of the test system a standard substrate, i.e. testosterone, was incubated.

Incubation

The final concentrations of the incubates were 1 mg/mL microsome protein of both test species and 15 µM [phenyl-UL-^{14}C]propoxycarbazone-sodium.
Each incubation mixture comprised the appropriate volume of the respective substrate stock solution 25 µL of the microsomal suspension and phosphate buffer. The incubation mixtures were pre-warmed at 37±1°C for 2 minutes with shaking at 1000 rpm. The reaction was started by adding 50 µL of 10 mM NADPH. All incubations were performed at 37±1°C. The final volume of each incubate was 500 µL and each experiment was performed in triplicate.

For the positive control the final concentrations of the incubates were 15 mg/mL microsome protein and 125 µM testosterone.
Each incubation mixture comprised the appropriate volume of the respective substrate stock solution filled up with phosphate buffer to 400 µL and 3 µL of the microsomal suspension. The incubation mixtures were pre-warmed at 37±1°C for 2 minutes with shaking at 1000 rpm. The reaction was started by adding 40 µL of 10 mM NADPH. All incubations were performed at 37±1°C.

Table CA 5.1-3: Incubation with rat and human liver microsomes

Sample no.	Substrate concentration	Microsome concentration (source)	Incubation time
[phenyl-UL-^{14}C]propoxycarbazone-sodium			
R-0-1	15 µM	1 mg/mL (RLM)	0 hour
R-0-2			
R-0-3			
R-60-1			1 hour
R-60-2			
R-60-3			
H-0-1	15 µM	1 mg/mL (HLM)	0 hour
H-0-2			
H-0-3			
H-60-1			1 hour
H-60-2			
H-60-3			
stability control	15 µM	–	1 hour
testosterone			
R-TST-1	125 µM	0.15 mg/mL (RLM)	5 min
R-TST-2			
R-TST-3			
H-TST-1	125 µM	0.15 mg/mL (HLM)	5 min
H-TST-2			
H-TST-3			

Sampling

At the end of the respective incubation time with [phenyl-UL-^{14}C]propoxycarbazone-sodium 0.5 mL acetonitrile at room temperature were added to the respective tube, inducing protein precipitation. Thereafter the supernatant was separated by centrifugation (16,000 x g/15 min/20°C). After centrifugation, 100 µL of each supernatant were diluted with 400 µL of HPLC mobile phase A. The samples were directly analysed by HPLC-RAD without any further extraction procedure.

At the end of the respective incubation time with testosterone 0.4 mL acetonitrile at room temperature were added to the respective tube, inducing protein precipitation.
Afterwards, 32 µL of dexamethasone IS solution + 32 µL MeOH was added to each incubate to provide an internal standard for analysis.
Thereafter the supernatant was separated by centrifugation (16,000 x g/15 min/4°C). After centrifugation, 100 µL of each supernatant were diluted with 100 µL of 0.1% acetic acid. The samples were directly analysed by LC-MS/MS without any further extraction procedure.

Analysis

Radioactivity was measured by LSC on Beckman LS 6000 scintillation counters equipped for computing quench-corrected disintegrations per minute (dpm). HPLC analyses of ^{14}C-propoxycarbazone-sodium metabolite profile were carried out on a Waters Alliance HPLC system consisting of a separation module and a UV-detector. The radioactivity signal was monitored with a radioactivity flow-through detector connected to a personal computer. Quantification of the radio-HPLC-chromatograms was done by electronic integration with the MassLynx software 4.0.

LC-MS/MS analyses of 6β-hydroxytestosterone were carried out on a Waters Alliance HPLC system consisting of a separation module and a Quattro Premier mass detector (3000 amu upper mass limit) with ESI interface. Quantification was done with the MassLynx software 4.1.

Metabolite identification

For metabolite identification the final concentrations of the incubates were 3 mg/mL human microsome protein and 30 µM [phenyl-UL-^{14}C]propoxycarbazone-sodium.

Each incubation mixture comprised the appropriate volume of the respective substrate stock solution filled up with phosphate buffer to 500 µL. The incubation mixtures were pre-warmed at 37±1°C for 2 minutes with shaking at 1000 rpm. Thereafter the reaction was started by adding NADPH cofactor. After 120 min incubation time the reaction was quenched with 150 µL acetonitrile at room temperature.

Subsequently the supernatant was separated by centrifugation (16,000 x g/15 min/20°C) and diluted with HPLC-RAD mobile phase A. Afterwards, the samples were subjected to solid phase extraction in disposable OASIS HLB Cartridges (3 mL, 60 mg), and the extract was dried under nitrogen, re-dissolved and injected to the HPLC-UV-MS system.

II. RESULTS AND DISCUSSION

A. RECOVERY AFTER INCUBATION

The mean recovery of radioactivity after microsome incubations and sample preparation (i.e. protein precipitation with acetonitrile and centrifugation) at T=0 was found to be 100.3% and 105.5% in RLM and HLM, respectively, while after 1 hour incubation the recoveries were 99.5% in RLM and 103.0% in HLM.

B. ENZYME ACTIVITY OF THE TEST SYSTEM

^{14}C-propoxycarbazone-sodium was found to be reasonably stable when incubated with rat and human microsomes. However, a small ^{14}C-containing degradation product (named as Pr-1) was detected in the rat and human microsome incubations at 0 hours accounting for 4.5% and 6.2% of the radioactivity, respectively. Compound Pr-1 was also found after 1 hour incubation without microsomes (5.5%). In addition, Pr-1 was found in the 1 h microsome incubations from both species and accounted for 4.5% (RLM) and 5.8% (HLM) of the radioactivity, indicating a little instability of the test item in the RLM and HLM testing systems. Incubation with the positive control 6β-hydroxytestosterone proved the metabolic competence of both test systems.

95.5% and 91.8% of the radioactivity remained unchanged when incubated with RLM and HLM, respectively, after 1 hour incubation in the presence of NADPH cofactor. No metabolites were detected in RLM incubations strongly suggesting that ^{14}C-propoxycarbazone-sodium is not metabolized by cytochrome P450 in rats.

In human liver microsome incubations, a single and minor metabolite was detected (namely Pr-2) that was not present neither in t=0 hour samples nor in buffer stability samples. Pr-2 accounted for 2.3% of the relative percentage of peak area, and was not detected in RLM, suggesting that Pr-2 could be a human-specific metabolite of ^{14}C-propoxycarbazone-sodium.

C. METABOLITE IDENTIFICATION

For metabolite Pr-2 identification, ^{14}C-propoxycarbazone-sodium was incubated with HLM under experimental conditions designed to maximize metabolite formation.

Figure CA 5.1-2 shows the MS spectra of ^{14}C-propoxycarbazone-sodium (upper panel) and metabolite Pr-2 (lower panel) obtained by electrospray ionisation (ESI) in the positive-ion mode, using a capillary voltage of 3.5 KV and a cone voltage of 25 V.

The MS spectrum of ^{14}C-propoxycarbazone-sodium shows a molecular ion at [m+1]/z 399.3, which fits to the molecular mass of unlabelled propoxycarbazone (398.3 Da). The Na^+ and K^+ adducts of propoxycarbazone ([m+1]/z 416.2 and 437.1, respectively) are also detected and confirm the identity of the compound. The MS spectrum of Pr-2 shows a molecular ion at [m+1]/z 385.3 that fits to a molecular mass of 384.3 Da. The presence of its Na^+ and K^+ adducts ([m+1]/z 407.2 and 423.1, respectively) confirms the identity of the compound. The isotopic distribution of ^{14}C-propoxycarbazone, metabolite Pr-2

and the ion at [m]/z 367.2 (see Figure CA 5.1-2) clearly shows an intense ion with a shift of 4 mass units from their molecular ions that correspond to their ^{14}C-labeled counterparts (i.e. two ^{14}C atoms in the phenyl ring), indicating that there the entire phenyl ring is contained in their chemical structure. As a conclusion, the MS analysis of metabolite Pr-2 shows a loss of 14 mass units as compared to the parent compound, strongly suggesting that metabolite Pr-2 could correspond to a demethylated form of propoxycarbazone. Two potential demethylation positions in the propoxycarbazone molecule are likely candidates from a metabolism perspective: the O-methyl located in the phenyl moiety and the N-methyl in the triazolinone moiety.

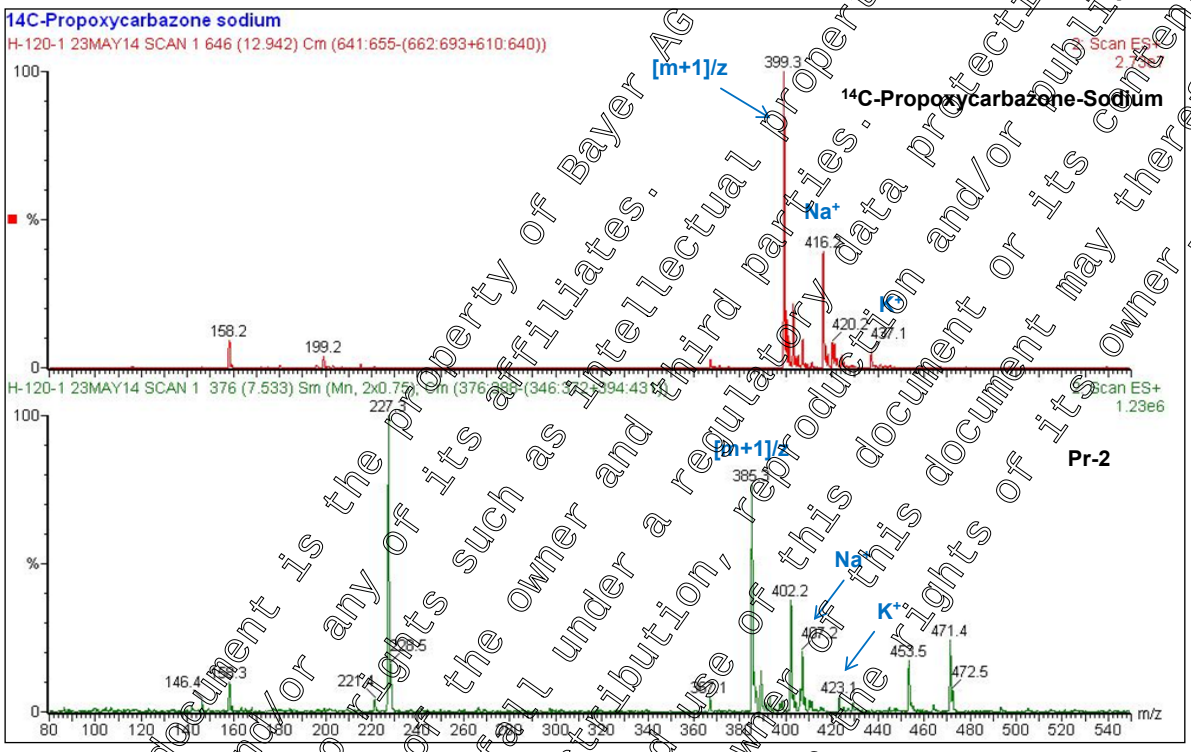


Figure CA 5.1-2: LC-MS spectra of ^{14}C-propoxycarbazone-sodium and metabolite Pr-2. (Capillary Voltage: 3.5 KV; Cone Voltage: 25 V)

Figure CA 5.1-3 and Figure CA 5.1-4 show the respective MS spectra of ^{14}C-propoxycarbazone-sodium and metabolite Pr-2 obtained by decreasing the cone voltage to 15 V in the same sample. The molecular ions for ^{14}C-propoxycarbazone and metabolite Pr-2 are visible together with their respective Na^+ and K^+ adducts and their ^{14}C-containing counterparts. Under these conditions, three characteristic ions were detected for propoxycarbazone at [m]/z 367.2, [m]/z 158.3 and [m]/z 115.9 (see Figure CA 5.1-3). The first ion could be explained by a potential fragmentation consisting in the loss of one of the methyl moieties of propoxycarbazone and a further de-hydration. The second and third ions were explained by the fragmentations shown in Figure CA 5.1-3, i.e. formation of the N-methyl propoxy triazolinone ion ([m]/z 158.3), and the further loss of its propyl residue ([m]/z 115.9).

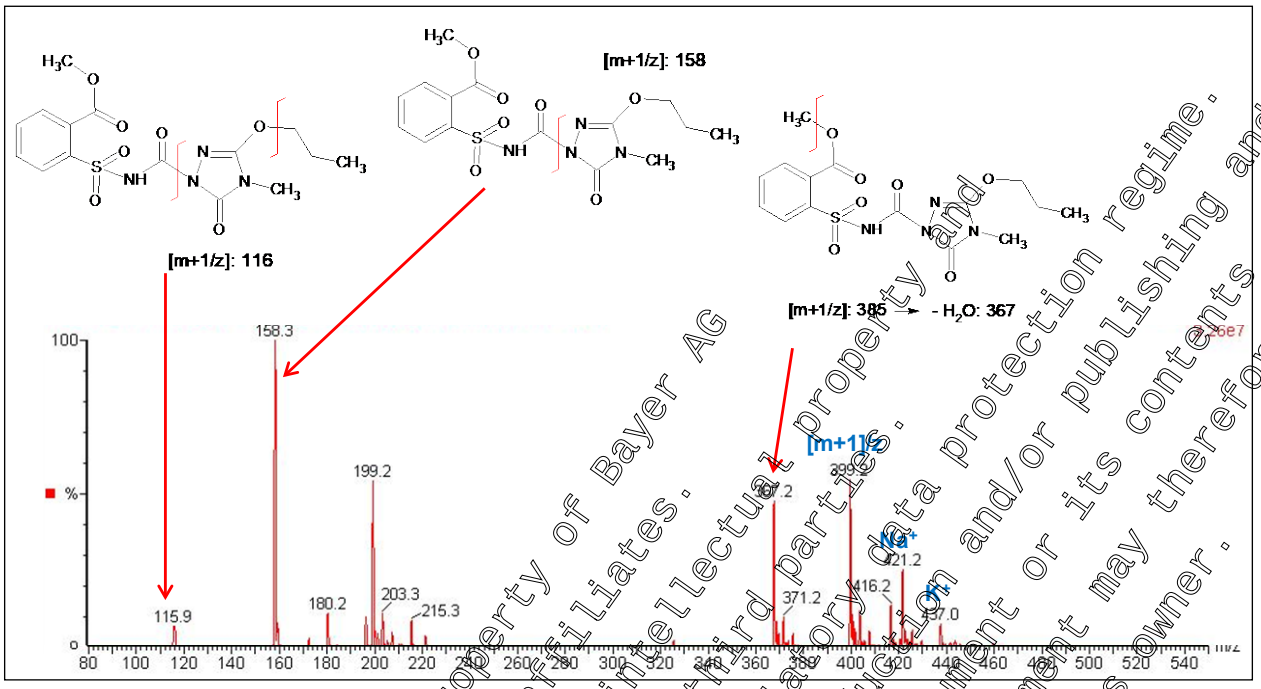


Figure CA 5.1-3: LC-MS spectra of ^{12}C-propoxycarbazone-sodium (Capillary Voltage: 3.5 KV; Cone Voltage: 15 V)

The same ions described for propoxycarbazone were also detected in the mass spectra of metabolite Pr-2 (Figure CA 5.1-4), indicating that the N-methyl propoxy triazolinone moiety was present and intact in the chemical structure of metabolite Pr-2. Therefore, the O-demethylation of propoxycarbazone leading to propoxycarbazone carboxylic acid metabolite is the most likely explanation for metabolite Pr-2 structure. The presence of the ion at [m]/z 367.2 would be explained by a de-hydration of metabolite Pr-2.

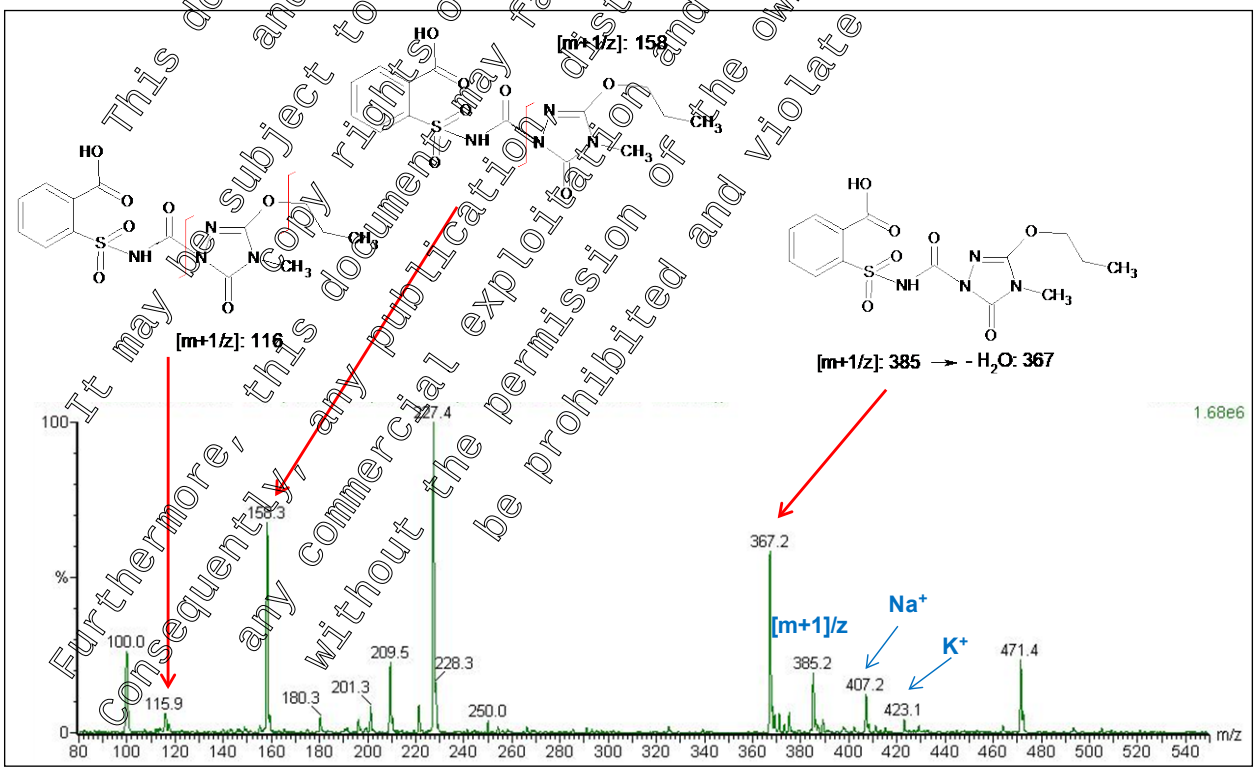


Figure CA 5.1-4: LC-MS spectra of metabolite Pr-2. (Capillary Voltage: 3.5 KV; Cone Voltage: 15 V)

No further information was obtained in the MS/MS experiments since the low amount of metabolite Pr-2 prevented the obtaining of a clear product-ion spectrum.

III. CONCLUSION

The results of the test with ^{14}C-propoxycarbazone-sodium demonstrated that the test item was highly stable when incubated with liver microsomes from rats and humans.

Two small ^{14}C-containing compounds could be detected (Pr-1 and Pr-2). Pr-1 HPLC peak was considered as abiotic degradation product of ^{14}C-propoxycarbazone-sodium since it was present in microsome incubations at t=0 hours incubation and/or in incubations at 37±1°C with buffer only.

Metabolite Pr-2 that accounted for 2.3% in HLM of the total relative percentage was tentatively identified by means of LC-MS as the O-demethylated form of propoxycarbazone (i.e. propoxycarbazone carboxylic acid metabolite; M04).

CA 5.2 Acute toxicity

Propoxycarbazone-sodium has a very low acute oral (LD_{50} > 5000 mg/kg bw, unspecific clinical signs), percutaneous (LD_{50} > 5000 mg/kg bw, no clinical signs) and inhalative (LC_{50} > 5.03 mg/L air, no clinical signs) toxicity in male and female rats. Propoxycarbazone-sodium is neither an eye- nor a skin irritant, and has no skin sensitizing potential (Table CA 5.2-1).
No new studies have been performed for this endpoint.

Table CA 5.2-1: Acute toxicity studies with propoxycarbazone-sodium

Study Type	Species	Effects	Results	Reference
Acute oral toxicity	rat	Soft faeces and moist anus	LD_{50} > 5000 mg/kg bw	[redacted] (1994) M-001552-01-1
Acute dermal toxicity	rat	No effects	LD_{50} > 5000 mg/kg bw	[redacted] (1994) M-001543-02-1
Acute inhalation toxicity	rat	No effects	LC_{50} > 5.03 mg/L air (4 h)	[redacted] (1995) M-001557-01-1
Skin irritation	rabbit	No effects	Non-irritant	[redacted] (1994) M-001525-01-1
Eye irritation	rabbit	No effects	Non-irritant	[redacted] (1994) M-001525-01-1
Skin sensitization (Magnusson & Kligman)	Guinea pig	No effects	Non-sensitizer	[redacted] (1994) M-001555-01-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

CA 5.2.1 Oral

All studies for this endpoint have been previously submitted and evaluated.

CA 5.2.2 Dermal

All studies for this endpoint have been previously submitted and evaluated.

CA 5.2.3 Inhalation

All studies for this endpoint have been previously submitted and evaluated.

CA 5.2.4 Skin irritation

All studies for this endpoint have been previously submitted and evaluated.

CA 5.2.5 Eye irritation

All studies for this endpoint have been previously submitted and evaluated.

CA 5.2.6 Skin sensitization

All studies for this endpoint have been previously submitted and evaluated.

CA 5.2.7 Phototoxicity

The data requirements published in Commission Regulation (EU) No. 283/2013 stipulate a study on phototoxicity for active substances showing an extinction coefficient ≥ 10 L x mol^{-1} x cm^{-1} in the spectrum of 290-700 nm. Propoxycarbazone-sodium does not fulfil this criterion and thus, this data requirement does not apply.

CA 5.3 Short term toxicity

The short-term studies with repeated administration demonstrated a low toxicity of propoxycarbazone-sodium in rats, mice and dogs (Table CA 5.3-1). A **new subacute feeding study in rats** has been performed with a batch representing the impurity profile of batches produced after 2002 (Edition No. M-001308-02-1). The NOAEL for the new substance batch did not differ from the results obtained with a batch reflecting the original specification containing fewer impurities since the observed changes in enzyme activities measured in liver tissue homogenate were considered to be of no toxicological relevance.

The affected organs were the liver (microsomal enzyme induction) adrenals (increased absolute weights, enlarged zona fasciculata) and heart (decreased relative weight) in dogs, forestomach in rats (local irritation), feed/water intake and body weight gain. In dogs, effects on organs were interpreted as an indirect physiologic response to chronic treatment or to reduced nutritional intake and were not considered to result from a direct toxicological mechanism of propoxycarbazone-sodium. A relevant immunotoxic potential has been excluded in the rat. Lowest NOAELs were 56, 205 and 286 mg/kg bw/day in dogs, mice and rats, respectively.

Table CA 5.3-1: Short-term toxicity studies with propoxycarbazone-sodium

Study Type	Species	Doses tested	LOAEL / Effects	NOAEL	Reference
Oral feeding, 4 weeks	rat	0, 800, 4000, 10 000, 20 000 ppm	20 000 ppm (2146 mg/kg bw/day): slightly elevated immunological parameters	10 000 ppm (985 mg/kg bw/day)	[redacted] (1996) M-001578-01-1
Oral feeding, 4 weeks	rat	0, 800, 4000, 10 000 ppm	No effects	10 000 ppm (1074 mg/kg bw/day)	[redacted] (2004) M-001308-02-1
Oral feeding, 4 weeks (plaque assay)	rat	0, 4000, 10 000, 20 000 ppm	20 000 ppm (2144 mg/kg bw/day): increased water intake	immunotox: 20 000 ppm overall: 10 000 ppm (986 mg/kg bw/day)	[redacted] (1999) M-017696-01-1
Oral feeding, 8 weeks	dog	0, 1000, 5000, 10 000, 40 000 ppm	40 000 ppm (1181 mg/kg bw/day): decreased feed consumption (vomiting) and body weight gain, increased liver enzymes	10 000 ppm (286 mg/kg bw/day)	[redacted] (1997) M-001743-01-1
Oral feeding, 5 weeks	mouse	0, 100, 1000, 10 000 ppm	10 000 ppm (5579 mg/kg bw/day): decreased body weight gain, increased feed intake	1000 ppm (394 mg/kg bw/day)	[redacted] (1996) M-001593-01-1
Oral feeding, 14 weeks	rat	0, 250, 1000, 4000, 20 000 ppm	20 000 ppm (1508 mg/kg bw/day: increased water intake, decreased glucose and triglyceride levels, irritation of the forestomach epithelium)	4000 ppm (286 mg/kg bw/day)	[redacted] (1996) M-001597-02-1
Oral feeding, 14 weeks	mouse	0, 625, 2500, 10 000 ppm	2500 ppm (860 mg/kg bw/day: decreased body weights)	625 ppm (205 mg/kg bw/day)	[redacted] (1997) M-001660-02-1
Oral feeding, 1-year	dog	0, 2000, 10 000, 25 000 ppm	10 000 ppm (235 mg/kg bw/day: decreased feed consumption and heart weights)	2000 ppm (56 mg/kg bw/day)	[redacted] (1998) M-009947-01-1
Dermal, 4 weeks	rat	0, 1000 mg/kg bw/day	No effects	1000 mg/kg bw/day	[redacted] (1997) M-001652-01-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

CA 5.3.1 Oral 28-day study

Report:	[redacted]; [redacted];2004;M-001308-02; Amended: 2004-03-23
Title:	MKH 6561 (c.n. Propoxycarbazone-sodium) - Subacute oral toxicity study in rats (4 weeks, administration by diet)
Report No:	AT01074
Document No:	M-001308-02-1
Guidelines:	**EEC Guideline B 7; OECD 407**
Deviations:	none
GLP/GEP:	**yes**

Executive Summary
The subchronic toxicity of propoxycarbazone-sodium (MKH 6561) was evaluated in a subacute repeated dose toxicity study. 5 Wistar rats/sex/dose (one control and three treated groups) were administered the test article continuously in the diet at concentrations of 800, 4000, and 10,000 ppm for at least 28 days (control animals received plain diet). The actual mean daily intake of the test substance was calculated as 76.08, 398.61, and 1078.69 mg/kg bw/day for males and 83.86, 440.61, and 1073.74 mg/kg bw/day for females, respectively. There were no test article-related adverse effects noted in any of the dose groups tested throughout subacute treatment with MKH 6561. Thus, under the conditions of this test, the NOAEL for MKH 6561 in the rat is ≥ 10,000 ppm dietary level, i.e. ≥ 1078.69 mg/kg bw/day for males and ≥ 1073.74 mg/kg bw/day for females, respectively.

I. MATERIALS AND METHODS

A. MATERIALS

1.	**Test material:**	MKH 6561, MKH 6561 EU
	Identification:	(Propoxycarbazone-sodium)
	Description:	White powder
	Lot/Batch #:	05649/0054
	Purity:	96.3%
	Stability of test compound:	Approved until 2004-05-24
2.	**Vehicle and/ or positive control:**	Control animals were given plain diet
3.	**Test animals:**	
	Species:	Rat
	Strain:	Wistar (HsdCpb:WU)
	Source:	[redacted] Germany
	Age:	Approximately 7 weeks
	Sex:	Male and female
	Weight at dosing:	Males: 133-145 g, females: 132-141 g
	Acclimation period:	8 days
	Diet/Food:	Fixed-formula standard diet [redacted] 3883.9.25 meal ([redacted] SA, [redacted], Switzerland), ad libitum
	Water:	Tap water, ad libitum

Housing:	Individually in polycarbonate cages Type III h on low-dust wood granulate ([redacted], Germany)
Environmental conditions:	Temperature: 22 ± 3°C
	Humidity: 55 ± 5%
	Air changes: ≥ 10/hour
	12 hours light/dark cycle

B. STUDY DESIGN AND METHODS

In life dates
2003-12-08 to 2004-01-13

Animal assignment and treatment
Animal assignment and dose groups:
Following 8 days of acclimation, all rats were grouped by weight and assigned to dose groups using random lists. The following dose groups were employed:

Test Group	Conc. in Diet [ppm]	Male	Female	Mean daily substance intake [mg/kg bw/day]	
				Male	Female
Control	0	5	5	0.00	0.00
Low dose group	800	5	5	76.08	83.86
Mid dose group	4000	5	5	398.61	440.61
High dose group	10,000	5	5	1078.69	1073.74

Dose levels have been selected based on the results obtained in a previous subacute toxicity study with MKH 6561.

Diet Preparation and Analysis:
MKH 6561 was mixed into the diet using a mixing. Due to technical reasons, the diet was separated in three portions after preparation, of which one part was administered to the animals immediately, and the two other parts were deep frozen and administered at test Days 8 and 15, respectively. The test compound stability in the deep frozen diet was determined for 15 days. For Week 4 the diet was prepared separately.

Details on oral exposure:
Duration of treatment/exposure: At least 28 days.
Frequency of treatment: Daily.

Examinations

Clinical Signs - Mortality and Moribundity/General Daily Observations	Animals were observed for mortality and moribundity twice daily and clinical signs were recorded once daily.
Clinical Signs - Detailed Observations	Animals were submitted to detailed clinical observations once prior to dosing and weekly thereafter. Examinations (conducted outside of the home cage) included evaluation of changes in skin, fur, eyes, mucus membranes, occurrence of secretions and excretions, autonomic activity, changes in gait, posture, and response to handling, clonic/tonic movements, stereotypes and bizarre behaviour.
Body Weights	Body weights were determined once predose and weekly thereafter until necropsy.
Food and Water Consumption, Compound Intake	Food and water consumption was measured weekly. Compound intake was calculated on the basis of food consumption and body weights.
Ophthalmic Evaluation	Ophthalmic evaluation was conducted on all animals predose and on control and high dose animals at study termination. If changes were noted, all animals of the next lower dose group would be examined in to establish a NOEL. Examinations were made after pupil dilatation using a direct or indirect ophthalmoscope and a slit lamp.
Clinical Pathology	On Day 23 of the study all animals of each group were given 5 ml tap water/animal by stomach tube and the urine was collected for a subsequent approximately 16 h period. The animals had no access to diet or water during the collection period. Blood samples non-anaesthetized animals were the collected on the following the morning for glucose concentration measurement. Immediately thereafter, the blood samples for haematology and clinical chemistry were obtained from all animals of each group under CO_2/O_2 (80:20) narcosis.
Haematology	The following parameters were examined: erythrocyte counts, reticulocyte counts, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), Heinz bodies, leukocyte counts, differential blood count, platelet counts, Hepato-Quick.
Clinical Chemistry	The following parameters were examined: alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, glutamate dehydrogenase, albumin, bilirubin, cholesterol, creatinine, glucose, urea, total protein, trigiycerides, chloride, potassium, phosphorus, sodium, triiodothyronin, thyroxine, TSH.
Urinalysis	The following parameters were examined: pH, volume, glucose, specific gravity, ketones, protein, bilirubin, microscopy of sediment, urobilinogen, blood.

Gross Pathology	Gross pathological examination was conducted on all animals.
Organ Weights	Total and relative organ weights of all sacrificed rats were determined. The following organs were weighed: adrenals, brain, heart, kidneys, liver, lungs, ovaries, spleen, testes, thyroids.
Histopathology	Tissues from each animal in the control and high dose groups were examined microscopically. He following organs were examined: adrenal glands, aorta, brain (cerebrum, cerebellum, brain stem), epididymides, oesophagus, eyes, exorbital lacrimal glands, femur, Harderian glands, intestine (duodenum, jejunum, ileum, caecum, colon, rectum), kidneys, liver, lungs, lymph nodes (mandibular, mesenteric), ovaries, oviducts, pituitary gland, prostate, salivary glands (parotid, submandibular, sublingual), sciatic nerve, seminal vesicles (incl. coagulation glands), skeletal muscle (thigh), skin (mammary region), spinal cord (cervical, thoracal, lumbar), spleen, sternum, stomach (forestomach and glandular stomach), testes, thymus, thyroid glands (with parathyroids), trachea, urinary bladder, uterus (with cervix). Organs and tissues with macroscopic findings were examined for all animals of all dose groups.
Investigations in Rat Liver Tissue	The following parameters were examined: cytochrome P-450, aminopyrine-N-demethylase, p-nitroanisole-O-demethylase.

Statistical evaluation
The quantitative results for individual animals were used to calculate group means, medians and standard deviations. The results for the groups that received the test compound were compared with those for the control group. The statistical evaluation of data related to laboratory investigations, body and organ weights as well as feed and water intake is performed using SAS® routines.

II. RESULTS AND DISCUSSION

A. MORTALITY

No mortality occurred throughout the study period.

B. CLINICAL OBSERVATIONS

Clinical signs of toxicity attributable to the test substance were not observed throughout the study period.

C. BODY WEIGHT

No effects on body weights or body weight development were detected in any of the dose levels throughout the study period.

D. FOOD AND WATER CONSUMPTION

There were no toxicologically relevant changes to the controls in food and water consumption observed.

E. OPHTHALMOSCOPIC EXAMINATION

Ophthalmoscopy revealed no test article related findings in any of the animals.

F. HAEMATOLOGY AND CLINICAL CHEMISTRY

There were no toxicologically significant differences in red and white blood cell parameters as well as in coagulation parameters between treated and control groups in both sexes.
No toxicologically relevant or dose-related changes were noted for clinical chemistry parameters evaluated in any of the dose groups. All values measured were within the range of the historical control data.
Changes in enzyme activities measured in liver tissue homogenate were considered to be of no toxicological relevance.

G. URINALYSIS

There were no toxicologically significant differences noted in urinalysis parameters checked for any dose group.

H. NECROPSY

There were no treatment-related macroscopic changes noted in any of the animals. Determination of organ weights revealed no test-article-related changes. Histopathological examination revealed no treatment-related findings.

III. CONCLUSION

There were no test article-related adverse effects noted in any of the dose groups tested throughout subacute treatment with propoxycarbazone-sodium (MKH 6561). Thus, under the conditions of this test, the NOAEL for propoxycarbazone-sodium in the rat is ≥ 10,000 ppm dietary level, i.e. ≥ 1078.69 mg/kg bw/day for males and ≥ 1073.74 mg/kg bw/day for females, respectively.

CA 5.3.2 Oral 90-day study

All studies for this endpoint have been previously submitted and evaluated.

CA 5.3.3 Other routes

All studies for this endpoint have been previously submitted and evaluated.

CA 5.4 Genotoxicity testing

Propoxycarbazone-sodium (MKH 6561) was tested for point-mutagenic activity, for its potential to induce chromosome aberration *in-vitro* and *in-vivo* and for DNA repair (Table CA 5.4-1).
The impurity profile present in batches produced in the years 2003 and later differed from that of the batches used for the tests submitted for the 2001 evaluation of propoxycarbazone-sodium.
A **new *in-vitro* chromosomal aberration study** has been conducted with a batch representing the altered impurity profile (Edition No. M-059611-01-1).
The previously submitted gene mutation test in bacteria featured only four tester strains, according to contemporary guideline requirements. To fulfil the requirements of the most recent version of the OECD TG 471, **a new test in the Salmonella strain, TA102, was conducted** (Edition No. M-461842-01-1).
Since all tests were negative, it can be concluded that propoxycarbazone-sodium has no mutagenic/genotoxic potential under *in-vitro* or *in-vivo* conditions.

The data requirements published in Commission Regulation (EU) No. 283/2013 stipulate a study on photomutagenicity for active substances and their major metabolites showing an extinction coefficient ≥

1000 L x mol^{-1} x cm^{-1} in the spectrum of 290-700 nm. Propoxycarbazone-sodium does not fulfil this criterion and thus, this data requirement does not apply.

Table CA 5.4-1: Genotoxicity/mutagenicity tests with propoxycarbazone-sodium

Test system	Concentration / Dose	Batch no. Purity (%)	Results	Reference
Ames test (TA 1535, 100, 1537, 98, +/-S9 mix)	up to 5000 µg/plate	NLL 5551-3 95.7	negative	[redacted] (1994) M-001520-01-1
Ames test (TA 102, +/-S9 mix)	0.316 to 100 µg/plate	2012-000352 95.1	negative	[redacted] (2012) M-461842-01-1
HPRT test (+/-S9 mix)	500 - 4000 µg/mL	NLL 5551-22.1 97.8	negative	[redacted] (1996) M-001590-01-1
ChrAb test (+/-S9 mix)	500 - 5000 µg/mL	NLL 5551-22.1 98.8	negative	[redacted] (1996) M-001563-01-1
ChrAb test (+/-S9 mix)	500 – 2000 µg/mL	05649/0054 96.3	negative	[redacted] (2004) M-059611-01-1
UDS test in primary rat hepatocytes	25 - 4000 µg/mL	NLL 5551-22.1 97.8	negative	[redacted] (1996) M-001585-01-1
Micronucleus test in mice	2500 mg/kg bw ip	NLL 5551-22.1 97.8/98.8	negative	[redacted] (1995) M-001560-01-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

CA 5.4.1 *In-vitro* studies

Report:	[redacted];[redacted];2012;M-461842-01
Title:	Mutagenicity study of propoxycarbazone-sodium in the Samonella typhimurium reverse mutation assay (in vitro)
Report No:	LPT 28508
Document No:	M-461842-01-1
Guidelines:	**According to Council Regulation (EC) no. 440/2008 part B.13/14 and OECD Guideline 471**
Deviations:	Only TA 102 was tested for mutagenicity; 2-AA was the only positive control used in this study (with metabolic activation)
GLP/GEP:	**yes**

Executive Summary

Propoxycarbazone-sodium was examined in the *Salmonella typhimurium* strain TA 102 in two independent experiments, each carried out without and with metabolic activation (a microsomal preparation derived from Aroclor 1254-induced rat liver). The first experiment was carried out as a plate incorporation test and the second as a preincubation test.

In a preliminary test, propoxycarbazone-sodium was examined in two preliminary cytotoxicity tests (plate incorporation test without and with metabolic activation) in test strain TA 100. Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. Pronounced cytotoxicity was noted starting at a concentration of 100 µg/plate in both experiments. Hence, 100 µg/plate was chosen as top concentration for the main study in the plate incorporation test and in the preincubation test.

In the main study, six concentrations ranging from 0.316 to 100 µg/plate were employed in the plate incorporation test and in the preincubation test, each carried out without and with metabolic activation. Pronounced cytotoxicity was noted at the top concentration of 100 µg/plate. No increase in revertant colony numbers as compared with control counts was observed for propoxycarbazone-sodium, tested up to a cytotoxic concentration of 100 µg/plate, in test strain TA 102 in two independent experiments without

and with metabolic activation, respectively (plate incorporation and preincubation test). The positive control item showed a significant increase in the number of revertant colonies of the test strain and confirmed the validity of the test conditions and the sensitivity of the test system.
In conclusion, propoxycarbazone-sodium is not mutagenic to the *Salmonella typhimurium* strain TA 102 up to cytotoxic concentrations, neither in the presence nor absence of a metabolic activation system under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	Propoxycarbazone-sodium
Description:	White solid/powder
Lot/Batch #:	2012-000352
Purity:	95.1%
Isomer distribution	Not reported
Stability of test compound:	Not reported

2. Vehicle and/ or positive control:
Vehicle: DMSO
Positive controls: Methyl methane sulfonate (MMS), -S9
2-Amino-anthracene (2-AA), +S9

3. Test system:

Organism:	*Salmonella typhimurium*
Strains:	TA 102 (main study), TA 100 (preliminary cytotoxicity test)
Source:	[redacted]
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	Preliminary cytotoxicity test (-S9 and +S9): 0.316, 1.0, 3.16, 10.0, 31.6, 100, 316, 1000, 3160 and 5000 µg/plate (plate incorporation) Main study (-S9 and +S9): 0.316, 1.0, 3.16, 10.0, 31.6 and 100 µg/plate (plate incorporation and pre-incubation)
Pre-incubation period:	20 min
Exposure duration:	48-72 h

B: STUDY DESIGN AND METHODS

Experimental dates
2012-04-26 through 2012-07-07

Experimental procedure

Preliminary study

Propoxycarbazone-sodium was examined in two preliminary cytotoxicity tests (plate incorporation test without and with metabolic activation) in tester strain TA 100. Cytotoxicity was evaluated by means of scarce background lawn and reduction of the number of revertants by more than 50%. Based on the outcome of this test, the doses for the main study were selected.

Main study

Two independent mutagenicity experiments were carried out with propoxycarbazone-sodium in the tester strain TA 102, each without and with metabolic activation. Six concentrations ranging from 0.316 to 100 µg/plate were employed, and each experiment consisted of 3 plates/concentration. Appropriate positive and solvent (DMSO) controls were included into the test to confirm its sensitivity.

In the plate incorporation test, 100 mL sterile top agar was mixed with 10 mL of a sterile solution of 0.5 mM L-histidine HCl/0.5 mM biotin, 0.1 mL Salmonella cell suspension (containing approximately 108 viable cells in the late exponential or early stationary phase) and 0.1 mL of the test item solution or solvent or positive control solution, respectively. In the experiment with metabolic activation, 0.5 mL of S9 mix was added, whereas in the experiments without metabolic activation, 0.5 mL of phosphate buffer was added. After pouring onto a minimal glucose agar plate and solidification, the plates were inverted and placed in a dark 37°C incubator for 48 to 72 hours. The revertant colonies on the test plates and on the control plates were counted with a colony counter, and the presence of the background lawn on all plates was confirmed.

The independent repeat was performed as pre-incubation in a water bath at 37°C for 20 minutes prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Tubes were aerated during preincubation by using a shaker. The remaining steps were the same as described for the plate incorporation method.

The following doses of propoxycarbazone-sodium were evaluated in the main study: 0.316, 1.0, 3.16, 10.0, 31.6 and 100 µg/plate.

The following doses were used for the positive controls:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
MMS	100	DMSO	TA102, -S9
2-AA	2	DMSO	TA102, +S9

Quality criteria

Quality criteria are tested by [redacted], Germany. The genotypes of the test strains are regularly confirmed in the following way:

- Histidine and biotin requirement ((his-) (bio-)):
  Each of the strains is streaked onto two Minimal glucose agar plate (Minimal Glucose Agar medium E) in the following way:
  1) with 0.1 mM L-histidine and 0.5 mM biotin (100 µL/each)
  2) with 0.5 mM biotin (100 µL/each)

  After incubation at 37°C for 24 hours, none of the strains should grow on plate 2; all strains should show excessive growth on plate 1.
- (rfa-) deep rough character:
  10 µL of 0.1% crystal violet applied with a paper disc should give zones of inhibition in the test strains after incubation at 37°C for 24 hours.
- UV-sensitivity (uvr B-):
  Plates are covered partly with black paper and placed under germicidal UV-irradiation. After incubation at 37°C for 24 hours TA 100 should grow only under the covered portion of each plate. TA 102 should also grow under the uncovered area.
- Ampicillin-resistance (pKM 101):
  0.8 mg ampicillin/plate is placed onto plates seeded with bacteria: Absence of zones of inhibition around the discs indicates resistance to ampicillin (TA 100 and TA 102).
- Ampicillin- and tetracycline-resistance

The pAQ1 strain (TA 102) is tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates.

Assessment Criteria

A test item is considered to show a positive response if

- The number of revertants is significantly increased ($p \leq 0.05$, U-test according to MANN and WHITNEY) compared with the solvent control to at least 2-fold of the solvent control in both independent experiments;
- In addition, a significant ($p \leq 0.05$) concentration (log value)-related effect (Spearman's rank correlation coefficient) is observed;
- Positive results have to be reproducible and the histidine independence of the revertants has to be confirmed by streaking random samples on histidine-free agar plates.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary study

Pronounced cytotoxicity (scarce background lawn and reduction of the number of revertants by more than 50%) was noted starting at a concentration of 100 µg/plate in both experiments. Hence, 100 µg propoxycarbazone-sodium/plate was chosen as top concentration for the main study in the plate incorporation test and in the preincubation test.

Main study

Pronounced cytotoxicity (scarce background lawn and reduction of the number of revertants by more than 50%) was noted at the top concentration of 100 µg/plate.

No increase in revertant colony numbers as compared with control counts was observed for Propoxycarbazone-sodium, tested up to a cytotoxic concentration of 100 µg/plate, in test strain TA 102 in two independent experiments without and with metabolic activation, respectively (plate incorporation and preincubation test). The positive control item showed a significant increase in the number of revertant colonies of the test strain and confirmed the validity of the test conditions and the sensitivity of the test system. The results of the main study are presented in Table 5.4-1.

Table 5.4-1: Results of the mutagenicity tests with TA 102 – without and with metabolic activation

Test item	Concentration [µg or µL/plate]	Number of revertants [mean number of colonies per plate ± SD]			
		Plate incorporation		Preincubation	
		-S9 mix	+S9 mix	-S9 mix	+S9 mix
Solvent control (DMSO)	100.0	279.7 ± 3.1	270.0 ± 27.0	279.0 ± 7.0	251.7 ± 1.5
Propoxycarbazone-sodium	0.316	274.3 ± 3.2	265.3 ± 2.3	270.7 ± 2.1	282.0 ± 7.5
	1.0	277.0 ± 3.0	282.0 ± 2.0	266.0 ± 6.0	276.3 ± 15.9
	3.16	284.3 ± 2.5	271.3 ± 2.1	253.0 ± 3.6	261.0 ± 13.0
	10.0	262.3 ± 5.0	257.0 ± 26.9	260.0 ± 1.7	251.0 ± 3.6
	31.6	271.7 ± 7.4	284.7 ± 7.2	267.0 ± 8.9	250.7 ± 5.1
	100.0	27.7 ± 14.0[#]	15.0 ± 2.0[#]	26.0 ± 10.6[#]	38.0 ± 26.9[#]
Positive control (MMS)	2.0	1104.0 ± 33.8	-	1105.3 ± 19.1	-
Positive control (2-AA)	100.0	-	1076.0 ± 33.8	-	1077.3 ± 2.3

[#] scarce background lawn

III. CONCLUSION

The test substance was not mutagenic in the Salmonella/microsome assay, with and without metabolic activation.

Report:	[redacted]; [redacted];2004;M-059611-01
Title:	MKH 6561 - In vitro chromosome aberration test with Chinese hamster V79 cells
Report No:	AT01101
Document No:	M-059611-01-1
Guidelines:	**Directive 2000/32/EC, method B.10.; OECD 473; US-EPA 712-C-98-223**
Deviations:	none
GLP/GEP:	**yes**

Executive Summary
An *in-vitro* cytogenicity study was conducted with propoxycarbazone-sodium using Chinese hamster V79 cells cultures both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254 induced animals.
The concentrations employed in the main study were 500, 1000, and 2000 µg/mL and based on solubility properties of the test item and the results of a preliminary cytotoxicity test. The test was carried out employing 2 exposure times without S9 mix (4 and 18 h) and 1 exposure time with S9 mix (4 h). Cells were generally harvested 18 h after start of exposure; however, for treatment with 2000 µg/mL in the absence of metabolic activation, a further test was conducted with a harvest time of 30 h after start of exposure.
No biologically relevant and statistically significant increases of metaphases with aberrations were detected at any time point in any of the concentrations tested, with or without metabolic activation (S9 mix). Appropriate solvent (DMSO) and positive controls (mitomycin C (-S9 mix) and cylcophosphamide (+S9 mix)) gave the expected results and thus proved the sensitivity of the test.
The test item did not relevantly reduce the mitotic index at any time point in any of the concentrations tested with or without metabolic activation (S9 mix). However, cytotoxicity in form of reduced survival was noted in parallel cultures treated with 2000 µg/mL for 18 h without metabolic activation. The positive control substance mitomycin C (-S9 mix) slightly reduced cell survival in the parallel cultures, but not the mitotic index of the cultures evaluated for clastogenicity. The positive control substance cylcophosphamide (+S9 mix) reduced both survival and mitosis rates after 4 of exposure (+S9 mix).
In conclusion, propoxycarbazone-sodium was not clastogenic to mammalian cells *in-vitro* under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	MKH 6561, MKH 6561 EU (Propoxycarbazone-sodium; Benzoic acid, 2-[[[(4,5-dihydro-4-methyl-5-oxo-3-propoxy-1 H-1,2,4-triazol-1-yl)carbonyl]amino] sulfonyl], methylester, sodium salt)
Description:	Fine white powder
Lot/Batch#:	05649/0054
Purity:	96.3%
Isomer distribution	Not reported
Stability of test compound:	Approved until May 24, 2004

2. Vehicle and/ or positive control:	Vehicle: DMSO Positive controls: Mitomycin C (MMC), -S9 Cyclophosphamide (CP), +S9

3. Test system:

Organism:	Mammalian cells in culture
Strains:	Chinese hamster V79 cells
Source	[redacted], Germany
Media:	Eagle's minimal essential medium (MEM, Earle) supplemented with nonessential amino acids, L-glutamine (2 mM), MEM-vitamins, $NaHCO_3$ solution (final concentration: 0.225%), penicillin (50 units/mL), streptomycin (50 µg/mL), heat-inactivated foetal calf serum
Properly maintained?	Yes
Periodically checked for karyotype stability?	Yes
Periodically checked for Mycoplasm?	Yes
Metabolic activation system:	Cofactor supplemented post-mitochondrial fraction (S9 mix) from livers of male Sprague Dawley rats treated with Aroclor 1254
Test concentrations:	–S9: 500, 1000 and 2000 µg/mL (4 h exposure, 18 h harvest time) and 2000 µg/mL (4 h exposure, 30 h harvest time) +S9: 500, 1000 and 2000 µg/mL (18 h exposure, 18 h harvest time)

B: STUDY DESIGN AND METHODS

Experimental dates
2003-12-16 through 2004-02-20

Experimental procedure

Determination of cytotoxicity:

Cytotoxicity of the test item was determined in a pre-test, as well as in additional cultures and by means of mitotic index determination during the main study.

Main study

Cells were treated with the test substance dissolved in DMSO and the respective positive and solvent controls both in the presence and absence of a metabolic activation system (S9 mix).
The following positive controls were employed for the study:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
MMC	0.1 (4 h exposure) 0.03 (18 h exposure)	Hanks' balanced salt solution (HBSS)	-S9
CP	2.0 (4 h exposure)	Hanks' balanced salt solution (HBSS)	+S9

Following cell treatment with the test item and the respective positive and solvent controls for 4 h, the cells were washed with PBS, given fresh medium and cultured for further 14 h. Medium of cells treated for 18 h was not replaced. Two hours prior to the end of incubation, the spindle inhibitor Colcemid was added to the cultures. At least two slides were generated per culture.
Following harvested, the slides were fixed in ethanol/acetic acid (3:1) and stained with 3% Giemsa solution.

Slide evaluation
The mitotic index was determined by counting 1000 cells per culture, whereas duplicate cultures were processed and examined.
For evaluation of clastogenicity, chromosomes of approximately 200 metaphases per concentration, 100 metaphases from each of two parallel cultures, were examined. Only metaphases containing the modal chromosome number (22) were analysed unless exchanges were detected. In this case, metaphases were evaluated even if the chromosome number was not equal to 22. The chromosome aberrations observed were characterised as gap, break, fragment, deletion, exchange, or multiple aberration. In addition to these aberrations, metaphases showing chromosome disintegration as an indication of a cytotoxic effect were also recorded if they were observed. They were counted separately and were not included among the cells that were assessed for aberrations. "Chromosome disintegration" was recorded if fewer than half of the chromosomes reveal characteristic structural features within a given metaphase. Among chromosome aberrations, polyploidy was also recorded.

Statistical evaluation
Statistical analysis was only conducted, if the results indicated an increase (chromosome aberrations) or decrease (mitotic index) as compared to the respective solvent control.
The mitotic index was statistically analysed (provided that it was reduced compared to the mean of the corresponding solvent control) using the one-sided chi^2-test. The numbers of metaphases with aberrations (including and excluding gaps) and of metaphases with exchanges were compared. Fisher's exact test was used for the statistical evaluation. A difference was considered to be significant, if the probability of error was <5%.

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.
A test was considered equivocal, if there was an increase above the range of historical negative controls which was statistically significant but not considered relevant, or if an increase occurred, which was considered relevant, but which was not statistically significant.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Genotoxicity
No biologically relevant and statistically significant increases of metaphases with aberrations were detected at any time point in any of the concentrations tested with or without metabolic activation (S9 mix). Appropriate solvent and positive controls gave the expected results and thus proved the sensitivity of the test.

Cytotoxicity

The test item did not relevantly reduce the mitotic index at any time point in any of the concentrations tested with or without metabolic activation (S9 mix). The positive control substance mitomycin C (MMC, -S9 mix) did not reduce the mitotic index; however, cyclophosphamide (CP, +S9 mix) reduced the mitosis rate.
Cytotoxicity determined in parallel cultures during the main study was not observed after 4 h exposure duration with or without metabolic activation at any concentration tested. However, relevant cytotoxicity was noted in cultures treated with 2000 µg/mL for 18 h without metabolic activation. MMC induced slight cytotoxicity only after 18 h of exposure (-S9 mix), whereas CP reduced the survival rate after 4 of exposure (+S9 mix).

A summary of the results is given in Table 5.4-2 through Table 5.4-3.

Table 5.4-2: Chromosome analysis in cultured Chinese hamster V79 cells *in-vitro* – without metabolic activation

Treatment	Concentration [µg/mL]	Mitotic index [%]	No. of metaphases scored	Aberrations [%]			Polyploid metaphases (mean out of 2 cultures)
				incl. gaps	excl. gaps	exchanges	
4 h exposure – 18 h harvest time							
DMSO	-	100.0	200	6.0	6.0	1.5	17.5
Test item	500	76.9	200	3.5	3.5	0.5	14.0
	1000	84.0	200	6.0	6.0	2.5	12.5
	2000	116.0	200	4.5	4.5	1.0	18.5
MMC	0.1	92.9	200	47.0**	47.0**	30.5**	17.0
4 h exposure – 30 h harvest time							
DMSO		100.0	200	7.0	7.0	2.0	13.5
Test item	2000	112.9	200	3.5	3.5	0.5	10.5
18 h exposure – 18 h harvest time							
DMSO	-	100.0	200	3.0	3.0	1.0	17.0
Test item	500	138.3	200	3.0	3.0	1.0	10.5
	1000	118.1	200	4.5	4.5	1.5	13.0
	2000	147.9	200	4.5	4.5	1.0	17.0
MMC	0.05	121.3	200	24.0**	22.5**	10.5**	13.5

MMC = Mitomycin C
* $p < 0.05$; ** $p < 0.01$

Table 5.4-3: Chromosome analysis in cultured Chinese hamster V79 cells *in-vitro* - with metabolic activation

Treatment	Concentration [µg/mL]	Mitotic index [%]	No. of metaphases scored	Aberrations [%]			Polyploid metaphases (mean out of 2 cultures)
				incl. gaps	excl. gaps	exchanges	
4 h exposure – 18 h harvest time							
DMSO	-	100.0	200	8.0	8.0	1.0	9.5
Test item	500	101.7	200	11.5	11.5	4.0	15.0
	1000	110.5	200	6.0	6.0	2.0	16.5
	2000	111.0	200	9.0	9.0	1.5	12.5
CP	2	75.6**	200	51.5**	50.0**	28.5**	18.0

4 h exposure – 30 h harvest time							
DMSO	-	100.0	200	7.5	7.0	2.0	14.5
Test item	2000	100.5	200	9.0	9.0	2.0	13.0

CP = cyclophosphamide
* $p < 0.05$; ** $p < 0.01$

III. CONCLUSION

The test substance was not clastogenic to mammalian cells in the chromosome aberration assay, with and without metabolic activation.

CA 5.4.2 *In-vivo* studies in somatic cells

All studies for this endpoint have been previously submitted and evaluated.

CA 5.4.3 *In-vivo* studies in germ cells

Since all studies for this endpoint have been previously submitted and evaluated as negative this data requirement does not apply.

CA 5.5 Long-term toxicity and carcinogenicity

In both long-term studies, the toxicity of propoxycarbazone-sodium proved to be very low; NOAELs were 43 and 369 mg/kg bw/day in rats and mice, respectively. The predominant effect was a decreased body weight gain. In the rat, this was accompanied by an increased urinary pH and by renal pelvic mineralization in the highest dose. There were no indications for an oncogenic potential of propoxycarbazone-sodium.

No new studies have been performed for this endpoint.

Table CA 5.5-1: Long-term toxicity studies with propoxycarbazone-sodium

Study Type	Species	Doses tested	LOAEL / Effects	NOAEL	Reference
Combined chronic toxicity /carcinogenicity study	rat	0, 1000, 10 000, 20 000 ppm	10 000 ppm (459 mg/kg bw/day): decreased body weight gain, increased urinary pH	1000 ppm (43 mg/kg bw/day)	[redacted], [redacted] (1999) M-007286-03-1
Carcinogenicity study	mouse	0, 280, 1400, 7000 ppm	7000 ppm (1881 mg/kg bw/day): decreased body	1400 ppm (369 mg/kg bw/day)	[redacted] (1999) M-015495-05-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

CA 5.6 Reproductive toxicity

The reproductive toxic potential of propoxycarbazone-sodium was tested in a two-generation study in rats and in developmental toxicity studies in rats and rabbits (Table CA 5.6-1). The results of these studies do not indicate a risk of reproductive toxicity. The two-generation study demonstrates the absence of any reproductive and neonatal toxicity up to 16000 ppm, a parentally toxic dose clearly exceeding the limit dose of 1000 mg/kg bw/day. The developmental study in rats determined a NOAEL of 1000 mg/kg bw/day, both for maternal and developmental toxicity.

Rabbits are more sensitive than rats, resulting in clear maternal toxicity including a secondary effect on gestation rate at 500 and 1000 mg/kg bw/day. The NOAEL for maternal toxicity in rabbits was 100 mg/kg bw/day, the NOAEL for embryo/foetal development was 500 mg/kg bw/day. Thus, a primary

developmental toxic potential in rabbits has been excluded. Both developmental toxicity studies did not reveal a teratogenic potential of propoxycarbazone-sodium.
No new studies have been performed for this endpoint.

Table CA 5.6-1: Reproductive toxicity studies with propoxycarbazone-sodium

Study Type	Species	Doses tested	LOAEL / Effects	NOAEL	Reference
1-generation (pilot study)	rat	0, 1000, 5000, 20 000 ppm	20 000 ppm (litter weight, litter size at birth, number of pups born, viability index, number of males born per litter reduced)	5000 ppm	[redacted] (1997) M-001656-01-1
2-generation	rat	0, 1000, 4000, 16 000 ppm	parental: 4000 ppm (323 mg/kg bw/day: focal vacuolation of the forestomach epithelium in F1 males, dilated caeca in F0 females) reproductive/ neonatal: no findings	parental: 1000 ppm (80 mg/kg bw/day) reproductive/ neonatal toxicity: 16 000 ppm (1231 mg/kg bw/day)	[redacted] (1999) M-012427-03-1
Developmental toxicity (gavage)	rat	0, 100, 300, 1000 mg/kg bw/day	No findings	1000 mg/kg bw/day	[redacted] (1997) M-001686-03-1
Developmental toxicity (gavage)	rabbit	0, 20, 100, 500, 1000 mg/kg bw/day	maternal: 500 mg/kg bw/day (clinical signs, abortions, effects on feed, water intakes, body weights, gastrointestinal tract, liver, thyroid hormones) embryo/foetal development: 1000 mg/kg bw/day (effects on placentas, post-implantation loss, number of foetuses, foetal weight, skeletal ossification, liver lobulation)	maternal: 100 mg/kg bw/day embryo/foetal development: 500 mg/kg bw/day	[redacted] (1998) M-005522-02-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

CA 5.6.1 Generational studies

All studies for this endpoint have been previously submitted and evaluated.

CA 5.6.2 Developmental toxicity studies

All studies for this endpoint have been previously submitted and evaluated.

CA 5.7 Neurotoxicity studies

Propoxycarbazone-sodium is not structurally related to known neurotoxicants such as organophosphates or carbamates.
The results of the acute and subchronic neurotoxicity studies in rats (Table CA 5.7-1) confirm the generally very low toxicity of propoxycarbazone-sodium and demonstrate the absence of any specific neurotoxicity at limit doses of 2000 mg/kg bw/day (acute) and greater than 1000 mg/kg bw/day (subchronic).
No new studies have been performed for this endpoint.

Table CA 5.7-1: Neurotoxicity studies with propoxycarbazone-sodium

Study Type	Species	Doses tested	LOAEL / Effects	NOAEL	Reference
Acute neurotoxicity, oral gavage	rat	0, 200, 800, 2000 mg/kg bw	No effects	2000 mg/kg bw/day	[redacted], [redacted] (1998) M-001679-01-1
Subchronic neurotoxicity, 13-week feeding	rat	0, 1000, 4000, 20 000 ppm	No effects	20 000 ppm (1321/1651 mg/kg bw/day, ♂/♀)	[redacted] (1998) M-009922-02-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

CA 5.7.1 Neurotoxicity studies in rodents

All studies for this endpoint have been previously submitted and evaluated.

CA 5.7.2 Delayed polyneuropathy studies

Not a data requirement in the EU.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

Six major plant or soil metabolites of propoxycarbazone-sodium were identified that were not (< 0.1%) present in the batches of technical propoxycarbazone-sodium used for toxicological testing and did not occur in relevant amounts in animal metabolism studies. A limited toxicological profile of these metabolites has been established (Table CA 5.8-1). For structures and code numbers please refer to Table CA 5.1-2 on page 9.

The soil metabolites had a low to moderate (**M09**) or a very low (**M04, M08**) acute oral toxicity in the rat. In the Ames test, all three soil metabolites were negative.

The soil metabolites **M10 and M11** were identified as potentially relevant after the initial Annex-I inclusion of propoxycarbazone-sodium.
For the soil metabolite **M10**, a battery of *in-vitro* genotoxicity studies was conducted. M10 was not mutagenic, with and without metabolic activation, in an Ames test, a chromosomal aberration test and a HPRT assay. Additionally, a 90-day feeding study in rats (preceded by a 14-day pilot study) was conducted. A subchronic NOAEL of 600 ppm (corresponding to 36.5 and 44.6 mg/kg bw/day for male and female rats, respectively) was established for M10, based on reduced body weights at 6000 ppm the highest dose tested. The effect level and the nature of the effect observed after dietary application of M10 are comparable to those observed in subchronic studies conducted with the active substance. Thus, M10 is considered to be of no toxicological concern.

M11 is the methyl ether derivative of M08. The acute oral toxicity of M11 was expected to be similar to the non-toxic M08 and thus no acute toxicity testing is deemed necessary.
To verify that the methylation does not confer mutagenic properties, a battery of *in-vitro* genotoxicity study was run with M11. M11 was not mutagenic, with and without metabolic activation, in the Ames test, a chromosomal aberration test as well as an HPRT assay. M11 is considered to be of no toxicological concern.

The plant metabolite **M01** had a very favourable toxicological profile in all investigated studies, comparable to that of the parent compound propoxycarbazone-sodium. It was practically non-toxic in the acute oral rat study, not genotoxic in bacterial reverse mutation assay and chromosome aberration studies, and caused no effects in the rat after subacute feeding of 10 000 ppm (approaching the 1000 mg/kg bw/day limit dose).

Taken together, these data indicate the absence of any critical toxicity of the investigated plant and soil metabolites of propoxycarbazone-sodium.

Table CA 5.8-1: Toxicity tests with metabolites of propoxycarbazone-sodium (new studies in bold)

Metabolite / Occurrence	Study type	Dose / Concentration tested	Results	Reference
M01 Plant	Acute oral toxicity, rat	5000 mg/kg bw Limit test	LD_{50} > 5000 mg/kg bw	[redacted] (1999) M-009960-02-1
	Ames	up to 5000 µg/plate	Negative	[redacted] (1999) M-006203-01-1
	ChrAb in V79 cells	up to 2500 µg/mL	Negative	[redacted] (1999) M-010058-01-1
	Oral 28-day, rat	0, 800, 4000, 10 000 ppm	No effects NOAEL: 10 000 ppm (880 mg/kg bw/day)	[redacted] (1999) M-013866-01-1
M04 Soil	Acute oral toxicity, rat	5000 mg/kg bw Limit test	LD_{50} > 5000 mg/kg bw	[redacted] (1999) M-010089-01-1
	Ames	up to 5000 µg/plate	Negative	[redacted] (1999) M-010077-01-1
M08 Soil	Acute oral toxicity, rat	5000 mg/kg bw Limit test	LD_{50} > 5000 mg/kg bw	[redacted] (1999) M-015583-03-1
	Ames	up to 5000 µg/plate	Negative	[redacted] (1999) M-010287-01-1
M09 Soil	Acute oral toxicity, rat	1000, 2000, 5000 mg/kg bw	♂ LD_{50} = 2633 mg/kg bw ♀ LD_{50} = 1756 mg/kg bw	[redacted] (1999) M-009698-01-1
	Ames	up to 5000 µg/plate	Negative	[redacted] (1999) M-010034-01-1
M10 Soil	Ames	up to 5000 µg/plate	Negative	[redacted] (2012) M-461843-01-1
	HPRT test in V79 cells	up to 5000 µg/mL	Negative	[redacted] (2012) M-461844-01-1
	ChrAb in CHO cells	up to 5000 µg/mL	Negative	[redacted] (2012) M-461845-01-1
	14-day feeding study, rat (range-finder)	0, 800, 8000, 16000 ppm	Spilling behaviour NOAEL: < 800 ppm (60.5 mg/kg bw/day)	[redacted] (2012) M-487113-01-1
	90-day feeding study, rat	0, 60, 600, 6000 ppm	Reduced body weight NOAEL: 600 ppm (36.5 mg/kg bw/day)	[redacted] (2013) M-461866-01-1
M11 Soil	Ames	up to 3160 µg/plate	Negative	[redacted] (2013) M-461846-01-1
	HPRT test in V79 cells	up to 5000 µg/mL	Negative	[redacted] (2013) M-466474-01-1
	ChrAb in human lymphocytes	up to 5000 µg/mL	Negative	[redacted] (2013) M-462692-01-1

Studies shaded in grey have been reviewed as part of the first EU review of propoxycarbazone-sodium (in Baseline Dossier for the active substance P 010245-01).

Report:	[redacted];[redacted];2012;M-487113-01
Title:	Technical grade BCS-AB10736: A 14 day range-finding toxicity study in the wistar rat
Report No:	11N-P72-VH
Document No:	M-487113-01-1
Guidelines:	**No guideline followed (range finding study for study according to OECD 408).**
Deviations:	Not applicable
GLP/GEP:	**yes**

Executive Summary

A range-finding study was conducted with BCS-AB10736. 5 Wistar Crl:WI(Han) rats/sex/dose (one control and three treated groups) were administered BCS-AB10736 continuously in the diet at concentrations of 800, 8000 and 16,000 ppm for 14 days (control animals received plain diet). Examined parameters included: clinical observations, mortality and moribundity checks, body weights, food consumption, serum chemistry, haematology, coagulation, gross pathology and organ weights.

All animals survived until scheduled sacrifice. An increased incidence of spilling behaviour observed in 800, 8000 and 16,000 ppm animals was the only clinical sign attributable to the test substance and occurred primarily on study Day 7. There was no clear dose relationship and appeared to be more persistent in females.

Marked changes in body weight (and decreased body weight gain) attributable to the test substance were noted in 8000 and 16,000 ppm animals. Based on final body weight (treated relative to control), decreases of 15 and 22% were noted in 8000 ppm males and females, and 32 and 30% in 16,000 ppm males and females, respectively.

Food consumption data were somewhat erratic in a few cases (likely due to spilling) but did not appear to be affected. Although there is an indication of decreased food consumption the first week of the study, a clear conclusion cannot be drawn due to the incidence of spilling.

Changes in the haematology/coagulation profile attributable to the test substance included increased erythrocyte counts, haemoglobin, and haematocrit in 8000 ppm males and in both sexes at 16,000 ppm. Increased prothrombin time was also noted in 16,000 ppm females. It is unclear if these changes are a direct effect of the test substance, and may reflect a degree of dehydration.

Changes in the clinical chemistry profile attributable to the test substance were noted in 8000 and/or 16,000 ppm animals and included increased urea nitrogen (8000 and 16,000 ppm females; slight in 16,000 ppm males), glucose (16,000 ppm females), and cholesterol in (8000 ppm and 16,000 ppm males and females). Decreased triglyceride concentration was noted in 8000 ppm males and in both sexes at 16,000 ppm. It is likely that some of the changes in the clinical chemistry profile were the result of or exacerbated by the marked decrease in body weight noted in the same animals.

Gross lesions attributable to the test substance were noted in 3/5 16,000 ppm males, and included reduced prostate and seminal vesicle size. These findings correlate with reduced organ weight in the prostate and seminal vesicles. Further changes in organ weights were not considered to be test material related.

Following 14 days of continuous and repeated exposure to the test substance, which exceeded a limit dose of 1000 mg/kg bw/day at the highest dietary concentration, the toxicological response of the rat was principally characterized by decreased body weight, as well as changes in the haematology, serum chemistry, and organ weight profiles. Based on these findings, dietary concentrations of 60, 600, and 6000 ppm were selected for a follow up 90 day toxicity study in the rat.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

	BCS-AB10736
Identification:	(4-methyl-5-propoxy-2,4-dihydro-3H-1,2,4-triazol-3-one)
	CAS # 145027-96-9
Description:	White crystals

Lot/Batch #:	NLL 5797-6-5
Expiration:	2012-05-20
Purity:	99.0%
Stability of test compound:	Not reported

2. Vehicle and/ or positive control: None (dry-mix)

3. Test animals:

Species:	Rat
Strain:	Wistar: Crl:WI(Han)
Source:	[redacted] (Kansas City, MO)
Age:	9 weeks
Sex:	Male/female
Weight at dosing:	238–272 g (males) 170–214 g (females)
Acclimation period:	7 days: November 15, 2011 (receipt)–November 22, 2011 (released for study).
Diet/Food:	Purina Mills Certified Rodent Diet 5002 in "meal" form, ad libitum. Grated feeders were used for all animals.
Water:	Tap water (municipal water supply of Kansas City, MO) via pressure-activated water nipples and/or water bottles, ad libitum.
Housing:	Individually in suspended stainless steel cages, with deotized (sanitized) cage board in the bedding trays.
Environmental conditions:	Temperature: 18 - 26°C Humidity: 30 - 70% Air changes: min. 10/hour 12 hours light/dark cycle

B. STUDY DESIGN AND METHODS

In life dates
2011-11-15 through 2011-09-12

Animal assignment and treatment
Animal assignment and dose groups
Following seven days of quarantine/acclimation, all rats were randomly assigned to dose groups using a weight stratification-based computer program (DATATOX, Instem Computer Systems, Version rC10, P/C, [redacted] England). The following dose groups were employed:

Test group	Conc. in diet [ppm]	Target dose [mg/kg bw/day]	Male	Female
Control	0	0	5	5
Low dose group	800	50	5	5
Mid dose group	8000	500	5	5
High dose group	16,000	1000	5	5

Dietary levels for this study (800, 8000 and 16,000 ppm) were selected by the sponsor based on the results of previous studies with the parent compound.

Route of Administration
The possible route of human exposure to BCS-AB10736 is via ingestion of foodstuffs that might contain low residues of the test substance. Thus, formulation with feed was an appropriate route of administration to further establish the toxicological profile of the test substance in a given test species.

Diet Preparation and Analysis
The test substance was mixed directly with the feed. Adjustments were not made for percentage purity of less than 100%. 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 the same as the 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. A single batch of each admixture was prepared for each treatment group and stored under freezer conditions until presented to the animals (weekly). Homogeneity and stability analysis, as well as concentration analysis were not required.

Details on oral exposure

Duration of treatment/exposure: 14 days

Frequency of treatment: Daily

Examinations

Clinical Signs - Mortality and Moribundity/General Daily Observations

Each animal was observed for mortality, abnormalities, and signs of pain and distress at least once daily (nominally twice daily during the normal work week and once on weekends and holidays).

Clinical Signs - Detailed Observations

Animals were submitted to detailed clinical observations at least once during the predose phase (e.g., at randomization), before dosing or immediately after dosing on Day 0, and weekly thereafter.

Examinations (conducted outside of the home cage) included evaluation of external surface areas (visual inspection and palpation for externally detectable "masses"), orifices, posture, respiration, and excretory products. During the examination, animals were also observed for common signs relating to neurological effects, including but not limited to piloerection, respiratory abnormalities, posture, involuntary motor movements (clonic or tonic), stereotypy, bizarre behavior, gait abnormalities, and vocalizations. Abnormal findings or an indication of normal were recorded.

Body Weights	Body weights were determined at least once during the predose phase, before initiation of exposure on Day 0, and weekly thereafter.
Food Consumption	Food consumption was measured Weekly (quantitative) during the exposure phase. Using specifically defined criteria, food consumption data were corrected, as conditions dictated, to account for misleading indications of food intake (i.e., excessive spillage, etc.).
Clinical Pathology	All surviving animals were examined for clinical pathology on the day of scheduled sacrifice. Rats were fasted overnight (≈14–18◦h) for scheduled blood collections. Blood was collected via the orbital sinus (rats were anesthetized with isoflurane), and approximately 500 µL for haematology, 1000 µL for serum chemistry and 1800 µL for coagulation were collected. The anticoagulants were sodium citrate for coagulation tests and K$_3$EDTA for haematology tests. Samples for clinical chemistry were collected without anticoagulant.
Haematology	The following parameters were examined: total red blood cell (erythrocyte) count, red cell distribution width, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, total platelet count, haemoglobin distribution width, total white blood cell (leukocyte) count, differential blood cell count, reticulocyte count, cytologic morphology.
Coagulation	The following parameters were examined: prothrombin time, activated partial thromboplastin time.
Clinical Chemistry	The following parameters were examined: glucose, urea nitrogen, creatinine, total protein, albumin, globulin, albumin/globulin ratio, cholesterol, total bilirubin, alanine aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, aspartate aminotransferase, calcium, inorganic phosphorus, sodium, potassium, chloride, triglycerides.
Necropsy	After at least 14 days of treatment, all rats were submitted to gross pathological examination.
Gross Pathology	The gross pathological examination included an examination of the external features of the carcass; external body orifices; the abdominal, thoracic, and cranial cavities; organs; and tissues. The following tissues (when present) were collected from each animal: Adrenal gland (2), brain, epididymis (2), heart, kidney (2), liver, lung with large bronchi, ovary (2), pituitary gland, prostate, seminal vesicle (with coagulating gland) (2), spleen, testis (2), thymus, thyroid with parathyroid (2), uterus.

Organ Weights	Organs weights of all sacrificed rats were determined. Paired organs were hereby weighed together. The following organs (when present) were weighed: adrenal (2), pituitary gland, brain, prostate, epididymis (2), seminal vesicle (with coagulating gland) (2), heart, spleen, kidney (2), testis (2), liver, thymus, lung, thyroid (with parathyroid) (2), ovary (2), uterus. Absolute and relative (organ-body weight ratio; percentage) organ weights were reported.
Histopathology	Microscopic evaluation of the collected was deemed unnecessary.

Statistical evaluation

BCS-AB10736 treated-groups were compared to the control group. Continuous data (e.g., body weight, food consumption, clinical chemistry, haematology, etc.) that were examined statistically were evaluated for equality or homogeneity of variance using Bartlett's Test. Group means were analysed by a one-way variance analysis (ANOVA) followed by Dunnett's Test. Alternatively, continuous data (e.g., clinical chemistry, haematology, organ and terminal body weight, etc.) determined to be nonhomogeneous, were analysed using a Kruskal-Wallis Analysis of Variance followed by a pairwise Mann-Whitney U Test. Frequency data (i.e., clinical observational incidence, etc.) that were examined statistically were evaluated using the Fisher Exact Tests. For the Bartlett Test, a probability (p) level ≤ 0.001 was considered significant. For all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. Statistical analyses described above were performed using DATATOX software, Version rC.10.

For the purpose of data interpretation, statistical significance was not automatically considered to imply toxicological significance. Conversely, the absence of a statistically significant comparison was not considered to imply the lack of a biologically important effect.

II. RESULTS AND DISCUSSION

A. MORTALITY

All animals survived until scheduled sacrifice.

B. CLINICAL OBSERVATIONS

With exception of spilling behaviour (spilling feed), clinical signs of toxicity attributable to the test substance were not observed. An increased incidence of spilling behaviour was noted in 800, 8000 and 16,000 ppm animals, primarily on study Day 7. There was no clear increase in the incidence of spilling with dose (number of animals, number of incidences), although the effect did appear to be more persistent in females (2 of the 5 animals in the 8000 and 16,000 ppm groups spilled on Day 7 and 14). Given the profile of spilling activity, the length of study, and the relatively small group size, this effect does appear to be related to the test substance.

C. BODY WEIGHT

Marked changes in body weight (and decreased body weight gain) attributable to the test substance were noted in 8000 and 16,000 ppm animals. Based on final body weight (treated relative to control), decreases of 15 and 22% were noted in 8000 ppm males and females, and 32 and 30% in 16,000 ppm males and females, respectively. Although the final body weights measured on study Day 14 were influenced by fasting, the degree of body weight loss noted in 8000 and 16,000 ppm animals was marked given the relatively short in-life phase.

Table 5.8-1: Average body weight and body weight gain

Dose level [mg/kg bw/day]	Body weight [g] (% of control)			Cumulative body weight gain [g]	
Male					
	Day 0	**Day 7**	**Day 14**	**Day 7**	**Day 14**
Control	265.7	298.1	294.8	32.5	29.1
800	257.6 (97)	278.8 (94)	271.4 (92)	21.2	13.8
8000	256.6 (97)	244.8* (82)	234.9* (80)	11.8*	-21.7*
16,000	263.3 (99)	230.1* (77)	220.7* (75)	-33.2*	-42.5*
Female					
	Day 0	**Day 7**	**Day 14**	**Day 7**	**Day 14**
Control	190.7	205.6	193.2	14.9	2.5
800	193.5 (101)	198.9 (97)	187.8 (97)	5.5*	-5.7*
8000	195.0 (102)	175.0* (85)	164.2* (85)	-20.0*	-30.8*
16,000	191.1 (100)	161.9* (79)	144.4* (75)	-29.1*	-46.7*

* $p < 0.05$

D. FOOD CONSUMPTION AND TEST SUBSTANCE INTAKE

Food consumption was assessed for each treated group in terms of both grams consumed per animal/day and grams consumed per kg bw/day, compared to controls. Food consumption data were somewhat erratic in a few cases (likely due to spilling) but did not appear to be affected in either sex at any dose tested. Although there is an indication of decreased food consumption during the first week of the study, a clear conclusion cannot be drawn due to the incidence of spilling. Note: Using specifically defined criteria, food consumption data were corrected to account for misleading indications of food intake (excessive spillage).

The mean daily intake of the test substance (mg BCS-AB10736/kg bw/day), calculated from feed consumption and body weight, for animals administered the test substance for 14 days at nominal concentrations of 800, 8000 and 16,000 ppm, respectively, is presented in the table below:

Table 5.8-2: Active ingredient intake

Dose level	Control	800 ppm	8000 ppm	16,000 ppm
Dietary concentration [ppm]	0	800	8000	16,000
Males				
Mean food consumption [g/kg/bw]	75.7	80.4	80.2	69.5
Dose [mg/kg bw/day]	0	64.3	642	1112
Females				
Mean food consumption [g/kg/bw]	85.8	75.7	99.8	84.0
Dose [mg/kg bw/day]	0	60.5	799	1344

E. HAEMATOLOGY AND CLINICAL CHEMISTRY

Changes in the haematology/coagulation profile included decreased white blood cell and absolute lymphocyte counts at 16,000 ppm males; decreased absolute eosinophil counts at 8000 and 16,000 ppm females; increased erythrocyte counts, haemoglobin, and haematocrit in 8000 ppm males and in both sexes at 16,000 ppm; and decreased reticulocyte counts at 8000 ppm males and in both sexes at 16,000 ppm. Increased prothrombin time was also noted in 16,000 ppm females. It is unclear if these changes are a direct effect of the test substance, and may reflect a degree of dehydration.

Changes in the clinical chemistry profile attributable to the test substance were noted in 8000 and/or 16,000 ppm animals and included increased urea nitrogen in 8000 and 16,000 ppm females (slight increase in 16,000 ppm males), increased glucose in 16,000 ppm females, and increased cholesterol in 8000 ppm and 16,000 ppm males and females. Decreased triglyceride concentration was noted in 8000 ppm males and in both sexes at 16,000 ppm. It is likely that some of the changes in the clinical chemistry profile (increase in total protein and albumin at 8000 and 16,000 ppm females; increase in albumin: globulin at 16,000 ppm females) were the result of or exacerbated by the marked decrease in body weight noted in the same animals and/or dehydration.

F. NECROPSY

Gross Pathology
Gross lesions attributable to the test substance were noted in 3/5 16,000 ppm males and included reduced prostate and seminal vesicle size. These findings correlate with reduced organ weight.

Organ Weights
Numerous organ weight changes attributable to the test substance were noted, primarily in 8000 and 16,000 ppm animals. However, given the magnitude of the body weight decrease noted in these animals, the changes in the organ weight profile are likely secondary to the marked changes in the body weight, and not a direct effect.

III. CONCLUSION

Through 14 days of continuous dietary exposure to the test substance, the toxicological response of the rat was principally characterized by decreased body weight, as well as changes in the haematology, serum chemistry, and organ weight profiles. Based on these findings, dietary concentrations of 60, 600, and 6000 ppm were selected for a follow up 90 day toxicity study in the rat.

Report:	[redacted]; [redacted]; 2013-M-461866-01
Title:	Technical grade BCS-AB10736: A subchronic toxicity testing study in the wistar rat
Report No:	11-S72-VS
Document No:	M-461866-01-1
Guidelines:	**OPPTS Guideline No. 870.3100: 90-Day Oral Toxicity in Rodents** **OECD Guideline No. 408 Subchronic Oral Toxicity - Rodent: 90-day Study** **MAFF Guideline 59 NohSan No. 4200 Subchronic Oral Toxicity Study**
Deviations:	none
GLP/GEP:	**yes**

Executive Summary
The subchronic toxicity of BCS-AB10736 was evaluated in a 90 day repeated dose toxicity study. 10 Wistar Crl:WI(Han) rats/sex/dose (one control and three treated groups) were administered BCS-AB10736 continuously in the diet at concentrations of 60, 600, and 6000 ppm for at least 90 days (control animals received plain diet). Examined parameters included: clinical observations, mortality and

moribundity checks, body weights, food consumption, eye exams, serum chemistry, haematology, coagulation, urinalysis, gross pathology, organ weights, and microscopic pathology.

All animals survived until scheduled sacrifice, and clinical observations attributable to the test substance were not observed.

The actual mean daily intake of the test substance was calculated on the basis of concentration analysis of the diets together with the animals' body weights and food consumption. The doses (mg/kg bw/day) over 13 weeks for dietary concentrations of 60, 600, and 6000 ppm were determined to amount 3.3, 36.5, and 337 for males and 4.0, 44.6, and 424 for females.

Food consumption was decreased during the first week of the study (likely due to an initial palatability problem). By Week 2, food consumption on a per animal basis had increased, but generally remained decreased relative to controls throughout the in-life phase of the study (more so in males than females). On a per body weight basis, treated animals consumed similar amounts of food, relative to controls, indicating that utilization was not affected.

Changes in body weight (and decreased body weight gain) attributable to the test substance were noted in both sexes at the high dose (6000 ppm). By the end of the in-life phase (Day 91), body weights were decreased 17% in both males and females (relative to controls).

No test material related adverse effects were noted in ophthalmologic examination, in the haematology/coagulation profile, in the clinical chemistry profile or urinalysis profile.

At necropsy, no gross lesions attributable to the test substance were observed. Organ weight changes directly attributable to the test substance were not observed in this study. A number of organ weights were changed relative to controls; however these changes were consistent with an effect secondary to relatively marked changes in body weight, and not a direct effect. Microscopic findings attributable to the test substance were observed in the liver. However, the effects noted ("Hypertrophy, hepatocellular, centrilobular and/or midzonal") were of relatively mild severity. Together with the lack of other effects, these liver effects were considered to represent an adaptive response of the liver to the test substance.

Based on the observations of this study, NOAEL values of 36.5 and 44.6 mg BCS-AB10736/kg bw/day for male and female rats, respectively, can be deduced.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	BCS-AB10736 (4-methyl-5-propoxy-2,4-dihydro-3H-1,2,4-triazol-3-one)
Description:	White crystals
Lot/Batch #:	NLL 5797-6-5
Expiration:	2012-05-20
Purity:	99.0%
Stability of test compound:	Not reported.

2. Vehicle and/ or positive control: None (dry-mix)

3. Test animals:

Species:	Rat
Strain:	Wistar: Crl:WI(Han)
Source:	[redacted]. ([redacted], NC)

Age:	Approximately 9 weeks at initiation of exposure
Sex:	Male/female
Weight at dosing:	215–276 g (males) 155–201 g (females)
Acclimation period:	7 days: November 14, 2011 (receipt)–November 21, 2011 (released for study)
Diet/Food:	Purina Mills Certified Rodent Diet 5002 in "meal" form, ad libitum. Grated feeders were used for all animals.
Water:	Tap water (municipal water supply of Kansas City, MO) via pressure-activated water nipples and/or water bottles, ad libitum.
Housing:	Individually in suspended stainless steel cages, with deotized (sanitized) cage board in the bedding trays.
Environmental conditions:	Temperature: 18 - 26°C Humidity: 30 - 70% Air changes: min. 15.92/hour 12 hours light/dark cycle

B. STUDY DESIGN AND METHODS

In life dates
2011-11-14 through 2012-02-28

Animal assignment and treatment
Animal assignment and dose groups
Following seven days of quarantine/acclimation, all rats were randomly assigned to dose groups using a weight stratification-based computer program (DATATOX, Instem Computer Systems, Version rC10, P/C, [redacted], England). The following dose groups were employed:

Test Group	Conc. in Diet [ppm]	Target dose [mg/kg bw/day]	Male	Female
Control	0	0	10	10
Low dose group	60	4	10	10
Mid dose group	600	40	10	10
High dose group	6000	400	10	10

Dietary levels for this study (60, 600, and 6000 ppm) were selected based on the results of a 14 day repeat-dose toxicity study in Wistar rats with BCS-AB10736.

Route of Administration
The possible route of human exposure to BCS-AB10736 is via ingestion of foodstuffs that might contain low residues of the test substance. Thus, formulation with feed was an appropriate route of administration to further establish the toxicological profile of the test substance in a given test species.

Diet Preparation and Analysis
The test substance was mixed directly with the feed. Adjustments were not made for percentage purity of less than 100%. Treated diet was mixed at room temperature; the entire batch of test substance (or aliquots from the original batch) was transferred to the mixing area. The control test diet was prepared the same as the 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. A single batch of each admixture was

prepared for each treatment group and stored under freezer conditions until presented to the animals (every two weeks). The homogeneity and stability of BCS-AB10736 when mixed in the dietary carrier was confirmed prior to study start. The concentration of BCS-AB10736 in the various test diets was analytically verified from samples collected at preparation for batches to be used during Weeks 1/2 and 3/4, and at approximately 1 ± 0.5 month of the experimental midpoint and termination of the in-life phase of the study.

Details on oral exposure

Duration of treatment/exposure	At least 90 days.
Frequency of treatment	Daily.

Examinations

Clinical Signs - Mortality and Moribundity/General Daily Observations	Each animal was observed for mortality, abnormalities, and signs of pain and distress at least once daily (nominally twice daily during the normal work week and once on weekends and holidays).
Clinical Signs - Detailed Observations	Animals were submitted to detailed clinical observations at least once during the predose phase (e.g., at randomization), before dosing or immediately after dosing on Day 0, and weekly thereafter. Examinations (conducted outside of the home cage) included evaluation of external surface areas (visual inspection and palpation for externally detectable "masses"), orifices, posture, respiration, and excretory products. During the examination, animals were observed for common signs relating to neurological effects, including, but not limited to, piloerection, respiratory abnormalities, posture, involuntary motor movements (clonic or tonic), stereotypy, bizarre behavior, gait abnormalities, and vocalizations. This included observation of each individual animal in the open field (standard arena) for approximately 30–60 seconds during Weeks 1 and monthly thereafter. Abnormal findings or an indication of normal were recorded.
Body Weights	Body weights were determined at least once during the predose phase, before initiation of exposure on Day 0, and weekly thereafter (terminal body weight was considered the second body weight during the last week of the study).
Food Consumption	Food consumption was measured weekly (quantitative) during the exposure phase. Using specifically defined criteria, food consumption data were corrected, as conditions dictate, to account for misleading indications of food intake (i.e., excessive spillage, clogged feeders, etc.).

Ophthalmic Evaluation	Ophthalmic evaluation was conducted once during the predose phase (all animals; prior to randomization) and during Week 13 of the dosing phase (control and high-dose only). The anterior portions of the rat's eyes were examined using a Finnoff trans-illuminator. The retina was examined using an indirect ophthalmoscope. The eyes were dilated with a mydriatic agent (MYDRIACYL® 1% containing 1.0% w/v Tropicamide) prior to retinal examination.
Clinical Pathology	All surviving animals were examined for clinical pathology on the day of scheduled sacrifice. Rats were fasted overnight (~16–19 h) for scheduled blood collections. Blood was collected via the orbital sinus (rats were anesthetized with isoflurane), and approximately 500 µL for haematology, 1000 µL for serum chemistry and 1800 µL for coagulation were collected. The anticoagulants were sodium citrate for coagulation tests and K_2EDTA for haematology tests. Samples for clinical chemistry were collected without anticoagulant. Urine was collected at room temperature during the overnight period (animals were housed in cages fitted with urine collection trays).
Haematology	The following parameters were examined: total red blood cell (erythrocyte) count, red cell distribution width, haemoglobin, haematocrit, mean corpuscular volume, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, total platelet count, haemoglobin distribution width, total white blood cell (leukocyte) count, differential blood cell count, blood smear, reticulocyte count, cytologic morphology.
Coagulation	The following parameters were examined: prothrombin time, activated partial thromboplastin time.
Clinical Chemistry	The following parameters were examined: glucose, urea nitrogen, creatinine, total protein, albumin, globulin, albumin/globulin ratio, cholesterol, total bilirubin, alanine aminotransferase, alkaline phosphatase, gamma-glutamyltransferase, aspartate aminotransferase, calcium, inorganic phosphorus, sodium, potassium, chloride, triglycerides.
Urinalysis	The following parameters were examined: clarity, colour, specific gravity, blood, ketones, protein, microscopic examination of sediment, urobilinogen, bilirubin, glucose, pH, leukocytes, nitrites.
Necropsy	After at least 90 days of treatment, all rats were submitted to gross pathological examination.

Gross Pathology	The gross pathological examination included an examination of the external features of the carcass; external body orifices; the abdominal, thoracic, and cranial cavities; organs; and tissues. The following tissues (when present) were collected from each animal: Adrenal gland (2), brain, epididymis (2), heart, kidney (2), liver, lung with large bronchi, ovary (2), pituitary gland, prostate, seminal vesicle (with coagulating gland) (2), spleen, testis (2), thymus, thyroid with parathyroid (2), uterus.
Organ Weights	Organ weights of all sacrificed rats were determined. Paired organs were hereby weighed together. The following organs (when present) were weighed: adrenal (2), pituitary gland, brain, prostate, epididymis (2), seminal vesicle (with coagulating gland) (2), heart, spleen, kidney (2), testis (2), liver, thymus, lung, thyroid (with parathyroid) (2), ovary (2), uterus. Absolute and relative (organ-body weight ratio; percentage) organ weights were reported.
Histopathology	Tissues from each animal in the control and high dose groups were examined microscopically. Additionally, gross lesions from all animals were examined. The liver was identified as the only target organ and was also examined microscopically from low and mid dose animals to identify the NOAEL.

Statistical evaluation

BCS-AB10736 treated-groups were compared to the control group. Continuous data (e.g., body weight, food consumption, clinical chemistry, haematology, etc.) that were examined statistically were evaluated for equality or homogeneity of variance using Bartlett's Test. Group means were analysed by a one-way variance analysis (ANOVA) followed by Dunnett's Test. Alternatively, continuous data (e.g., clinical chemistry, haematology, organ and terminal body weight, etc.) determined to be nonhomogeneous, were analysed using a Kruskal-Wallis Analysis of Variance followed by a pairwise Mann-Whitney U Test. Frequency data (i.e., clinical observational incidence, etc.) that were examined statistically were evaluated using the Fisher Exact Tests. For the Bartlett Test, a probability (p) level ≤ 0.001 was considered significant. For all other statistical tests, differences with p values ≤ 0.05 were considered statistically significant. Statistical analyses described above were performed using DATATOX software, Version rC.10.

For the purpose of data interpretation, statistical significance was not automatically considered to imply toxicological significance. Conversely, the absence of a statistically significant comparison was not considered to imply the lack of a biologically important effect.

II. RESULTS AND DISCUSSION

A. MORTALITY

No mortality occurred throughout the study period.

B. CLINICAL OBSERVATIONS

Clinical signs of toxicity attributable to the test substance were not observed throughout the study period.

C. BODY WEIGHT

Changes in body weight (and decreased body weight gain) attributable to the test substance were noted in both sexes at the high dose (6000 ppm). By the end of the in-life phase (Day 91), body weights were decreased 17% in both males and females (relative to controls).

Table 5.8-3: Average body weight and body weight gain

Dose level [mg/kg bw/day]	Body weight [g]			Cumulative body weight gain [g]	
Male					
	Day 0	Day 28	Day 91	Day 28	Day 91
Control	248.6	333.6	393.1	85.0	144.5
4	246.6	335.6	394.9	89.0	148.3
40	249.4	334.7	395.4	85.3	146.0
400	248.7	287.2*	324.7*	38.5*	76.0*
Female					
	Day 0	Day 28	Day 91	Day 28	Day 91
Control	179.2	219.8	233.7	40.6	54.5
4	178.4	217.3	233.0	38.8	54.5
40	174.0	207.4	224.6	33.4	50.6
400	176.8	186.4*	194.9*	9.6*	18.1*

* $p < 0.05$

D. FOOD CONSUMPTION AND TEST SUBSTANCE INTAKE

Food consumption was assessed for each treated group in terms of both grams consumed per animal/day and grams consumed per kg bw/day, compared to controls. Based on these criteria, changes in food consumption attributable to the test substance were noted in both sexes at the high-dose. Food consumption was decreased during the first week of the study. It is not clear why the animals consumed less; however, it is likely due to an initial palatability problem (i.e., the animals did not want to eat the dietary admixture). By Week 2, food consumption on a per animal basis had increased, but generally remained decreased relative to controls throughout the in-life phase of the study (more so in males than in females). On a per body weight basis, treated animals consumed similar amounts of food, relative to controls, indicating that utilization was not affected (i.e., lighter animals eat less food).

The mean daily intake of the test substance (mg BCS-AB10736/kg bw/day), calculated from feed consumption and body weight, for animals administered the test substance for 13 weeks at nominal concentrations of 60, 600, or 6000 ppm, respectively, is presented in the table below:

Table 5.8-4: Active ingredient intake

Dose Level	Control	60 ppm	600 ppm	6000 ppm
Dietary Concentration [ppm]	0	52.3	573	5504
Males				
Mean Food Consumption [g/kg/bw]	62.3	62.8	63.7	61.2
Dose [mg/kg bw/day]	0	3.3 ± 0.6	36.8 ± 6.8	337 ± 55
Females				
Mean Food Consumption [g/kg/bw]	74.8	75.5	77.8	77.1
Dose [mg/kg bw/day]	0	4.0 ± 0.6	44.6 ± 6.0	424 ± 73

E. OPHTHALMOSCOPIC EXAMINATION

Ophthalmologic findings attributable to exposure to the test substance were not observed during the post-exposure examination.

F. HAEMATOLOGY AND CLINICAL CHEMISTRY

Changes in the haematology/coagulation profile attributable to the test substance were not observed in this study.

Changes in the clinical chemistry profile attributable to the test substance were observed in high dose males and females and included increased urea nitrogen, cholesterol, and phosphorus in both sexes, and increased calcium in males only. All of the changes described above were considered to be non-adverse.

G. URINALYSIS

Changes in the urinalysis profile attributable to the test substance were not observed in this study.

H. NECROPSY

Gross Pathology
Gross lesions attributable to the test substance were not observed in this study.

Organ Weights
Organ weight changes directly attributable to the test substance were not observed in this study. A number of organ weight changes were noted in high dose animals. Decreases in absolute organ weights were noted in the thymus, spleen, lung, pituitary, liver, and adrenals in males and/or females. However, in every case, the changes in the organ weight profile are consistent with an effect secondary to relatively marked changes in body weight, and not a direct effect. In most cases, absolute weights were decreased with no corresponding decrease in relative weight. For a few organs (liver, kidney, testes, thyroid, and brain), an increase in relative weight change was noted. This is a common result of the maintenance of organs (priority) in the face of decreasing body weight, with the exception of the liver, which responds to the subchronic challenge by the test substance with an increase in hepatocyte size, which correlates with higher weight. Moreover, with the exception of the liver, microscopic findings attributable to the test substance were not observed in any other organ.

Histopathology
Microscopic findings attributable to the test substance were observed in the liver of high dose animals. Minimal to slight (or mild) centrilobular and/or midzonal hypertrophy of the liver was noted in high dose males (9/10) and females (6/10). The lesion was coded as “Hypertrophy, hepatocellular, centrilobular and/or midzonal” and was characterized by enlarged hepatocytes primarily involving the cytoplasm of centrilobular areas and also occasionally involving the midzonal areas of the liver. The cytoplasmic

appearance of hepatocytes varied from granular or intensively eosinophilic or pale. Given the relatively mild severity of the lesion and the lack of other effects indicating otherwise, these liver effects likely represent an adaptive response of the liver to the test substance.

III. CONCLUSION

Through approximately 13 weeks of continuous dietary exposure to the test substance, the toxicological response of the rat was principally characterized in both sexes by decreased body weight (adverse), as well as structural and/or functional changes in the liver (adaptive, non-adverse) at dietary concentrations of 6000 ppm.
Based on a lack of a similar adverse test substance-related effects at 600 ppm, a systemic subchronic toxicity NOAEL of 36.5 mg BCS-AB10736/kg bw/day was established for the rat (specifically, 36.5 and 44.6 mg BCS-AB10736/kg bw/day for male and female rats, respectively).

Report:	[redacted];[redacted];2012;M-461843-01
Title:	Mutagenicity study of BCS-AB10736 in the Salmonella typhimurium reverse mutation assay (in vitro)
Report No:	LPT 28509
Document No:	M-461843-01-1
Guidelines:	**According to Council Regulation (EC) no. 440/2008 part B.13/14 and OECD Guideline 471**
Deviations:	None
GLP/GEP:	**yes**

Executive Summary

BCS-AB10736 was examined in the 5 *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 in two independent experiments, each carried out without and with metabolic activation (a microsomal preparation derived from Aroclor 1254-induced rat liver). The first experiment was carried out as a plate incorporation test and the second as a preincubation test.
In a preliminary test, BCS-AB10736 was examined in two preliminary cytotoxicity tests (plate incorporation test without and with metabolic activation) in tester strain TA 100. Ten concentrations ranging from 0.316 to 5000 µg/plate were tested. No signs of cytotoxicity were noted in the experiments without and with metabolic activation up to the top concentration of 5000 µg/plate. Hence, 5000 µg/plate was chosen as top concentration for the main study in the plate incorporation test and in the preincubation test.
In the main study, six concentrations ranging from 31.6 to 5000 µg/plate were employed in the plate incorporation test and in the preincubation test, each carried out without and with metabolic activation. No signs of cytotoxicity were noted in the plate incorporation test without and with metabolic activation up to the top concentration of 5000 µg/plate in all test strains. No increase in revertant colony numbers as compared with control counts was observed for BCS-AB10736, tested up to a concentration of 5000 µg/plate, in any of the 5 test strains of the two independent experiments without and with metabolic activation, respectively (plate incorporation and preincubation test). The positive control items showed a significant increase in the number of revertant colonies of the respective test strain and confirmed the validity of the test conditions and the sensitivity of the test system. In conclusion, BCS-AB10736 is not mutagenic to the *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 up to the limit concentration, neither in the presence nor absence of a metabolic activation system under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	BCS-AB10736 (4-methyl-5-propoxy-2,4-dihydro-3H-1,2,4-triazol-3-one)
Description:	White, crystals
Lot/Batch #:	NLL 5797-6-5
Purity:	99.0%
Isomer distribution	Not reported
Stability of test compound:	Not reported

2. Vehicle and/ or positive control:

Vehicle: Dimethylsulfoxide (DMSO)
Positive controls:
Sodium azide (NaN_3), TA 100/TA 1535, -S9
2-Nitro-fluorene (2-NF), TA 98, -S9
9-Amino-acridine (9-AA), TA 1537, -S9
Methyl methane sulfonate (MMS), TA 102, -S9
2-Aminoanthracene (2-AA), TA 98/TA 102/TA 1537, +S9
Cyclophosphamide (CP), TA 100/TA 1535, +S9

3. Test system:

Organism:	*Salmonella typhimurium*
Strains:	TA 1535, TA 100, TA 1537, TA 98, and TA 102
Source:	[redacted]
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	– S9 and +S9: 31.6, 100, 316, 1000, 3160 and 5000 µg/plate
Pre-incubation period:	20 min
Exposure duration:	48 - 72 h

B: STUDY DESIGN AND METHODS

Experimental dates
2012-04-26 through 2012-06-24

Experimental procedure

Preliminary study

BCS-AB10736 was examined in two preliminary cytotoxicity tests (plate incorporation test without and with metabolic activation) in tester strain TA 100. Cytotoxicity was evaluated by means of scarce background lawn and reduction of the number of revertants by more than 50%. Based on the outcome of this test, the doses for the main study were selected.

Main study

Two independent mutagenicity experiments were carried out with BCS-AB10736 in the tester strains TA 1535, TA 100, TA 102, TA 98 and TA 1537 each without and with metabolic activation. Six concentrations ranging from 31.6 to 5000 µg/plate were employed, and each experiment consisted of 3 plates/concentration. Appropriate positive and solvent (DMSO) controls were included into the test to confirm its sensitivity.

In the plate incorporation test, 100 mL sterile top agar was mixed with 10 mL of a sterile solution of 0.5 mM L-histidine HCl/0.5 mM biotin, 0.1 mL Salmonella cell suspension (containing approximately 108 viable cells in the late exponential or early stationary phase) and 0.1 mL of the test item solution or solvent or positive control solution, respectively. In the experiment with metabolic activation, 0.5 mL of S9 mix was added, whereas in the experiments without metabolic activation, 0.5 mL of phosphate buffer was added. After pouring onto a minimal glucose agar plate and solidification, the plates were inverted and placed in a dark 37°C incubator for 48 to 72 hours. The revertant colonies on the test plates and on the control plates were counted with a colony counter, and the presence of the background lawn on all plates was confirmed.

The independent repeat was performed as pre-incubation in a water bath at 37°C for 20 minutes prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Tubes were aerated during preincubation by using a shaker. The remaining steps were the same as described for the plate incorporation method.

The following doses of BCS-AB10736 were evaluated in the main study: 31.6, 100, 316, 1000, 3160 and 5000 µg/plate.

The following doses were used for the positive controls:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
NaN_3	10	*aqua ad iniectabilia*	TA 1535/TA 100, -S9
2-NF	10	DMSO	TA 98, -S9
9-AA	100	ethanol, abs.	TA 1537, -S9
MMS	100	DMSO	TA 102, -S9
2-AA	2	DMSO	TA 98/ TA 102/TA 1537, +S9
CP	1500	*aqua ad iniectabilia*	TA 100/TA 1535, +S9

Quality criteria

Quality criteria are tested by [redacted], Germany. The genotypes of the test strains are regularly confirmed in the following way:

- Histidine and biotin requirement ((his-) (bio-)):

  Each of the strains is streaked onto two Minimal glucose agar plate (Minimal Glucose Agar medium E) in the following way:

  1) with 0.1 mM L-histidine and 0.5 mM biotin (100 µL/each)
  2) with 0.5 mM biotin (100 µL/each)

  After incubation at 37°C for 24 hours, none of the strains should grow on Plate 2; all strains should show excessive growth on Plate 1.
- (rfa-) deep rough character:

10 µL of 0.1% crystal violet applied with a paper disc should give zones of inhibition in the test strains after incubation at 37°C for 24 hours.

- UV-sensitivity (uvr B-):
  Plates are covered partly with black paper and placed under germicidal UV-irradiation. After incubation at 37°C for 24 hours TA 100 should grow only under the covered portion of each plate. TA 102 should also grow under the uncovered area.
- Ampicillin-resistance (pKM 101):
  0.8 mg ampicillin/plate is placed onto plates seeded with bacteria: Absence of zones of inhibition around the discs indicates resistance to ampicillin (TA 100 and TA 102).
- Ampicillin- and tetracycline-resistance
  The pAQ1 strain (TA 102) is tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates.

Assessment Criteria

A test item is considered to show a positive response if

- The number of revertants is significantly increased ($p \leq 0.05$, U-test according to MANN and WHITNEY) compared with the solvent control to at least 2-fold of the solvent control in both independent experiments;
- In addition, a significant ($p \leq 0.05$) concentration (log value)-related effect (Spearman's rank correlation coefficient) is observed;
- Positive results have to be reproducible and the histidine independence of the revertants has to be confirmed by streaking random samples on histidine-free agar plates.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary Test

No signs of cytotoxicity were noted in the experiments without and with metabolic activation up to the top concentration of 5000 µg BCS-AB10736/plate. Hence, 5000 µg BCS-AB10736/plate were chosen as top concentration for the main study.

Main study:

No signs of cytotoxicity were observed without and with metabolic activation up to the limit concentration of 5000 µg/plate in all tester strains.

No increase in revertant colony numbers as compared with control counts was observed for BCS-AB10736, tested up to the limit concentration of 5000 µg/plate, in any of the 5 tester strains in two independent experiments without and with metabolic activation, respectively (plate incorporation and preincubation test).

A summary of the results is given in Table 5.8-5 through Table 5.8-8.

Table 5.8-5: Plate incorporation method - without metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
–	0	21.7 ± 6.1	126.0 ± 12.8	298.3 ± 16.7	34.0 ± 0.0	7.3 ± 3.5
–	31.6	20.3 ± 1.5	118.7 ± 1.5	269.0 ± 4.6	36.3 ± 0.6	3.3 ± 0.6
–	100	15.0 ± 6.1	120.3 ± 14.6	293.3 ± 6.8	35.0 ± 1.7	7.7 ± 3.2
–	316	15.0 ± 3.0	125.7 ± 7.5	271.3 ± 8.4	37.7 ± 0.6	7.3 ± 1.5
–	1000	20.7 ± 4.0	134.3 ± 9.5	270.7 ± 6.4	35.0 ± 1.7	2.7 ± 0.6
–	3160	20.3 ± 1.5	105.3 ± 5.5	285.0 ± 5.6	33.7 ± 8.5	5.0 ± 2.6
–	5000	16.0 ± 3.6	129.7 ± 1.5	273.3 ± 7.4	31.0 ± 6.6	4.0 ± 2.6
Pos controls -S9	Name	NaN_3	NaN_3	MMS	2-NF	9-AA
	Conc. [µg/plate]	10	10	100	10	100
	Revertants per plate	247.3 ± 4.9	951.3 ± 14.0	1125.7 ± 20.1	247.7 ± 5.1	180.0 ± 3.6

Table 5.8-6: Pre-incubation method - without metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
–	0	18.0 ± 2.6	121.7 ± 2.3	295.0 ± 28.2	39.7 ± 8.1	4.7 ± 2.1
–	31.6	20.3 ± 6.8	119.7 ± 3.1	305.3 ± 4.0	31.7 ± 5.1	6.3 ± 4.2
–	100	27.0 ± 2.0	118.7 ± 7.2	283.7 ± 16.2	29.7 ± 6.8	3.7 ± 2.3
–	316	22.3 ± 8.5	134.0 ± 6.1	293.3± 10.2	37.3 ± 12.4	2.0 ± 1.0
–	1000	22.0 ± 5.0	123.7 ± 10.1	292.7 ± 22.2	40.3 ± 7.0	6.0 ± 3.6
–	3160	21.7 ± 1.5	130.0 ± 13.1	279.0 ± 6.2	38.7 ± 13.8	7.0 ± 3.6
–	5000	20.0 ± 1.7	112.7 ± 11.0	255.0 ± 3.5	36.3 ± 6.7	5.0 ± 4.4
Pos controls -S9	Name	NaN_3	NaN_3	MMS	2-NF	9-AA
	Conc. [µg/plate]	10	10	100	10	100
	Revertants per plate	246.3 ± 6.1	946.0 ± 12.0	954.7 ± 29.0	266.0 ± 6.2	173.3 ± 2.9

Table 5.8-7: Plate incorporation method - with metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
+	0	17.0 ± 6.1	122.7 ± 4.6	298.3 ± 4.5	35.0 ± 1.7	6.7 ± 0.6
+	31.6	20.3 ± 2.3	161.3 ± 10.1	286.3 ± 0.6	37.0 ± 2.6	4.0 ± 0.0
+	100	21.7 ± 8.4	162.7 ± 11.6	300.7 ± 5.1	27.7 ± 5.7	8.0 ± 2.6
+	316	24.0 ± 3.0	164.0 ± 8.9	253.7 ± 3.8	32.3 ± 2.9	8.0 ± 1.0
+	1000	19.7 ± 5.5	129.0 ± 40.0	254.7 ± 5.5	29.3 ± 4.5	8.0 ± 1.0
+	3160	20.0 ± 2.6	112.7 ± 3.8	269.7 ± 5.0	35.7 ± 1.5	8.7 ± 1.2
+	5000	19.7 ± 4.0	120.0 ± 4.0	269.3 ± 6.7	36.7 ± 1.5	7.0 ± 3.0
Pos controls +S9	Name	CP	CP	2-AA	2-AA	2-AA
	Conc. [µg/plate]	1500	1500	2	2	2
	Revertants per plate	235.0 ± 7.8	954.3 ± 18.6	945.0 ± 15.5	250.3 ± 9.1	235.7 ± 8.3

Table 5.8-8: Pre-incubation method - with metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
+	0	25.7 ± 4.2	110.7 ± 8.5	284.0 ± 3.0	42.7 ± 0.6	6.7 ± 4.2
+	31.6	18.7 ± 5.0	101.0 ± 1.7	270.3 ± 2.5	43.7 ± 0.6	3.7 ± 2.1
+	100	20.0 ± 5.0	106.3 ± 4.9	269.7 ± 4.9	45.7 ± 1.5	4.3 ± 0.6
+	316	17.3 ± 6.7	126.3 ± 7.8	275.3 ± 3.5	30.0 ± 13.9	6.7 ± 1.5
+	1000	21.0 ± 8.7	121.7 ± 7.4	273.0 ± 7.8	28.3 ± 12.7	4.3 ± 3.1
+	3160	16.3 ± 4.2	105.3 ± 5.8	283.7 ± 9.8	35.7 ± 12.7	4.0 ± 3.6
+	5000	24.7 ± 1.2	113.0 ± 5.6	289.7 ± 2.1	37.3 ± 12.4	6.7 ± 4.2
Pos controls +S9	Name	CP	CP	2-AA	2-AA	2-AA
	Conc. [µg/plate]	1500	1500	2	2	2
	Revertants per plate	252.3 ± 7.0	965.0 ± 11.0	928.0 ± 32.6	261.7 ± 1.2	171.3 ± 4.0

III. CONCLUSION

The test substance was not mutagenic in the Salmonella/microsome assay, with and without metabolic activation.

Report:	[redacted];[redacted];2012;M-461844-01
Title:	Mutagenicity study of BCS-AB10736 in mammalian cells (V79) in the in vitro gene mutation assay (HPRT test)
Report No:	LPT 28510
Document No:	M-461844-01-1
Guidelines:	**According to Council Regulation (EC) no. 440/2008 part B.17 and OECD Guideline 476 (1997)**
Deviations:	none
GLP/GEP:	**yes**

Executive Summary

BCS-AB10736 was tested for mutagenic potential in a gene mutation assay in cultured mammalian cells (V79, genetic marker HPRT) both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254-induced animals. The duration of the exposure with the test item was 4 hours or 24 hours in the experiments without S9 mix and 4 hours in the experiments with S9 mix.

The concentrations employed were chosen based on the results of a preliminary cytotoxicity study. In this study no signs of cytotoxicity were noted in the experiments without and with metabolic activation (24 h or 4 h exposure) up to the top concentration of 5000 µg/mL. Hence, 5000 µg/mL was employed as the top concentration for the main mutagenicity test without and with metabolic activation.

In the main study, five concentrations ranging from 312.5 to 5000 µg/mL were selected for the experiments without and with metabolic activation, respectively. In the main study, no signs of cytotoxicity in form of decreased plating efficiency were noted in the experiments in the absence or presence of metabolic activation up to the top concentration of 5000 µg/mL. In experiments both with and without metabolic activation, the mutation frequency of the negative control DMSO was well within the expected range and the mutation frequency of the cultures treated with BCS-AB10736 at concentrations up to 5000 µg/plate were within the normal range of the negative controls. The positive controls EMS (-S9) and DMBA (+S9) caused a pronounced increase in the mutation frequencies, indicating the validity of this test system.

BCS-AB10736 is therefore concluded not to be mutagenic to mammalian cells *in-vitro* under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	BCS-AB10736 (4-methyl-5-propoxy-2,4-dihydro-3H-1,2,4-triazol-3-one)
Description:	White crystals
Lot/Batch #:	NLL 5797-6-5
Purity:	99.0%
Isomer distribution	Not reported.
Stability of test compound:	2012-05-20 (expiry date)

2. Vehicle and/ or positive control:

Vehicle: Dimethylsulfoxide (DMSO)
Positive controls:
Ethyl methanesulfonate (EMS), -S9
9,10-Dimethyl-1,2-benzanthracene (DMBA), +S9

3. Test system:

Organism:	Mammalian cells in culture
Strains:	V79 cells
Source	[redacted], Germany
Media:	The cells were maintained and exposed to the test item in the absence of S9 in DMEM-FCS (Dulbecco's modified Eagle-Medium supplemented with 10% foetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL)). Exposure to the test item in the presence of S9 mix was performed in PBS-HEPES (Dulbecco's phosphate buffered saline (PBS) which additionally contained 20 mM HEPES (N'-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid), pH 7.4)
Properly maintained?	yes
Periodically checked for Mycoplasma contamination?	yes
Periodically "cleansed" against high spontaneous background?	yes
Locus Examined:	hprt locus
Selection agent:	6-thioguanine
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	–S9 and +S9: 312.5, 625, 1250, 2500 and 5000 µg/mL

B: STUDY DESIGN AND METHODS

Experimental dates
2012-04-26 through 2012-07-19

Experimental procedure
Preliminary cytotoxicity test
To determine the cytotoxicity, the same procedure was used as employed for the mutagenicity experiments (see below), except that no mutant selection was carried out. No signs of cytotoxicity were noted in the experiments without and with metabolic activation (24 h or 4 h exposure) up to the limit concentration of 5000 µg/mL. Hence, 5000 µg/mL were employed as the top concentration for the main mutagenicity test without and with metabolic activation.

Main study
Cell treatment: On Day 1 of the experiment, approximately 1,500,000 cells were placed in 30 mL DMEM-FCS per 150 mm diameter dish. On the following day, the cells were exposed to the selected concentrations of the test item. In the absence of S9 mix, the cells were exposed in DMEM-FCS to the test item for 4 hours (Experiment 1) or 24 hours (Experiment 2). In the experiments with S9 mix, the medium was replaced by 18 mL S9 mix and the exposure limited to 4 hours. The negative control was treated with DMSO (the vehicle) in the same way. In addition, the following positive controls were employed:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
EMS	600, 700	DMSO	-S9
DMBA	20, 30	DMSO	+S9

Expression of new genotype: At the end of the exposure period, the cells were trypsinised and a relative plating efficiency (PE_1) was determined for each dose to obtain an accurate measure of the toxic effect of the chemical. Three replicate plates were used with a known number of cells. The remaining cells were re-plated and the culture incubation continued until Day 8 with 30 mL normal DMEM-FCS with one sub-cultivation on Day 5.

Treatment with selecting agent: Following the expression time, the cells were harvested by trypsinisation and re-plated at a density of 1,000,000 cells, in DMEM-FCS containing 6-thioguanine (10 µg/mL) for selection of mutants (5 replicate plates), or at approx. 100 to 150 cells (exact number known) in medium without 6-thioguanine for the estimation of plating efficiencies (PE_2), (3 replicate plates). The plates were fixed and stained after about 8 days (plating efficiency plates) or 12 days (6-thioguanine plates).

Acceptance criteria
Solvent control: As the total number of colonies is normally low and as a single mutation may cause several colonies due to cell division during the expression period, a relatively large variation of the mutation frequency may result. This is especially true, if a low spontaneous mutation frequency is forced by cloning (in order to achieve a high sensitivity of the test).
The historical background mutation frequency in this system has been reported to be 1 to 44 mutants per 106 survivors in non-activation solvent controls and 6 to 46 per 106 survivors in S9 activation solvent controls. The background data obtained at LPT are given at the end of this chapter. The spontaneous mutation frequency may be variable from experiment to experiment, but should normally lie within the above-mentioned range. The positive controls EMS (600 and 700 µg/mL) and DMBA (20 and 30 µg/mL) should cause a 10-fold or greater increase in mutation frequency.
The background mutation frequency at LPT ranges from 1.30 to 38.36 x 10^{-6} cloneable cells for the negative controls. The mutation frequency of the positive controls at LPT ranges from 112.1 to 1708.4 x 10^{-6} cloneable cells for EMS and 130.0 to 2693.3 x 10^{-6} cloneable cells for DMBA.

Assessment Criteria

So far no satisfactory mathematical methods are available for the statistical analysis of mammalian cell mutagenicity experiments such as those performed. However, the following pre-determined descriptive criteria are considered the most useful for interpretation of the results:

- If in both independent experiments solvent and positive controls show results within the norm and if the test item does not increase the mutation frequency 2-fold above the mean of the solvent controls under any condition, or if the mutation frequency is always lower than 40 x 10^{-6} and if at least 1,000,000 cells per condition have been evaluated, the item is considered as negative in the test.
- In case of a dose-dependent increase of the mutation frequency in both independent experiments (at similar concentrations) to at least 2-fold solvent control and at least 40 x 10^{-6} both in the presence and/or absence of S9 mix, the item is considered as positive in the test.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary study

No signs of cytotoxicity were noted in the experiments without and with metabolic activation (24 h or 4 h exposure) up to the top concentration of 5000 µg/mL. Hence, 5000 µg/mL was employed as the top concentration for the main mutagenicity test without and with metabolic activation.

Main study

Cytotoxicity: No signs of cytotoxicity in form of decreased plating efficiency (PE_1) and (PE_2) were noted in the experiments in the absence or presence of metabolic activation up to the top concentration of 5000 µg/mL.

Experiments with metabolic activation: The mutation frequency of the negative control DMSO was 15.69 and 22.62 x 10^{-6} cloneable cells and was therefore well within the expected range. The mutation frequency of the cultures treated with BCS-AB10736 at concentrations of 312.5, 625, 1250, 2500 or 5000 µg/mL culture medium ranged from 13.46 to 32.27 x 10^{-6} cloneable cells and were therefore within the normal range of the negative controls. The positive control EMS caused a pronounced increase in the mutation frequency ranging from 367.47 to 728.35 x 10^{-6} cloneable cells, indicating the validity of this test system.

Experiments with metabolic activation: The mutation frequency of the negative control DMSO was 16.20 and 18.05 x 10^{-6} cloneable cells and was therefore well within the expected range. The mutation frequency of the cultures treated with BCS-AB10736 at concentrations of 312.5, 625, 1250, 2500 or 5000 µg/mL culture medium ranged from 15.34 to 32.82 x 10^{-6} cloneable cells and were therefore within the normal range of the negative controls. The positive control DMBA caused a pronounced increase in the mutation frequency ranging from 141.00 to 309.38 x 10^{-6} cloneable cells, indicating the validity of this test system.

A summary of the results is given in Table 5.8-9 through Table 5.8-12.

Table 5.8-9: Experiment 1 - without metabolic activation – 4 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.59	0.80	3.99	18.05
BCS-AB10736	312.5	0.87	0.62	3.08	32.82

	625	0.60	0.62	3.09	19.42
	1250	0.74	0.55	2.76	27.22
	2500	0.75	0.62	3.11	23.14
	5000	0.48	0.84	4.19	20.77
EMS	600	0.32	0.31	1.54	486.67
	700	0.28	0.28	1.39	469.44

Table 5.8-10: Experiment 1 - with metabolic activation – 4 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.59	0.80	4.03	22.62
BCS-AB10736	312.5	0.69	0.68	3.39	21.84
	625	0.69	0.61	3.06	27.16
	1250	0.65	0.56	2.79	32.22
	2500	0.77	0.61	3.03	30.33
	5000	0.63	0.55	2.74	27.69
DMBA	20	0.15	0.47	2.33	141.00
	30	0.58	0.51	2.56	292.30

Table 5.8-11: Experiment 2 - without metabolic activation – 24 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.61	0.78	3.89	16.20
BCS-AB10736	312.5	0.69	0.80	3.98	15.34
	625	0.61	0.66	3.30	18.48
	1250	0.59	0.70	3.48	19.84
	2500	0.68	0.62	3.14	24.81
	5000	0.56	0.54	2.69	24.17
EMS	600	0.23	0.48	2.41	367.47
	700	0.31	0.22	1.08	728.35

Table 5.8-12: Experiment 2 - with metabolic activation – 4 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.62	0.73	3.63	15.69
BCS-AB10736	312.5	0.76	0.61	3.03	14.48
	625	0.64	0.67	3.34	13.46
	1250	0.73	0.63	3.13	17.23
	2500	.0.75	0.65	3.24	14.18
	5000	0.62	0.62	3.10	14.84
DMBA	20	0.19	0.57	2.84	309.38
	30	0.17	0.54	2.68	301.74

III. CONCLUSION

The test substance was not mutagenic to mammalian cells in the HPRT assay, with and without metabolic activation.

Report:	[redacted];[redacted];2012;M-461845-01
Title:	In vitro assessment of the clastogenic activity of BCS-AB10736 in cultured CHO cells
Report No:	LPT 28511
Document No:	M-461845-01-1
Guidelines:	**According to Council Regulation (EC) no. 440/2008 part B.10. and OECD Guideline 473 (1997)**
Deviations:	none
GLP/GEP:	**yes**

Executive Summary

An *in-vitro* cytogenicity study was conducted with BCS-AB10736 using CHO cell cultures both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254 induced animals.

The test was carried out employing 2 exposure times without S9 mix (3 and 21 h) and 1 exposure time with S9 mix (3 h). The experiment with S9 mix was carried out twice. The concentrations employed in the main study were chosen based on the results of a preliminary cytotoxicity study, in which no signs of cytotoxicity were noted in the experiments without and with metabolic activation (21 h or 3 h exposure) up to the limit concentration of 5000 µg/mL. Hence, the top concentration employed in the main cytogenicity study was 5000 µg/mL.

In the main study, no signs of cytotoxicity were noted in any of the experiments up to the limit concentration tested. No test material related increase in chromosomal aberrations was noted up to 5000 µg/mL. No test item-related polyploidy or endoreduplication were noted in the experiments without or with metabolic activation. Appropriate solvent and positive controls were included into the test and gave the expected results. A significant increase in clastogenicity was noted for both cyclophosphamide and Mitomycin C in the presence or absence of a metabolic activation system, confirming therefore the sensitivity of the test.

BCS-AB10736 is therefore concluded not to be clastogenic to mammalian cells *in-vitro* under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. **Test material:**

Identification:	BCS-AB10736 (4-methyl-5-propoxy-2,4-dihydro-3H-1,2,4-triazol-3-one)
Description:	White, crystals
Lot/Batch #:	NLL 5797-6-5
Purity:	99.0%
Isomer distribution	Not reported.
Stability of test compound:	2012-05-20 (expiry date)

2. Vehicle and/ or positive control:	Vehicle: DMSO Positive controls: Mitomycin C (MMC), -S9 Cyclophosphamide (CP), +S9
3. Test system:	
Organism:	Mammalian cells in culture
Strains:	Chinese hamster ovary (CHO-K1) cells
Source	[redacted], MD, USA)
Media:	Ham's F-12 supplemented with 10% FCS (foetal calf serum)
Properly maintained?	yes
Periodically checked for karyotype stability?	yes
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	-S9 and +S9: 312.5, 625, 1250, 2500 and 5000 µg/mL

B: STUDY DESIGN AND METHODS

Experimental dates
2012-04-26 through 2012-07-06

Experimental procedure
Preliminary cytotoxicity test
To determine the cytotoxicity, the cells were treated with the test item at concentrations of 10, 25, 100, 250, 1000, 2500 and 5000 µg/mL in the presence of S9 mix for 3 h and in the absence of S9 mix for 21 h, respectively. No signs of cytotoxicity were noted in the experiments without and with metabolic activation up to the limit concentration of 5000 µg/mL. Hence, 5000 µg/mL were employed as the top concentration for the main cytogenicity test without and with metabolic activation.

Main study
Cell treatment: Prior to exposure, the cell culture medium was replaced by fresh medium, whereas one set of cells received medium with S9 mix and the other set received plain medium. The test item was dissolved in DMSO at various concentrations and added to the cell cultures at a standard volume of 100 µL/10 mL. Accordingly, the respective positive controls and DMSO as solvent control were added. The following positive controls were employed:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
MMC	0.4, 0.8	DMSO	-S9
CP	10, 20	DMSO	+S9

Every treatment was conducted in duplicate.
In Experiment 1, the cells were incubated for 3 h. Following treatment, the exposure medium was replaced by fresh cell culture medium and incubated for further 18 h. In Experiment 2, the cells treated in the presence of metabolic activation were handled in the same manner. Cells treated without S9 were

continuously exposed to the test chemicals for 21 h. The incubation time of 21 h corresponds to 1.5 cell cycles for this cell line.

Culture harvesting and slide preparation: 2 h before the end of the 21h incubation period, mitotic activity was arrested by addition of the spindle inhibitor Colcemid to each culture at a final concentration of 0.25 µg/mL. The cells were harvested and fixed first in methanol/glacial acetic acid (3:1) for 2-3 h, followed by a further fixative step in 30% glacial acetic acid by repeated aspiration through a Pasteur pipette. Two drops of this cell suspension were dropped onto a cold, pre-cleaned microscope slide. The slides were left to air-dry at room temperature, then stained in 10% Giemsa.

Slide evaluation

The slides received code numbers randomly chosen by a computer. The slides were examined under low power (x 100 magnification) and those areas judged to be of sufficient technical quality were located and examined under high power (x 1000 magnification, oil immersion objective).

For each treatment and culture 100 metaphases were examined, if possible. Observed aberrations were noted and scored according to J.R.K. Savage (1975):

Gap	Achromatic region in chromatid(s) not greater than the width of a chromatid; scored as single-stranded or double-stranded.
Break	Achromatic region in chromatid(s) greater than the width of a chromatid or a discontinuity with displacement; scored as chromatid or chromosomal.
Fragment	Any free displaced portion of chromatid material.
Exchange	Aberration arising from an exchange between two or more chromosomes which results in the products re-uniting to form a dicentric or polycentric structure. These may be chromosome or chromatid interchanges. In studies of this type, where full karyotyping is not undertaken and chromosome banding has not been performed, only asymmetrical or chromatid exchanges will normally be recognised.
Other types of aberrations	E.g. isochromatid/isochromatid exchanges such as dicentric chromosomes, centric rings, pulverised metaphases or polyploidy.

In addition, the total number of gaps was recorded in 100 metaphases for each culture.

Metaphases which differed from the normal diploid complement (20±2) were excluded from evaluation. However, test item-related variations of the normal chromosome number were noted (polyploidy / endoreduplication). In case of a positive or equivocal response on polyploidy, an additional 200 metaphases would have been evaluated for polyploidy, aneuploidy or endoreduplication on the slide(s) derived from the most appropriate test condition (treatment period, absence or presence of S9 mix and concentration).

Metaphases were assigned to one of the following 5 categories:

1	normal metaphases
2	metaphases with 1 - 2 aberrations
3	metaphases with multiple aberrations
4	pulverised metaphases: extreme (≥ 50%) fragmentation of chromosomal material
5a	polyploidy or
5b	endoreduplication

To examine the cytotoxicity of the test item, 1000 cells were scored and the mitotic index was calculated as the percentage of cells in metaphase.

The following concentrations were not evaluated, as it was thought that they would provide no further information:

- 312.5 µg BCS-AB10736/mL
  (in the first experiments without and with metabolic activation, 3-h exposure)
- 0.8 µg mitomycin C/mL since the lower dose of 0.4 µg/mL was sufficient to demonstrate the sensitivity of the test conditions

(in the experiments without metabolic activation, 3-h or 21-h exposure)

- 20 µg cyclophosphamide/mL since the lower dose of 10 µg/mL was sufficient to demonstrate the sensitivity of the test conditions
  (in the experiments with metabolic activation, 3-h exposure)

Statistical evaluation

The assessment was carried out by a comparison of the number of chromosome aberrations of the samples with those of the solvent control, using the exact test of R.A. FISHER ($p \leq 0.05$) as recommended by the UKEMS guidelines

It is generally accepted that chromatid gaps are not true chromosomal aberrations. In this study, therefore only the total numbers of cells with aberrations exclusive of gap damage were analysed. However, the numbers of cells with aberrations including gap damage are also tabulated.

Assessment criteria

The test item is judged to have mutagenic properties with respect to chromosomal or chromatid change, if the following criteria are fulfilled:

- the number of chromosomal aberrations is significantly (at $p \leq 0.05$) increased compared with the solvent control and outside of the historical background data
- the increase observed is concentration-dependent
- both duplicate cultures lead to similar results
- the increase should not occur in the severely cytotoxic range (mitotic index < 0.25), as it is known that high cytotoxicity causes artefacts in the form of aberrations in *in-vitro* chromosomal aberration tests.
- a reproducible increase in the number of cells with chromosomal aberrations.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary study

No signs of cytotoxicity were noted in the experiments without and with metabolic activation (21 h or 3 h exposure) up to the top concentration of 5000 µg/mL. Hence, 5000 µg/mL was employed as the top concentration for the main cytogenicity test without and with metabolic activation

Main study

No signs of cytotoxicity were noted in the experiments in the absence or presence of metabolic activation up to the top concentration of 5000 µg/mL.

Tests without metabolic activation (3- and 21-hour exposure): The mean incidence of chromosomal aberrations (excluding gaps) of the cells treated with BCS-AB10736 at concentrations from 625 to 5000 µg/mL medium (3-h or 21-h exposure) in the absence of metabolic activation ranged from 0.5% to 3.0%. The results obtained are considered to be within the normal range of the solvent control where a mean incidence of chromosomal aberrations (excluding gaps) of 2.0% or 0.0% was observed after a 3-hour and 21-hour exposure, respectively (historical range: 0 – 5%).

Test with metabolic activation (3-hour exposure): The mean incidence of chromosomal aberrations (excluding gaps) of the cells treated with BCS-AB10736 at concentrations from 625 to 5000 µg/mL medium in the presence of metabolic activation ranged from 0.5% to 2.0%. The results obtained are considered to be within the normal range of the negative control where a mean incidence of chromosomal aberrations (excluding gaps) of 0.0% or 1.5% was observed in the first and second experiment, respectively (historical range: 0 – 3%).

No test item-related polyploidy or endoreduplication were noted in the experiments without or with metabolic activation.

A summary of the results is given in Table 5.8-13 through Table 5.8-14.

Table 5.8-13: Chromosome analysis in cultured Chinese hamster ovary cells *in-vitro* - without metabolic activation

Treatment	Concentration [µg/mL]	Metabolic activation	Mitotic index	No. of metaphases scored	All aberrations		Polyploidy
					incl. gaps	excl. gaps	
Experiment 1 - 3 h exposure							
DMSO	-	-	1.00	200	7	4	0
BCS-AB10736	625	-	1.10	200	11	6	0
	1250	-	1.26	200	4	1	0
	2500	-	1.33	200	7	4	0
	5000	-	0.88	200	4	2	0
MMC	0.4	-	0.67	200	44	38	0
Experiment 2 - 21 h exposure							
DMSO	-	-	1.00	200	2	0	0
BCS-AB10736	625	-	1.07	200	5	2	0
	1250	-	0.89	200	8	3	0
	2500	-	0.87	200	11	4	0
	5000	-	0.67	200	5	1	0
MMC	0.4	-	0.22	200	56	40	0

Table 5.8-14: Chromosome analysis in cultured Chinese hamster ovary cells *in-vitro* - with metabolic activation

Treatment	Concentration [µg/mL]	Metabolic activation	Mitotic index	No. of metaphases scored	All aberrations		Polyploidy
					incl. gaps	excl. gaps	
Experiment 1 - 3 h exposure							
DMSO		+	1.00	200	3	0	0
BCS-AB10736	625	+	0.71	200	10	4	1[#]
	1250	+	0.61	200	9	3	0
	2500	+	0.86	200	7	3	0
	5000	+	0.82	200	5	1	0
CP	10	+	0.20	200	85	62	1[#]
Experiment 2 - 3 h exposure							
DMSO	-		1.00	200	7	3	0
BCS-AB10736	625	+	0.77	200	10	1	0
	1250	+	0.75	200	5	1	0
	2500	+	0.79	200	9	4	0
	5000	+	0.68	200	5	2	0
CP	10	+	0.14	200	72	63	0

[#] tetraploidy (excluded from evaluation)

III. CONCLUSION

The test substance was not clastogenic to mammalian cells in the chromosome aberration assay, with and without metabolic activation.

Report:	[redacted];2013;M-461846-01
Title:	Mutagenicity study of BCS-AG71018 in the Salmonella typhimurium reverse mutation assay (in vitro)
Report No:	LPT 29979
Document No:	M-461846-01-1
Guidelines:	**According to Council Regulation (EC) no. 440/2008 part B.13/14 and OECD Guideline 471**
Deviations:	None
GLP/GEP:	**yes**

Executive Summary
BCS-AG71018 was examined in the 5 *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 in two independent experiments, each carried out without and with metabolic activation (a microsomal preparation derived from Aroclor 1254-induced rat liver). The first experiment was carried out as a plate incorporation test and the second as a preincubation test.
In the main study, six concentrations of BCS-AG71018 ranging from 10.0 to 3160 µg/plate based on a preliminary cytotoxicity testing were employed in the plate incorporation test and in the preincubation test, each carried out without and with metabolic activation. Pronounced cytotoxicity was noted in the plate incorporation test without and with metabolic activation at the top concentration of 3160 µg/plate in all test strains. No increase in revertant colony numbers as compared with control counts was observed for BCS-AG71018, tested up to a concentration of 3160 µg/plate, in any of the tester strains in two independent experiments without and with metabolic activation respectively (plate incorporation and preincubation test). The positive control items showed a significant increase in the number of revertant colonies of the respective test strain and confirmed the validity of the test conditions and the sensitivity of the test system. In conclusion, BCS-AG71018 is not mutagenic to the *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535 and TA 1537 up to the limit concentration, neither in the presence nor absence of a metabolic activation system under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	BCS-AG71018 (4-methoxy-1,2-benzothiazol-3(2H)-one 1,1-dioxide)
Description:	Light yellow solid
Lot/Batch #:	BCOQ 6413-13-5
Purity:	99.7%
Isomer distribution	Not reported
Stability of test compound:	Until 2014-05-22 (expiry date)

2. Vehicle and/ or positive control:

Vehicle: Dimethylsulfoxide (DMSO)
Positive controls:
Sodium azide (NaN_3), TA 100/TA 1535, -S9
2-Nitro-fluorene (2-NF), TA 98, -S9
9-Amino-acridine (9-AA), TA 1537, -S9
Mitomycin C (MMC), TA 102, -S9
2-Aminoanthracene (2-AA), TA 100/TA 1535, +S9
Benzo(a)pyrene (B[a]P), TA 98/TA 102/TA 1537, +S9

3. Test system:

Organism:	*Salmonella typhimurium*
Strains:	TA 1535, TA 100, TA 1537, TA 98, and TA 102
Source:	[redacted]
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	– S9 and +S9: 10.0, 31.6, 100, 316, 1000 and 3160 µg/plate
Pre-incubation period:	20 min
Exposure duration:	48 - 72 h

B: STUDY DESIGN AND METHODS

Experimental dates
2013-05-27 through 2013-06-20

Experimental procedure
Preliminary study
BCS-AB10736 was examined in two preliminary cytotoxicity tests (plate incorporation test without and with metabolic activation) in tester strain TA 100. Cytotoxicity was evaluated by means of scarce background lawn and reduction of the number of revertants by more than 50%. Based on the outcome of this test, the doses for the main study were selected.

Main study
Two independent mutagenicity experiments were carried out with BCS-AB10736 in the tester strains TA 1535, TA 100, TA 102, TA 98 and TA 1537 each without and with metabolic activation. Six concentrations ranging from 10.0 to 3160 µg/plate were employed, and each experiment consisted of 3 plates/concentration. Appropriate positive and solvent (DMSO) controls were included into the test to confirm its sensitivity.
In the plate incorporation test, sterile top agar was mixed with a sterile solution of L-histidine HCl/biotin, Salmonella cell suspension and the test item solution or solvent or positive control solution, respectively. In the experiment with metabolic activation, S9 mix was added, whereas in the experiments without metabolic activation, phosphate buffer was added. After pouring onto a minimal glucose agar plate and solidification, the plates were inverted and placed in a dark 37°C incubator for 48 to 72 hours. The revertant colonies on the test plates and on the control plates were counted with a colony counter, and the presence of the background lawn on all plates was confirmed.
The independent repeat was performed as pre-incubation in a water bath at 37°C for 20 minutes prior to mixing with the overlay agar and pouring onto the surface of a minimal agar plate. Tubes were aerated

during preincubation by using a shaker. The remaining steps were the same as described for the plate incorporation method.

The following doses were used for the positive controls:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
NaN_3	10	*aqua ad iniectabilia*	TA 1535/TA 100, -S9
2-NF	10	DMSO	TA 98, -S9
9-AA	100	ethanol, abs.	TA 1537, -S9
MMC	10	DMSO	TA 102, -S9
2-AA	2	DMSO	TA 100/TA 1535, +S9
B[a]P	10	DMSO	TA 98/ TA 102/TA 1537, +S9

Quality criteria

Quality criteria are tested by [redacted], Germany. The genotypes of the test strains are regularly confirmed in the following way:

- Histidine and biotin requirement ((his-) (bio-)):
  Each of the strains is streaked onto two Minimal glucose agar plate (Minimal Glucose Agar medium E) in the following way:
  1) with 0.1 mM L-histidine and 0.5 mM biotin (100 µL each)
  2) with 0.5 mM biotin (100 µL each)

  After incubation at 37°C for 24 hours, none of the strains should grow on plate 2; all strains should show excessive growth on plate 1.
- (rfa-) deep rough character:
  10 µL of 0.1% crystal violet applied with a paper disc should give zones of inhibition in the test strains after incubation at 37°C for 24 hours.
- UV-sensitivity (uvr B-):
  Plates are covered partly with black paper and placed under germicidal UV-irradiation. After incubation at 37°C for 24 hours TA 100 should grow only under the covered portion of each plate. TA 102 should also grow under the uncovered area.
- Ampicillin-resistance (pKM 101):
  0.8 mg ampicillin/plate is placed onto plates seeded with bacteria: Absence of zones of inhibition around the discs indicates resistance to ampicillin (TA 100 and TA 102).
- Ampicillin- and tetracycline-resistance
  The pAQ1 strain (TA 102) is tested for both ampicillin and tetracycline resistance on ampicillin/tetracycline plates.

Assessment Criteria

A test item is considered to show a positive response if

- at one or more concentrations the number of revertants is reproducibly increased in at least one strain with or without metabolic activation. A 2-fold increase in comparison to the solvent control is regarded as being relevant for a positive response in the strains TA98, TA100 and TA102. For the strains TA1535 and TA1537 a 3-fold increase represents a biological relevant effect. The Mann and Whitney test ($p \leq 0.05$) may be used to determine statistical significance.

or

- a concentration-related increase over the range tested in the number of the revertants per plate is observed. The Spearman's rank correlation coefficient may be applied.

Biological relevance of the results should be considered first. Positive results have to be reproducible and the histidine independence of the revertants has to be confirmed by streaking random samples on histidine-free agar plates.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary study

Pronounced cytotoxicity was noted at concentrations of ≥ 3160 µg/plate. Hence, 3160 µg/plate were chosen as top concentration for the main study.

Main study

Pronounced cytotoxicity was noted in all tester strains at 3160 µg/plate in both the plate incorporation and preincubation test with and without metabolic activation.

No increase in revertant colony numbers as compared with control counts was observed for BCS-AG71018, tested up a cytotoxic concentration of 3160 µg/plate, in any of the tester strains in two independent experiments without and with metabolic activation, respectively (plate incorporation and preincubation test).

A summary of the results is given in Table 5.8-15 through Table 5.8-18.

Table 5.8-15: Plate incorporation method - without metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
–	0	20.0±1.0	157.0±33.4	270.0±11.3	26.0±1.7	7.0±0.0
–	10.0	19.0±1.0	142.3±22.7	273.3±13.8	21.3±1.2	5.3±0.6
–	31.6	19.3±6.5	125.7±21.2	264.7±7.4	28.3±3.1	7.7±2.5
–	100	18.7±4.0	126.3±6.5	271.3±17.1	26.3±6.1	6.0±1.0
–	316	15.0±5.6	128.3±10.7	270.3±11.6	27.0±2.0	5.3±3.8
–	1000	19.3±8.5	132.0±4.4	279.0±14.7	23.0±2.6	7.3±3.8
–	3160	6.0±1.0#	23.3±1.5#	101.7±3.5#	9.3±0.6#	1.0±0.0#
Pos controls -S9	Name	NaN_3	NaN_3	MMC	2-NF	9-AA
	Conc. [µg/plate]	10	10	10	10	100
	Revertants per plate	80.0±2.0	812.7±9.7	1263.3±21.0	121.7±9.7	39.7±4.2

Scarce background lawn

Table 5.8-16: Pre-incubation method - without metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
–	0	23.0±2.6	148.0±10.4	267.7±5.7	26.7±2.1	6.7±1.5
–	10.0	17.0±1.0	146.0±3.0	269.3±18.6	26.0±1.7	5.7±0.6
–	31.6	13.0±1.0	133.7±8.1	272.7±4.0	24.7±4.2	4.3±2.1
–	100	19.0±8.2	131.7±10.4	270.3±4.9	26.3±3.2	4.0±0.0
–	316	13.0±2.0	142.3±10.0	277.0±5.3	23.0±1.7	5.7±2.1
–	1000	22.3±7.0	150.0±5.3	254.3±7.6	23.0±1.7	4.0±0.0
–	3160	7.3±0.6#	27.3±5.0#	108.0±2.6#	10.0±1.0#	1.3±0.6#

Pos controls -S9	Name	NaN_3	NaN_3	MMC	2-NF	9-AA
	Conc. [µg/plate]	10	10	10	10	100
	Revertants per plate	108.3±2.3	1087.0±17.3	1111.7±1.5	136.7±4.0	50.0±6.1

[#] Scarce background lawn

Table 5.8-17: Plate incorporation method - with metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
+	0	17.7±5.1	156.0±5.2	274.7±7.4	27.0±2.6	7.3±0.6
+	10.0	14.7±4.7	152.3±7.0	275.3±4.6	23.7±5.5	7.7±2.3
+	31.6	14.3±0.6	142.0±2.0	265.3±10.7	28.0±0.0	8.3±0.6
+	100	18.3±4.9	137.7±3.1	274.3±1.2	25.0±3.6	8.0±1.7
+	316	15.7±3.1	139.7±17.2	278.0±16.1	23.0±1.0	7.7±1.2
+	1000	12.7±0.6	155.0±10.6	280.7±9.3	28.0±4.7	5.0±2.6
+	3160	6.3±2.1[#]	23.7±3.8[#]	103.0±3.6[#]	7.0±1.0[#]	1.0±0.0[#]
Pos controls +S9	Name	2-AA	2-AA	B[a]P	B[a]P	B[a]P
	Conc. [µg/plate]	2	2	10	10	10
	Revertants per plate	79.7±1.5	779.3±5.5	1199.0±30.2	124.3±15.0	45.7±14.2

[#] Scarce background lawn

Table 5.8-18: Pre-incubation method - with metabolic activation

S9 mix	Test substance concentration [µg/plate]	Number of revertants [mean number of colonies per plate ± SD]				
		Base-pair substitution type			Frameshift type	
		TA1535	TA100	TA102	TA98	TA1537
+	0	18.7±2.3	161.3±24.0	270.7±10.5	28.0±1.5	6.0±1.0
+	10.0	13.7±3.1	150.0±7.8	257.3±2.1	24.3±1.2	4.0±2.0
+	31.6	17.7±4.0	144.3±21.5	273.0±7.5	28.3±3.2	4.3±2.1
+	100	23.4±6.4	155.7±7.5	269.0±9.5	28.3±6.1	6.0±4.0
+	316	20.0±6.0	147.3±9.5	275.7±12.7	22.7±3.1	3.3±3.2
+	1000	25.7±0.6	164.3±8.1	260.7±7.4	29.7±8.5	3.3±0.6
+	3160	7.7±3.5[#]	26.3±2.1[#]	109.3±7.1[#]	8.3±1.5[#]	1.0±0.0[#]
Pos controls +S9	Name	2-AA	2-AA	B[a]P	B[a]P	B[a]P
	Conc. [µg/plate]	2	2	10	10	10
	Revertants per plate	110.0±1.0	1104.3±15.9	1103.0±12.5	143.3±3.2	54.7±0.6

[#] Scarce background lawn

III. CONCLUSION

The test substance was not mutagenic in the Salmonella/microsome assay, with and without metabolic activation.

Report:	[redacted];[redacted];2013;M-466474-01
Title:	Mutagenicity study of BCS-AG71018 in mammalian cells (V79) in the in vitro gene mutation assay (hprt test)
Report No:	LPT 29981
Document No:	M-466474-01-1
Guidelines:	**According to Council Regulation (EC) No. 440/2008 Method B.17 and OECD Guideline 476**
Deviations:	None
GLP/GEP:	**yes**

Executive Summary

BCS-AG71018 was tested for mutagenic potential in a gene mutation assay in cultured mammalian cells (V79, genetic marker HPRT) both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254-induced animals. The duration of the exposure with the test item was 4 hours or 24 hours in the experiments without S9 mix and 4 hours in the experiments with S9 mix.

The concentrations employed were chosen based on the results of a preliminary cytotoxicity study. In this study no signs of cytotoxicity were noted in the experiments without and with metabolic activation (24 h or 4 h exposure) up to the top concentration of 5000 µg/mL. Hence, 5000 µg/mL was employed as the top concentration for the main mutagenicity test without and with metabolic activation.

In the main study, five concentrations ranging from 312.5 to 5000 µg/mL were selected for the experiments without and with metabolic activation, respectively. In the main study, no signs of cytotoxicity in form of decreased plating efficiency were noted in the experiments in the absence or presence of metabolic activation up to the top concentration of 5000 µg/mL. In experiments both with and without metabolic activation, the mutation frequency of the negative control DMSO was well within the expected range and the mutation frequency of the cultures treated with BCS-AB10736 at concentrations up to 5000 µg/plate were within the normal range of the negative controls. The positive controls EMS (-S9) and DMBA (+S9) caused a pronounced increase in the mutation frequencies, indicating the validity of this test system.

BCS-AG71018 is therefore concluded not to be mutagenic to mammalian cells *in-vitro* under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	BCS-AG71018 (4-methoxy-1,2-benzothiazol-3(2H)-one 1,1-dioxide)
Description:	Light yellow crystalline powder
Lot/Batch #:	BCOO 6413-13-5
Purity:	99.7%
Isomer distribution	Not reported
Stability of test compound:	2014-05-22 (expiry date)

2. Vehicle and/ or positive control:

Vehicle: Dimethylsulfoxide (DMSO)

Positive controls:

Ethyl methanesulfonate (EMS), -S9

9,10-Dimethyl-1,2-benzanthracene (DMBA), +S9

3. Test system:

Organism:	Mammalian cells in culture
Strains:	V79 cells
Source	[redacted], Germany
Media:	The cells were maintained and exposed to the test item in the absence of S9 in DMEM-FCS (Dulbecco's modified Eagle-Medium supplemented with 10% foetal calf serum, penicillin (100 U/mL) and streptomycin (100 µg/mL)). Exposure to the test-item in the presence of S9 mix was performed in PBS-HEPES (Dulbecco's phosphate buffered saline (PBS) which additionally contained 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) pH 7.4.)
Properly maintained?	yes
Periodically checked for Mycoplasma contamination?	yes
Periodically "cleansed" against high spontaneous background?	yes
Locus Examined:	hprt locus
Selection agent:	6-thioguanine
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	-S9 and +S9: 312.5, 625, 1250, 2500 and 5000 µg/mL

B: STUDY DESIGN AND METHODS

Experimental dates
2013-05-27 through 2013-08-01

Experimental procedure
Preliminary cytotoxicity test
To determine the cytotoxicity, the same procedure was used as employed for the mutagenicity experiments (see below), except that no mutant selection was carried out. No signs of cytotoxicity were noted in the experiments without and with metabolic activation (24 h or 4 h exposure) up to the limit concentration of 5000 µg/mL. Hence, 5000 µg/mL were employed as the top concentration for the main mutagenicity test without and with metabolic activation.

Main study
Cell treatment: On Day -1 of the experiment, cells were maintained in DMEM-FCS. On the following day, the cells were exposed to the selected concentrations of the test item. In the absence of S9 mix, the cells were exposed in DMEM-FCS to the test item for 4 hours (Experiment 1) or 24 hours (Experiment 2). In the experiments with S9 mix, the medium was replaced by S9 mix and the exposure limited to 4 hours. The negative control was treated with DMSO (the vehicle) in the same way. In addition, the following positive controls were employed:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
EMS	600, 700	DMSO	-S9

DMBA	20, 30	DMSO	+S9

Expression of new genotype: At the end of the exposure period, the cells were trypsinised and a relative plating efficiency (PE_1) was determined for each dose to obtain an accurate measure of the toxic effect of the chemical. Three replicate plates were used with a known number of cells. The remaining cells were re-plated and the culture incubation continued until Day 8 with normal DMEM-FCS with one sub-cultivation on Day 4 or 5.

Treatment with selecting agent: Following the expression time, the cells were harvested and re-plated at a density of 1,000,000 cells, in DMEM-FCS containing 6-thioguanine (10 µg/mL) for selection of mutants (5 replicate plates), or at approximately 100 to 150 cells (exact number known) in medium without 6-thioguanine for the estimation of plating efficiencies (PE_2), (3 replicate plates). The plates were fixed and stained after about 8 days (plating efficiency plates) or 12 days (6-thioguanine plates).

Acceptance criteria
Solvent control: As the total number of colonies is normally low and as a single mutation may cause several colonies due to cell division during the expression period, a relatively large variation of the mutation frequency may result. This is especially true, if a low spontaneous mutation frequency is forced by cloning (in order to achieve a high sensitivity of the test).
The historical background mutation frequency in this system has been reported to be 1 to 44 mutants per 106 survivors in non-activation solvent controls and 6 to 46 per 106 survivors in S9 activation solvent controls. The background data obtained at LPT are given at the end of this chapter. The spontaneous mutation frequency may be variable from experiment to experiment, but should normally lie within the above-mentioned range. The positive controls EMS (600 and 700 µg/mL) and DMBA (20 and 30 µg/mL) should cause a 10-fold or greater increase in mutation frequency.
The background mutation frequency at LPT ranges from 1.30 to 38.36 x 10^{-6} cloneable cells for the vehicle controls. The mutation frequency of the positive controls at LPT ranges from 112.1 to 1708.4 x 10^{-6} cloneable cells for EMS and 130.0 to 2693.3 x 10^{-6} cloneable cells for DMBA.

Assessment criteria
So far no satisfactory mathematical methods are available for the statistical analysis of mammalian cell mutagenicity experiments such as those performed. However, the following pre-determined descriptive criteria are considered the most useful for interpretation of the results:

- If in both independent experiments solvent and positive controls show results within the norm and if the test item does not increase the mutation frequency 2-fold above the mean of the solvent controls under any condition, or if the mutation frequency is always lower than 40 x 10^{-6} and if at least 1,000,000 cells per condition have been evaluated, the item is considered as negative in the test.
- In case of a dose-dependent increase of the mutation frequency in both independent experiments (at similar concentrations) to at least 2-fold solvent control and at least 40 x 10^{-6} both in the presence and/or absence of S9 mix the item is considered as positive in the test.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary study
No signs of cytotoxicity were noted in the experiments without and with metabolic activation (24 h or 4 h exposure) up to the top concentration of 5000 µg/mL. Hence, 5000 µg/mL was employed as the top concentration for the main mutagenicity test without and with metabolic activation

Main study

Cytotoxicity: No signs of cytotoxicity in form of decreased plating efficiency (PE_1) and (PE_2) were noted in the experiments in the absence or presence of metabolic activation up to the top concentration of 5000 µg/mL. Test item precipitation was noted at 5000 µg/mL.

Experiments without metabolic activation: The mutation frequency of the negative control DMSO was 12.11 and 29.45 x 10^{-6} cloneable cells and was therefore well within the expected range. The mutation frequency of the cultures treated with BCS- AG71018 at concentrations of 312.5, 625, 1250, 2500 or 5000 µg/mL culture medium ranged from 12.39 to 34.92 x 10^{-6} cloneable cells and were therefore within the normal range of the negative controls. The positive control EMS caused a pronounced increase in the mutation frequency ranging from 378.39 to 1541.43 x 10^{-6} cloneable cells, indicating the validity of this test system.

Experiments with metabolic activation: The mutation frequency of the negative control DMSO was 15.48 and 15.91 x 10^{-6} cloneable cells and was therefore well within the expected range. The mutation frequency of the cultures treated with BCS- AG71018 at concentrations of 312.5, 625, 1250, 2500 or 5000 µg/mL culture medium ranged from 12.33 to 37.50 x 10^{-6} cloneable cells and were therefore within the normal range of the negative controls. The positive control DMBA caused a pronounced increase in the mutation frequency ranging from 195.71 to 655.88 x 10^{-6} cloneable cells, indicating the validity of this test system.

A summary of the results is given in Table 5.8-19 through Table 5.8-22.

Table 5.8-19: Experiment 1 - without metabolic activation – 4 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.66	1.14	5.70	12.11
BCS- AG71018	312.5	1.04	0.92	4.60	12.39
	625	1.03	0.71	3.55	16.34
	1250[#]	0.60	0.98	4.90	12.86
	2500[#]	0.30	1.13	5.65	13.63
	5000[##]	0.54	1.03	5.15	14.95
EMS	600	0.59	0.59	4.35	378.39
	700	0.70	0.70	4.60	380.43

[#] Change of colour of the pH indicator in the exposure medium from red to orange or yellow-orange
[##] Test item precipitation

Table 5.8-20: Experiment 1 - with metabolic activation – 4 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.72	1.24	6.20	15.48
BCS- AG71018	312.5	0.62	1.33	6.65	12.33
	625	0.64	1.05	5.25	21.90
	1250[#]	0.85	1.16	5.80	17.59
	2500[#]	0.73	0.94	4.70	21.70
	5000[##]	0.81	1.07	5.35	13.46
DMBA	20	0.21	0.67	3.35	267.76
	30	0.22	0.58	2.90	391.03

[#] Change of colour of the pH indicator in the exposure medium from red to orange or yellow-orange
[##] Test item precipitation

Table 5.8-21: Experiment 2 - without metabolic activation – 24 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.71	0.91	4.55	29.45
BCS- AG71018	312.5	0.64	0.62	3.10	24.19
	625	0.65	0.64	3.20	30.00
	1250[#]	0.71	0.59	2.95	34.92
	2500[#]	0.77	0.56	2.80	27.14
	5000[##]	0.79	0.73	3.65	28.49
EMS	600	0.29	0.63	3.15	447.62
	700	0.35	0.14	0.70	1541.43

[#] Change of colour of the pH indicator in the exposure medium from red to orange or yellow-orange
[##] Test item precipitation

Table 5.8-22: Experiment 2 - with metabolic activation – 4 h exposure duration

Test item	Concentration [µg/mL]	Plating efficiency		Mutants per 10^6 cells	Mutation frequency x 10^{-6}
		PE_1	PE_2		
DMSO	-	0.61	0.88	4.40	15.91
BCS- AG71018	312.5	0.73	0.96	4.80	20.42
	625	0.64	0.72	3.60	27.50
	1250[#]	0.82	0.94	4.70	15.74
	2500[#]	0.62	0.77	3.85	23.12
	5000[##]	0.70	0.83	4.15	14.70
DMBA	20	0.18	0.84	4.20	195.71
	30	0.23	0.68	3.40	655.88

[#] Change of colour of the pH indicator in the exposure medium from red to orange or yellow-orange
[##] Test item precipitation

III. CONCLUSION

The test substance was not mutagenic to mammalian cells in the HPRT assay, with and without metabolic activation.

Report:	[redacted]; [redacted];2013;M-462692-01
Title:	In vitro assessment of the castogenic activity of BCS-AG71018 in cultured human peripheral lymphocytes
Report No:	LPT 29980
Document No:	M-462692-01-1
Guidelines:	**According to OECD Guideline 473 and EC No. 440/2008 Method B.10Council Regulation (EC) no. 440/2008 part B.13/14 and OECD Guideline 471**
Deviations:	None
GLP/GEP:	**yes**

Executive Summary
An *in-vitro* cytogenicity study was conducted with BCS- AG71018 using cultured human peripheral lymphocytes both in the presence and absence of metabolic activation by a rat liver post-mitochondrial fraction (S9 mix) from Aroclor 1254 induced animals. The test was carried out employing 2 exposure times without S9 mix (4 and 24 h) and 1 exposure time with S9 mix (4 h). The concentrations of 312.5-5000 µg/mL employed in the main study were chosen based on the results of a preliminary cytotoxicity test.
In the main study, pronounced cytotoxicity was noted in all cultures treated with the limit concentration of 5000 µg/mL with or without metabolic activation and additionally in cultures treated with 2500 µg/mL without metabolic activation for 24 h. Haemolysis was further noted in all cultures treated with the top concentration. No test material related increase in chromosomal aberrations or polyploidy was noted up to 5000 µg/mL with or without metabolic activation. Appropriate solvent and positive controls were included into the test and gave the expected results. A significant increase in clastogenicity was noted for both cyclophosphamide and Mitomycin C in the presence or absence of a metabolic activation system, confirming therefore the sensitivity of the test.
BCS- AG71018 is therefore concluded not to be clastogenic to cultured human lymphocytes *in-vitro* under the conditions of this test.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material:

Identification:	BCS-AG71018 (4-methoxy-1,2-benzothiazol-3(2H)-one 1,1-dioxide)
Description:	Light yellow crystalline powder
Lot/Batch #:	BCOQ 6413-13-5
Purity:	99.7%
Isomer distribution	Not reported
Stability of test compound:	Until 2014-05-22 (expiry date)

2. Vehicle and/ or positive control:
Vehicle: Dimethylsulfoxide (DMSO)
Positive controls:
Mitomycin C (MMC), -S9
Cyclophosphamide (CP), +S9

3. Test system:

Organism:	Human
Strains:	Human peripheral lymphocytes in heparinised whole blood

Source:	Healthy donors known to be without any medication
Media:	Chromosome Medium 1A with Phytohemagglutinin supplemented with Penicillin/Streptomycin (10 000 IU/mL)
Properly maintained?	Not applicable
Periodically checked for karyotype stability?	Not applicable
Metabolic activation system:	Post-mitochondrial fraction (S9 fraction) from rats treated with Aroclor 1254 was prepared according to Maron and Ames (1983). S9 was collected from 20 – 30 rats.
Test concentrations:	–S9 and +S9: 312.5, 625, 1250, 2500 and 5000 µg/mL

B: STUDY DESIGN AND METHODS

Experimental dates
2013-05-27 through 2013-06-27

Experimental procedure
Preliminary cytotoxicity test
To determine the cytotoxicity, the cells were treated with the test item at concentrations of 10-5000 µg/mL in the presence of S9 mix for 4 h and in the absence of S9 mix for 24 h, respectively. No signs of cytotoxicity were noted in the experiments without and with metabolic activation up to the limit concentration of 5000 µg/mL. Hence, 5000 µg/mL were employed as the top concentration for the main cytogenicity test without and with metabolic activation.

Main study
The test item was dissolved in DMSO at various concentrations and added to the cell cultures at a standard volume of 100 µL/10 mL. Accordingly, the respective positive controls and DMSO as solvent control were added. The following positive controls were employed:

Positive control	Concentrations [µg/plate]	Vehicle	Remarks
MMC	0.1, 0.2	DMSO	-S9
CP	10, 20	DMSO	+S9

Every treatment was conducted in duplicate using blood from the same donors.
In Experiment 1, the cells were incubated for 4 h. Following treatment, the exposure medium was replaced by fresh cell culture medium and incubated for further 20 h. In Experiment 2, the cells treated in the presence of metabolic activation were handled in the same manner. Cells treated without S9 were continuously exposed to the test chemicals for 24 h.
Culture harvesting and slide preparation: 2 h prior to harvest, mitotic activity was arrested by addition of the spindle inhibitor Colcemid to each culture. The cells were harvested and fixed first in methanol/glacial acetic acid (4:1) 24 h after start of exposure. The prepared slides were left to air-dry at room temperature, then stained in 10% Giemsa.

Slide evaluation
For each treatment and culture 100 metaphases were examined, if possible. Observed aberrations were characterised as gap, break, fragment, exchange, and other types of aberrations. In addition, the total number of gaps was recorded in 100 metaphases for each culture. Metaphases which differed from the normal diploid complement (20±2) were excluded from evaluation. However, test item-related variations of the normal chromosome number were noted (polyploidy / endoreduplication). In case of a positive or equivocal response on polyploidy, an additional 200 metaphases would have been evaluated for

polyploidy, aneuploidy or endoreduplication on the slide(s) derived from the most appropriate test condition (treatment period, absence or presence of S9 mix and concentration).
To examine the cytotoxicity of the test item, 1000 cells were scored and the mitotic index was calculated as the percentage of cells in metaphase.

The following concentrations were not evaluated, as it was thought that they would provide no further information:

- 312.5 µg BCS-AG71018/mL
  (in the experiments without and with metabolic activation, 4-h exposure)
- 0.1 µg mitomycin C/mL since the lower dose of 0.4 µg/mL was sufficient to demonstrate the sensitivity of the test conditions
  (in the experiments without metabolic activation, 4-h or 24-h exposure)
- 10 µg cyclophosphamide/mL
  (in the experiments with metabolic activation, 4-h exposure)

Statistical evaluation
The assessment was carried out by a comparison of the number of chromosome aberrations of the samples with those of the solvent control, using the exact test of FISHER ($p \leq 0.05$). It is generally accepted that chromatid gaps are not true chromosomal aberrations. In this study, therefore only the total numbers of cells with aberrations exclusive of gap damage were analysed. However, the numbers of cells with aberrations including gap damage are also tabulated.

Assessment criteria
The test item is judged to have mutagenic properties with respect to chromosomal or chromatid change, if the following criteria are fulfilled:

- the number of chromosomal aberrations is significantly (at $p \leq 0.05$) increased compared with the solvent control and outside of the historical background data
- the increase observed is concentration-dependent
- both duplicate cultures lead to similar results
- the increase should not occur in the severely cytotoxic range (mitotic index < 0.25), as it is known that high cytotoxicity causes artefacts in the form of aberrations in *in-vitro* chromosomal aberration tests.
- a reproducible increase in the number of cells with chromosomal aberrations.

II. RESULTS AND DISCUSSION

A. REVERTANT FREQUENCIES

Preliminary study
Pronounced cytotoxicity and haemolysis were noted in the experiment without and with metabolic activation at the top concentration of 5000 µg BCS-AG71018/mL. In addition, cytotoxicity was noted at 2500 µg/mL medium in the experiment without metabolic activation. 5000 µg/mL was employed as the top concentration for the main cytogenicity test without and with metabolic activation

Main study
Treatment with BCS-AG71018 at concentrations of 625-2500 or 312.5-1250 µg/mL (4 h or 24 h exposure, respectively) without metabolic activation or at concentrations of 625-2500 µg/mL with metabolic activation did not increase the mean incidence of chromosomal aberrations (excluding gaps) over the historical control range of 0 - 4%. The top concentration of 5000 µg/mL in all experiments with and without metabolic activation and, in addition, 2500 µg/mL in the second experiment (24 h exposure) without metabolic activation led to almost complete cytotoxicity. Haemolysis was further noted in cultures treated with 5000 µg/mL with or without metabolic activation.
The positive and vehicle control data were within the historical control range; the positive control cultures had a significantly increased frequency of cells with aberrations.

No test item-related polyploidy or endoreduplication were noted in the experiments without or with metabolic activation.
A summary of the results is given in Table 5.8-23 through Table 5.8-24.

Table 5.8-23: Chromosome analysis in cultured human lymphocytes *in-vitro* - without metabolic activation

Treatment	Concentration [µg/mL]	Metabolic activation	Mitotic index	No. of metaphases scored	All aberrations		Polyploidy
					incl. gaps	excl. gaps	
Experiment 1 - 4 h exposure							
DMSO	-	-	1.00	200	3	0	0
BCS-AG71018	625	-	1.25	200	6	2	0
	1250	-	0.93	200	9	4	0
	2500	-	0.93	200	9	4	0
	5000[#]	-	0.03	11[##]	0	0	0
MMC	0.2	-	1.19	200	55	16[*]	0
Experiment 2 - 24 h exposure							
DMSO	-	-	1.00	200	2	0	0
BCS-AG71018	312.5	-	1.25	200	4	2	0
	625	-	1.03	200	4	2	0
	1250	-	1.30	200	8	4	0
	2500	-	0.35	15[##]	0	0	0
	5000[#]	-	0.00	4[##]	0	0	0
MMC	0.2	-	1.31	200	32	17[*]	0

[#] haemolysis
[##] no more metaphases of sufficient quality for evaluation due to cytotoxicity of the test item
* statistically significant from solvent control ($p \leq 0.05$)

Table 5.8-24: Chromosome analysis in cultured human lymphocytes *in-vitro* - with metabolic activation

Treatment	Concentration [µg/mL]	Metabolic activation	Mitotic index	No. of metaphases scored	All aberrations		Polyploidy
					incl. gaps	excl. gaps	
Experiment 1 - 4 h exposure							
DMSO	-	+	1.00	200	1	1	0
BCS-AG71018	625	+	1.13	200	9	2	0
	1250	+	0.91	200	4	1	0
	2500	+	0.69	200	6	1	0
	5000[#]	+	0.02	14[##]	0	0	0
CP	20	+	0.60	200	36	22[*]	0
Experiment 2 - 4 h exposure							
DMSO	-	+	1.00	200	2	0	0
BCS-AG71018	625	+	1.04	200	6	2	0
	1250	+	1.47	200	8	3	0
	2500	+	1.14	200	9	5	0
	5000[#]	+	0.01	5[##]	0	0	0
CP	20	+	0.52	200	35	18[*]	0

[#] haemolysis
[##] no more metaphases of sufficient quality for evaluation due to cytotoxicity of the test item
* statistically significant from solvent control ($p \leq 0.05$)

III. CONCLUSION

The test substance was not clastogenic to cultured human lymphocytes in the chromosome aberration assay, with and without metabolic activation.

CA 5.8.2 Supplementary studies on the active substance

No new studies have been performed for this endpoint.

CA 5.8.3 Endocrine disrupting properties

Designated studies on endocrine disrupting (ED) properties of propoxycarbazone-sodium have not been conducted by the applicant. The existing body of data is sufficient to exclude relevant ED-like potential of propoxycarbazone-sodium. This is based on the absence of effects on the weight of hormone-sensitive tissues like reproductive organs, thyroids and pituitary. In addition, the available fertility studies showed no effects on male or female fertility, which may be considered sensitive targets of ED-like activity.

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

No reports from medicinal surveillance on manufacturing personnel are available.

CA 5.9.2 Data collected on humans

From the experimental biological testing and from the field tests with propoxycarbazone-sodium formulations no negative effects on the health of the humans were reported.

CA 5.9.3 Direct observations

There is no exposure of the general population to propoxycarbazone-sodium up to now.

CA 5.9.4 Epidemiological studies

No information available.

CA 5.9.5 Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical tests

Human poisoning cases are not known. Based on the results of acute toxicity studies in rats it is unlikely that clinical signs may occur after uptake of higher doses. Results of special clinical studies are not known.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

A specific antidote is not known. In case of an oral uptake, first aid measures should consist of removal of ingested compound by gastric lavage or induction of vomiting and symptomatic treatment. Contaminated skin should be washed immediately with plenty of water.

CA 5.9.7 Expected effects of poisoning

No information available. Even after uptake of higher doses clinical signs are unlikely to occur. In the acute oral toxicity study in rats the only clinical signs observed at a very high dose of 5000 mg/kg bw/day were soft faeces and moist anus. From the laboratory animal studies a recovery from potential acute symptoms within a few days would be expected.

CA 5.10 Summary of mammalian toxicity and overall evaluation

Propoxycarbazone-sodium (MKH 6561), an herbicidal compound, belongs to the class of sulfonylaminocarbonyltriazolinones. The mode of action in plants is the inhibition of the enzyme acetolactate synthase.

Absorption, distribution, excretion and metabolism

The studies on rats using phenyl- as well as triazolinone-labelled active substance showed a low degree of absorption followed by fast elimination from the body. The low rate of absorption was confirmed by a bile fistulation experiment, where after intraduodenal administration only small amounts of radioactivity were detected in bile. After oral administration of propoxycarbazone-sodium, more than 88% of the administered radioactivity was excreted in all dose groups tested within 48 hours independent of the labelling position. The concentrations of radioactive residue in organs, tissues and GIT were very low indicating no tendency for accumulation of propoxycarbazone-sodium related residues. Due to the low degree of absorption the major portion of radioactivity was consequently excreted via faeces with more than 66% of the administered radioactivity after oral administration in all dose groups. Renal excretion accounted for approximately 21 to 31% of the administered dose. The excretion behaviour showed no significant differences with respect to dose rate and sex. Only a small amount of radioactivity was measured in the bile, corresponding to 3.4% of the dose administered to male rats. Less than 1% of the administered radioactivity was found in the expired air.
Propoxycarbazone-sodium was not intensively metabolised in rats. The major component in urine and faeces of all dose groups was unchanged parent compound accounting for 75 to 89% of the administered dose. Metabolisation took place via cleavage of the amide bond, resulting in sulfonamide methyl ester (M05) and N-methyl propoxy triazolinone (M10). Sulfonamide methyl ester (M05) was further metabolised yielding sulfonamide acid (M06) and saccharin (M07). Sulfonamide methyl ester (M05) was observed in the high dose experiment in faeces in a maximum amount of 8.8% of the administered dose.

Sulfonamide acid (M06) and saccharin (M07) did not exceed 4% of the given dose in any experiment. Identification rate ranged from 83 to approximately 100% of the administered dose.
These result are supported by the comparative *in-vitro* metabolism study of ^{14}C-propoxycarbazone-sodium in pooled rat liver microsomes (RLM; males), obtained from Wistar rats, and pooled human liver microsomes (HLM; males and females).
The results of the test with ^{14}C-propoxycarbazone-sodium demonstrated that the test item was moderately stable after incubation with RLM and HLM. Two small ^{14}C-containing products could be detected (namely Pr-1 and Pr-2). Pr-1 HPLC peak was considered as a degradation product of ^{14}C-propoxycarbazone-sodium since it was present either in microsome incubations at t=0 hours and in incubations at 37±1°C with buffer only, accounting for 4.5% to 6.2% of the total radioactivity in these samples. Pr-2 compound was formed after incubation of ^{14}C-propoxycarbazone-sodium with liver microsomes from humans but not from rats. Therefore, it was considered as an actual human-specific metabolite of ^{14}C-propoxycarbazone-sodium. The MS spectra of metabolite Pr-2 containing its characteristic fragment ions demonstrated that this metabolite is most likely formed by O-demethylation of propoxycarbazone leading to the propoxycarbazone carboxylic acid metabolite (M04; see Metabolites).

Acute toxicity
Propoxycarbazone-sodium has a very low acute oral, percutaneous and inhalative toxicity. Propoxycarbazone-sodium is neither an eye- nor a skin irritant, and has no skin sensitizing potential.

Short-term toxicity
The short-term studies with repeated administration demonstrated a low toxicity of propoxycarbazone-sodium in rats, mice and dogs. The lowest NOAEL was determined in the 1-year dog study (56 mg/kg bw/day). The targets were the liver (microsomal enzyme induction), adrenals (increased absolute weights, enlarged zona fasciculata) and heart (decreased relative weight) in dogs, forestomach in rats (local irritation), feed/water intake and body weight gain. Organ effects in dogs were interpreted as an indirect physiologic response to chronic treatment or reduced nutritional intake and were not considered to result from a direct toxicological mechanism of propoxycarbazone-sodium. A relevant immunotoxic potential has been excluded in the rat.
A new subacute feeding study in rats has been performed with a batch representing the impurity profile of batches produced after 2002. The NOAEL for the new substance batch did not differ from the results obtained with a batch reflecting the original specification containing fewer impurities.

Genotoxicity
Propoxycarbazone-sodium was tested for point-mutagenic activity, for chromosome aberration *in-vitro* and *in-vivo* and for DNA repair. Since these tests were all negative as well as the new tests conducted either to the different impurity profile since the 2001 evaluation or to fulfil the requirements of the most recent version of the OECD TG 471, it can be concluded that propoxycarbazone-sodium has no mutagenic/genotoxic potential under *in-vitro* or *in-vivo* conditions.

Long-term toxicity and carcinogenicity
In all long-term studies, the toxicity of propoxycarbazone-sodium proved to be very low; NOAELs were 43 or 369 mg/kg bw/day in rats or mice, respectively. The predominant effect was a decreased body weight gain. In the rat, this was accompanied by an increased urinary pH and by renal pelvic mineralization in the highest dose. In both rodent species, there were no indications for an oncogenic potential of propoxycarbazone-sodium.

Reproductive toxicity
The reproductive toxic potential of propoxycarbazone-sodium was tested in a two-generation study in rats and in developmental toxicity studies in rats and rabbits. The results of these studies do not indicate a risk of reproductive toxicity. The two-generation study demonstrates the absence of any reproductive and neonatal toxicity up to 16000 ppm, a parentally toxic dose clearly exceeding the limit dose of 1000 mg/kg bw/day. The developmental study in rats determined a NOAEL of 1000 mg/kg bw/day, both for maternal and developmental toxicity. Rabbits are more sensitive than rats, resulting in clear maternal toxicity

including a secondary effect on gestation rate at 500 and 1000 mg/kg bw/day. The NOAEL for maternal toxicity in rabbits was 100 mg/kg bw/day, the NOAEL for embryo/foetal development was 500 mg/kg bw/day. Thus, a primary developmental toxic potential in rabbits has been excluded. Both developmental toxicity studies did not reveal a teratogenic potential of propoxycarbazone-sodium.

Neurotoxicity

The results of the acute and subchronic neurotoxicity screening studies in rats confirm the generally very low toxicity of propoxycarbazone-sodium and demonstrate the absence of any specific neurotoxicity at limit doses of 2000 mg/kg bw/day (acute) and greater than 1000 mg/kg bw/day (subchronic).

Metabolites

Six major plant or soil metabolites of propoxycarbazone-sodium were identified that were not (< 0.1%) present in the batches of technical propoxycarbazone-sodium used for toxicological testing and did not occur in relevant amounts in animal metabolism studies. A limited toxicological profile of these metabolites has been established. For structures and code numbers please refer to Table CA 5.1-2 on page 9.

The soil metabolites had a low to moderate (M09) or a very low (M04, M08) acute oral toxicity in the rat. In the Ames test, all three soil metabolites were negative.
The soil metabolites M10 and M11 were identified as potentially relevant after the initial Annex-I inclusion of propoxycarbazone-sodium.
For the soil metabolite M10, a battery of in-vitro genotoxicity studies was conducted. M10 was not mutagenic, with and without metabolic activation, in an Ames test, a chromosomal aberration test and a HPRT assay. Additionally, a 90-day feeding study in rats (preceded by a 14-day pilot study) was conducted. A subchronic NOAEL of 600 ppm (corresponding to 36.5 and 44.6 mg/kg bw/day for male and female rats, respectively) was established for M10, based on reduced body weights at 6000 ppm the highest dose tested. The effect level and the nature of the effect observed after dietary application of M10 are comparable to those observed in subchronic studies conducted with the active substance. Thus, M10 is considered to be of no toxicological concern.
M11 is the methyl ether derivative of M08. The acute oral toxicity of M11 was expected to be similar to the non-toxic M08 and thus no acute toxicity testing is deemed necessary.
To verify that the methylation does not confer mutagenic properties, a battery of in-vitro genotoxicity study was run with M11. M11 was not mutagenic, with and without metabolic activation, in the Ames test, a chromosomal aberration test as well as an HPRT assay. M11 is considered to be of no toxicological concern.
The plant metabolite M01 had a very favourable toxicological profile in all investigated studies, comparable to that of the parent compound propoxycarbazone-sodium. It was practically non-toxic in the acute oral rat study, not genotoxic in bacterial reverse mutation assay and chromosome aberration studies, and caused no effects in the rat after subacute feeding of 10 000 ppm (approaching the 1000 mg/kg bw/day limit dose).

Taken together, these data indicate the absence of any critical toxicity of the investigated plant and soil metabolites of propoxycarbazone-sodium.

Acceptable Daily Intake (ADI)

Based on the comprehensive toxicological data base, a special hazard for the consumer of treated crops is not expected. The chronic studies did not indicate a carcinogenic potential, and the neurotoxicity test battery did not indicate a neurotoxic potential. Propoxycarbazone-sodium has no teratogenic and no primary reproduction toxic potential in rats and rabbits. All mutagenicity/genotoxicity tests with propoxycarbazone-sodium were negative.
An ADI is usually based on the data from the chronic feeding studies in rats and mice. Thus, the NOAEL of the chronic toxicity/carcinogenicity study in rats which was 1000 ppm (43 mg/kg bw/day) is used for the ADI calculation. Taking into account the favourable toxicological profile, the usual uncertainty factor of 100 is applicable. Therefore, the ADI for propoxycarbazone-sodium can be established at 0.43 mg/kg bw.

Acceptable Operator Exposure Level (AOEL)
Propoxycarbazone-sodium has a very low acute oral, dermal and inhalative toxicity. Propoxycarbazone-sodium is neither an eye- nor a skin irritant, has no skin sensitizing potential and is not a developmental or neurotoxicant. The most appropriate study to use as a basis for the sytemic AOEL is the oral developmental toxicity study in rabbits. Thus, the maternal NOAEL of this study which was 100 mg/kg bw/day is used for the systemic AOEL calculation. Taking into account the favourable toxicological profile and the proportion of dose absorbed from the intestinal lumen, the usual safety factor of 100 and an approximate absorption rate of 30% is applicable to calculate the systemic AOEL.
Therefore, the systemic AOEL for propoxycarbazone-sodium can be established at 0.3 mg/kg bw/d.

Setting a dermal AOEL based on the subacute dermal toxicity study in rats is not appropriate, because the number of animals of this study (5 per gender and dose group) is too low. Furthermore comparing the results of the developmental toxicity studies in rats and rabbits demonstrates that the rabbit was the more sensible species.

Acute Reference Dose (ARfD)
An ARfD is not allocated because on the basis of its toxicological profile, propoxycarbazone-sodium is considered unlikely to present an acute hazard. The acute oral and dermal toxicity of propoxycarbazone-sodium is low.

Drinking water limit
The maximum admissible concentration of an active substance is 0.1 µg/L, as established by Directive 89/778/EEC.