Bayer CropScience

Document Title

Summary of the toxicological and metabolism studies for Trifloxystrobin

Data Requirements

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

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

Date

2013-11-27

Author(s)

[redacted]

[redacted]

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The summaries and evaluations contained in this document are based on unpublished proprietary data submitted for the purpose of the assessment undertaken by the regulatory authority.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authorities should not grant, amend, or renew a registration on the basis of the summaries and evaluation of unpublished proprietary data contained in this document unless they have received the data on which the summaries and evaluation are based, either: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

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

Table of Contents

CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

A dossier on trifloxystrobin (CAS No. 141517-21-7) was submitted January 1998 by the Novartis Crop Protection UK Ltd to the EU RMS United Kingdom for agricultural use as a fungicide. The substance was subsequently transferred to Bayer CropScience. The RMS evaluated the data in a Monograph/DAR and distributed the DAR to the MSs and the European Commission. A final examination by the SCFCAH with participation of experts from the MSs was established by Standing Committee on April 2003. Finally, trifloxystrobin was included into Annex I of the Council Directive 91/414/EEC by the Commission Directive 2003/68/EC of 11 July 2003 as published in the Official Journal of the EU of 16 July 2003. This decision entered into force by 1 October 2003.

Comments with respect to the Annex I renewal process

This supplemental dossier contains only summaries of studies, which were not available at the time of the first Annex I inclusion of trifloxystrobin and were therefore not evaluated during the first EU review of this compound. The summaries on the different toxicological endpoints (information is taken from the Monograph/Addendum III to the DAR (December 2002)) were supplemented and adapted with the new information. In order to facilitate discrimination between new information and original paragraphs, the new information is written in bold letters. All other studies, which were already submitted by Novartis/Syngenta/Bayer for the first Annex I inclusion, are contained in the Monograph/Addendum III to the DAR (December 2002) and in the baseline dossier provided by Bayer CropScience.
A synonymous name for trifloxystrobin used at several locations in this delta dossier is CGA 279202 or the abbreviation TFS.

The following table provides an overview on the batches of trifloxystrobin used in all toxicological studies on this compound. Studies not evaluated during the first EU review are written in bold letters.

Table 5-1: Overview of trifloxystrobin batches used for toxicity studies

Batch Number	Purity (%)	Study type	Reference
EDFL006101	**99.1**	**Trifloxystrobin, isomers and metabolites - Cytotoxicity in rat hepatocytes**	[redacted] **(2013)** **M-463388-01-2**
EDFL006101	**99.1**	**Trifloxystrobin (CGA 279202), Isomers and Metabolites - Studies on potential interactions with the mitochondrial respiration of freshly isolated rat liver mitochondria**	[redacted] **(2013)** **M-463641-01-1**
EDFL006101	**99.1**	**Phototoxicity test**	[redacted] **(2013)** **M-463801-01-1**
EDFL006101	**99.1**	**Rat, 28-day immunotoxicity, oral (diet)**	[redacted] **(2012)** **M-429141-01-1**
EDFL006101	**99.1**	**Trifloxystrobin - Determination by high performance liquid chromatography analysis in ground rodent diet**	[redacted] **& Tahmasseb (2011)** **M-411302-01-1**

Batch Number	Purity (%)	Study type	Reference
GP-940801	95.9	Acute inhalation toxicity, rat	[redacted] (1995) M-040049-01-1
KGL 4617	96.2	Dog, 28-day, (capsule) (range finding)	[redacted] (1994) M-040122-01-1
KGL-4617/5	96.2	Rat, 28-days feeding (range finding)	[redacted] (1994) M-040074-01-1
KGL-4617/5	96.2	Mouse, 3-month feeding (range finding)	[redacted] (1994, amended 1997) M-040129-01-2
KGL-4617/5	96.2	Rat, 3-month feeding	[redacted] (1995, amended 1997) M-040135-01-1
M14415	99.9	Effects of trifloxystrobin (CGA 279202) and its metabolites CGA 321113, CGA 373466, NOA 413161 and NOA 413163 on succinate-supported rat liver mitochondrial respiration	[redacted] (2002, amended 2003) M-034840-02-1
M14415	99.9	Investigation of the hepatotoxic potential of trifloxystrobin and its metabolites on primary rat hepatocytes in an in vitro model	[redacted] (2002 amended 2002) M-090653-02-1
P.405009	96.0[1]	Acute oral toxicity, rats	[redacted] (1994) M-039034-01-1
P.405009	96.4	Acute oral toxicity, mouse (limit test)	[redacted] (1996, amended 1997) M-039046-02-1
P.405009	96.4	Acute dermal toxicity, rat	[redacted] (1995, amended 1997) M-040043-02-1
P.405009	96.0[1]	Acute dermal toxicity, rabbits	[redacted] (1994) M-039075-01-1
P.405009	96.0[1]	Skin irritation, rabbits	[redacted] (1994) M-040053-01-1
P.405009	96.0[1]	Eye irritation, rabbits	[redacted] (1994) M-040060-01-1
P.405009	96.0[1]	Skin sensitization, guinea pigs – (Buehler method)	[redacted] (1994) M-040068-01-1
P.405009	96.4	Skin sensitization test, guinea pig – (maximization test)	[redacted] (1994, amended 1997) M-040063-01-1
P.405009	**95.9**	**Skin sensitization, LLNA (mouse)**	[redacted] **(2003) M-104762-01-1**
P.405009	96.4	Dog, 3-month oral (capsule)	[redacted] (1996, amended 1997) M-040184-01-1
P.405009	96.4	Dog, 12-month chronic oral toxicity	[redacted] (1997) M-040217-01-1
P.405009	96.4	Rat, 28-day dermal toxicity	[redacted] (1996, amended 1997) M-040287-01-1
P.405009	96.4	Bacterial reverse mutation assay	[redacted] (1994) (M-040308-01-1
P.405009	96.4	In vitro mammalian chromosome aberration test, Chinese hamster cells	[redacted] (1994) M-040332-01-1

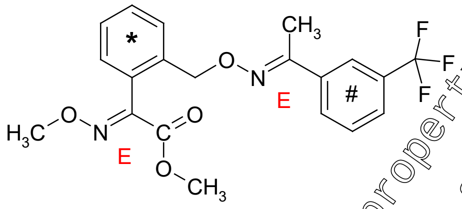
Batch Number	Purity (%)	Study type	Reference
P.405009	96.4	In vitro mammalian cell gene mutation test, Chinese hamster cells V79	[redacted] (1995) M-040439-01-1
P.405009	96.4	In vitro Unscheduled DNA synthesis (UDS), rat hepatocytes	[redacted] (1995) M-040338-01-1
P.405009	96.4	In vivo Micronucleus test, mouse	[redacted] (1994, amended 2000) M-040451-02-1
P.405009	96.4	Rat, 24-month feeding (carcinogenicity and chronic toxicity)	[redacted] (1997, amended 1998) M-040512-02-1
P.405009	96.4	Mouse, 18-months feeding (carcinogenicity)	[redacted], 1997 (amended 1999) M-039533-03-1
P.405009	96.4	Rat dietary two-generation reproduction study	[redacted] (1997, amended 2001) M-039264-02-1
P.405009	96.4	Rat, oral developmental	[redacted] (1995) M-039420-01-1
P.405009	96.4	Rabbit, oral developmental	[redacted] (1994, amended 1999) M-039377-03-1
P.405009	96.4	Rat, acute oral neurotoxicity study	[redacted] (1997, amended 1999) M-039223-03-1

[1] Batch based on FL No. 941274 (Batch code P.405009)

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

Trifloxystrobin (methyl (E)-methoxyimino-{(E)-α-[1-(α,α,α-trifluoro-m-tolyl)ethylidene-aminooxy]-o-toly}acetate, IUPAC) is a fungicide belonging to the group of strobilurin chemical compounds. The mode of action involves inhibition of mitochondrial respiration in fungi. Technical trifloxystrobin consists of a mixture of four diastereomers with the parent substance (EE configuration of the two C=N double bonds) being the dominant isomer. The four isomers in the technical product have a typical composition of parent-E/E : E/Z : Z/E : Z/Z = 95.8 : 1.3 : 1.2 : 0.7.

Trifloxystrobin was ^{14}C-labelled in both of the two phenyl rings of the molecule for investigation of metabolism studies in plants and animals.



* glyoxyl-phenyl-UL-^{14}C; abbr. [14C-GP]

trifluoromethyl-phenyl-UL-^{14}C; abbr. [14C-TP]

Five studies were submitted with the original dossier for evaluation by the RMS:

- An absorption, distribution and excretion study to OECD guideline 417 in the rat using both [Glyoxyl-phenyl-UL-^{14}C] and [Trifluoromethyl-phenyl-UL-^{14}C] labelled trifloxystrobin. This study includes investigations of biliary excretion.
- A follow up absorption, distribution and excretion study investigating some apparent label related differences in tissue distribution at high doses.
- A study investigating the metabolites present in the urine, faeces and bile samples taken from animals in the main absorption, distribution and excretion and its follow-up study
- An *in-vivo* dermal absorption study in rats using trifloxystrobin as an EC formulation.
- An *in-vitro* study comparing the dermal absorption study of the trifloxystrobin EC formulation in isolated rat and human epidermis.

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

Studies on absorption, distribution, metabolism and excretion (ADME) of orally administered trifloxystrobin in the rat were summarized by the RMS in Section B.6.1.3 of the "Summary, Scientific Evaluation and Assessment of Trifloxystrobin" dated April 2000. For a brief overview this summary is repeated in the following paragraphs.

"Summary of mammalian metabolism

After oral administration of trifloxystrobin, the extent of absorption was influenced by the dose level and the sex of the animals. Female rats absorbed about 65% of the low dose (0.5 mg/kg bw) from the GI tract based on urinary and biliary excretion, and tissues residues, whereas in male rats the

extent of absorption was only 56%. At the high dose (100 mg/kg bw), the absorbed portion decreased to about 55% and 45% of the dose in males and females, respectively. Furthermore, the AUC value increased only 130-times compared with a dose level ratio of 200:1.

After administration of [Glyoxyl-Phenyl-UL-^{14}C] trifloxystrobin maximal blood residues were found at 12 to 24 hours. Irrespective of the dose level and the sex of the animals, the residues in all tissues depleted with half-life times of 14 - 40 hours, except blood and spleen of female rats dosed with the high dose (82 and 68 hours respectively).

Seven days after administration of [Glyoxyl-Phenyl-UL-^{14}C] trifloxystrobin at the low dose (0.5 mg/kg), the tissue residues were very low (total residues <0.5% of the administered dose). At the high dose residues were about 126 and 108-fold higher than the low dose level, in males and females respectively. Some sex and label-specific differences in tissue residues were observed at the high dose level. Generally tissue residues were higher in females than males. Label-specific differences were noted in the fat, kidneys, liver and plasma.

Within 48 hours, 72 - 96% of the dose was eliminated with the urine and faeces independent of the dose level, pretreatment with non-radiolabelled trifloxystrobin, the site of label, and the sex of the animals. However, the routes of elimination were different in male and female rats. Within seven days, male rats eliminated approximately 15% of the dose via kidneys while females excreted 33% in the urine. Bile-duct cannulated rats demonstrate that the bile (ca. 44% of the dose) is the principal route of elimination in both males and females. There was evidence of the involvement of enterohepatic circulation in the excretion process.

The half-life times of the tissue residues after oral administration of trifloxystrobin at a low and high dose, were very similar with either of the two labels demonstrating that the small amount of label-specific metabolites formed do not influence the overall depletion kinetics.

Trifloxystrobin was extensively metabolised at the low dose level (0.5 mg/kg), since ca. 5% of the administered trifloxystrobin was found as unchanged parent in faeces, whereas at the high dose level (100 mg/kg bw) 31-47% of unchanged parent was found in faeces. The amount of parent found in faeces at the low dose does not correlate well with levels of absorption seen at this dose. This may be an artefact of the relatively low recoveries of radiolabel from the faeces at the low dose.

In most experimental animal groups the metabolites identified accounted for between ca. 60 – 70% of the administered dose. The extent of characterisation is considered acceptable given the extensive metabolism of trifloxystrobin.

The reactions involved in the major metabolic pathways of trifloxystrobin were:

(1) hydrolysis of the methyl ester to the corresponding acid,
(2) demethylation of the methoxyimino group yielding a hydroxyimino compound,
(3) oxidation of the methyl side chain to a primary alcohol, followed by partial oxidation to the respective carboxylic acid, and
(4) cleavage of the bridge between the glyoxyl-phenyl and trifluoromethylphenyl moiety.

The major metabolic pathways of trifloxystrobin operative in the rat were not significantly influenced by the dose and pretreatment but were by the sex of the animals resulting in some female specific urinary metabolites.

The sex dependent excretion pattern and the sex-related differences in tissue residues indicated quantitative and/or qualitative differences in the metabolism of trifloxystrobin in male and female rats. This gender based difference in elimination route was considered to be a result of unique metabolism

in females, and not related to differences in relative abundance and preferred route of elimination of common metabolites.

Based on the structures of the urinary, faecal, and biliary metabolites identified by spectroscopic means ('H-NMR and MS) or characterised the metabolic pathways operative in the rat were proposed as summarized in Figure 5.1.1- 1."

Figure 5.1.1- 1: Proposed metabolism of trifloxystrobin in rat, goat and hen

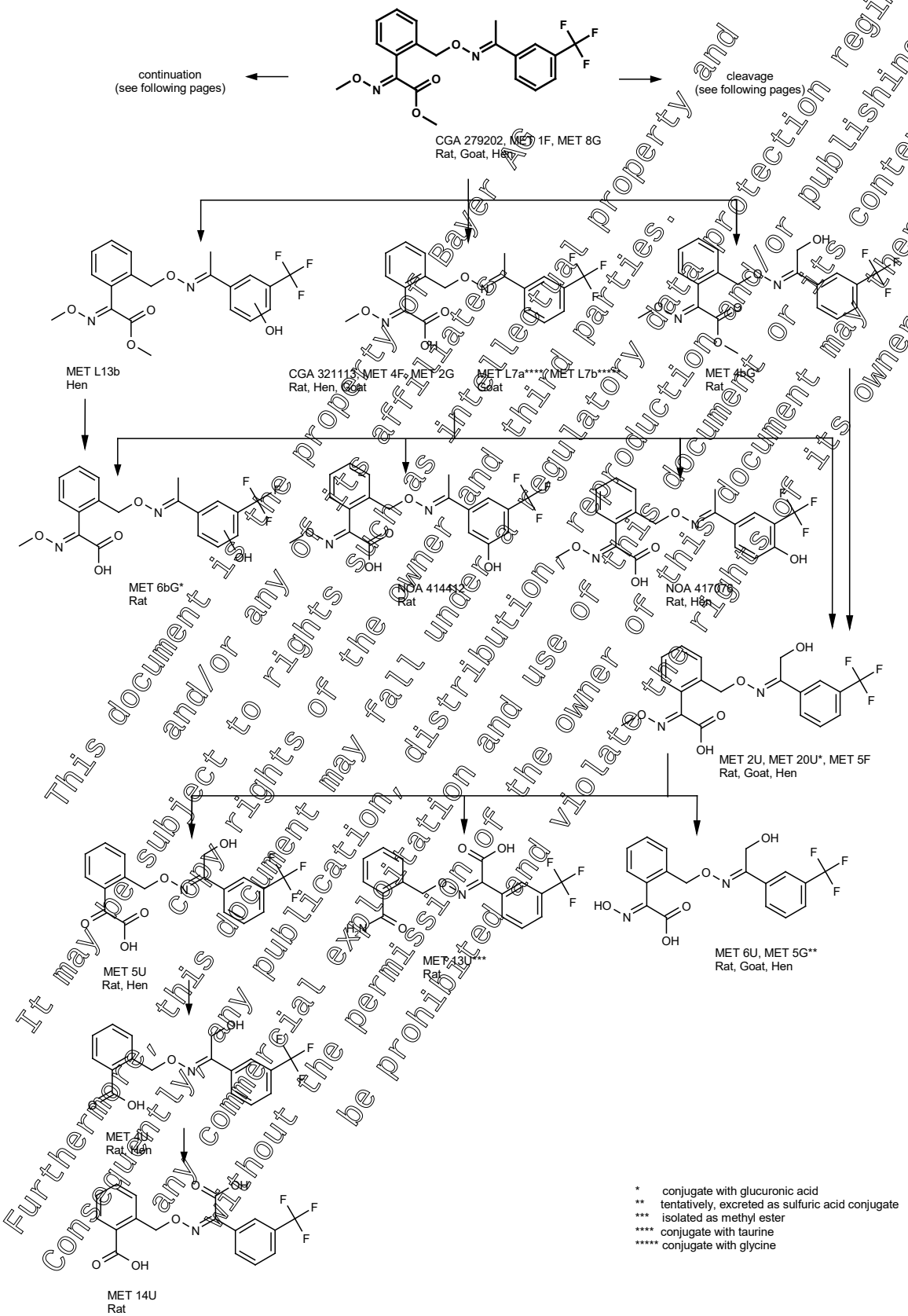


Figure 5.1.1- 1: Proposed metabolism of trifloxystrobin in rat, goat and hen (contd.)

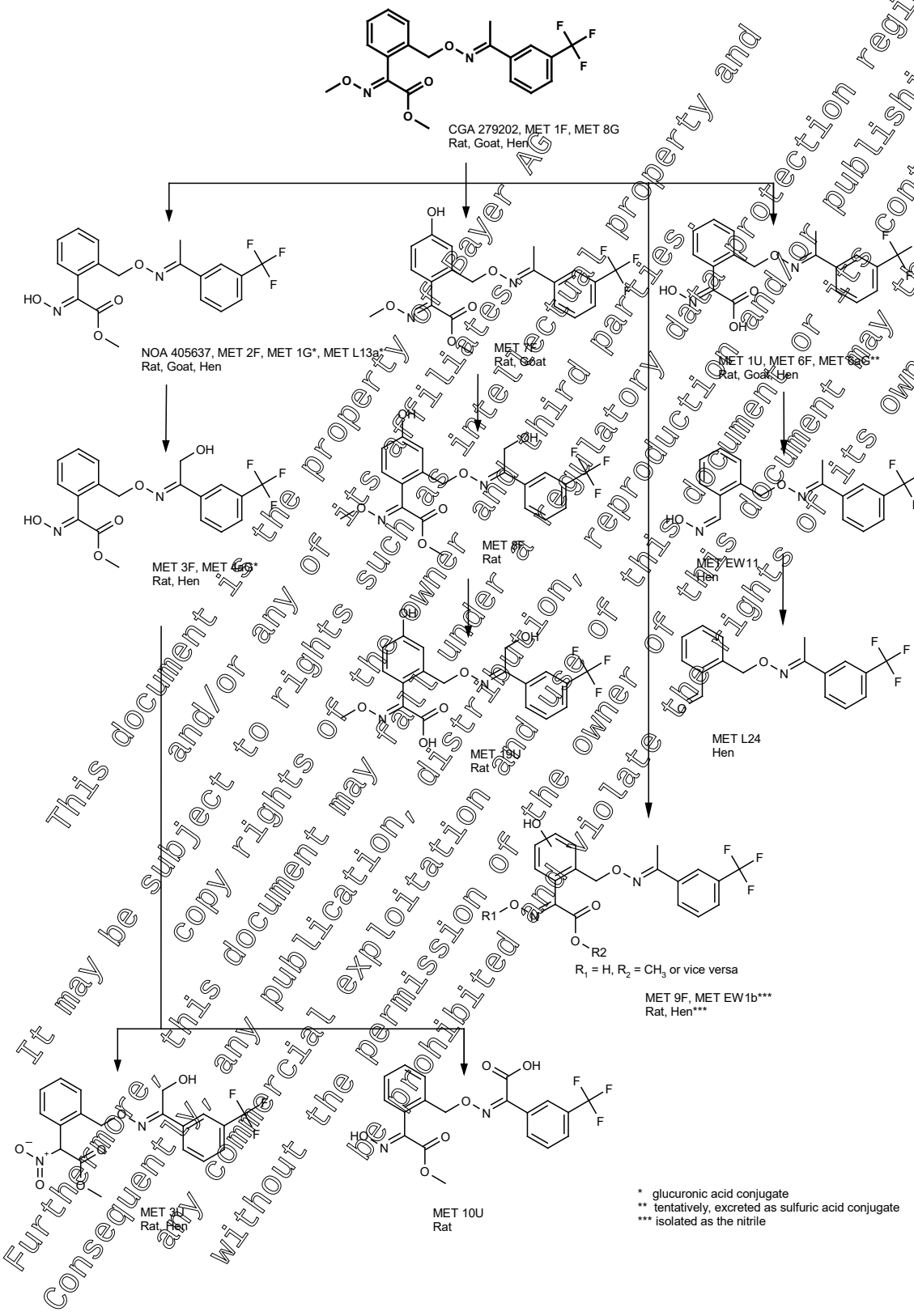
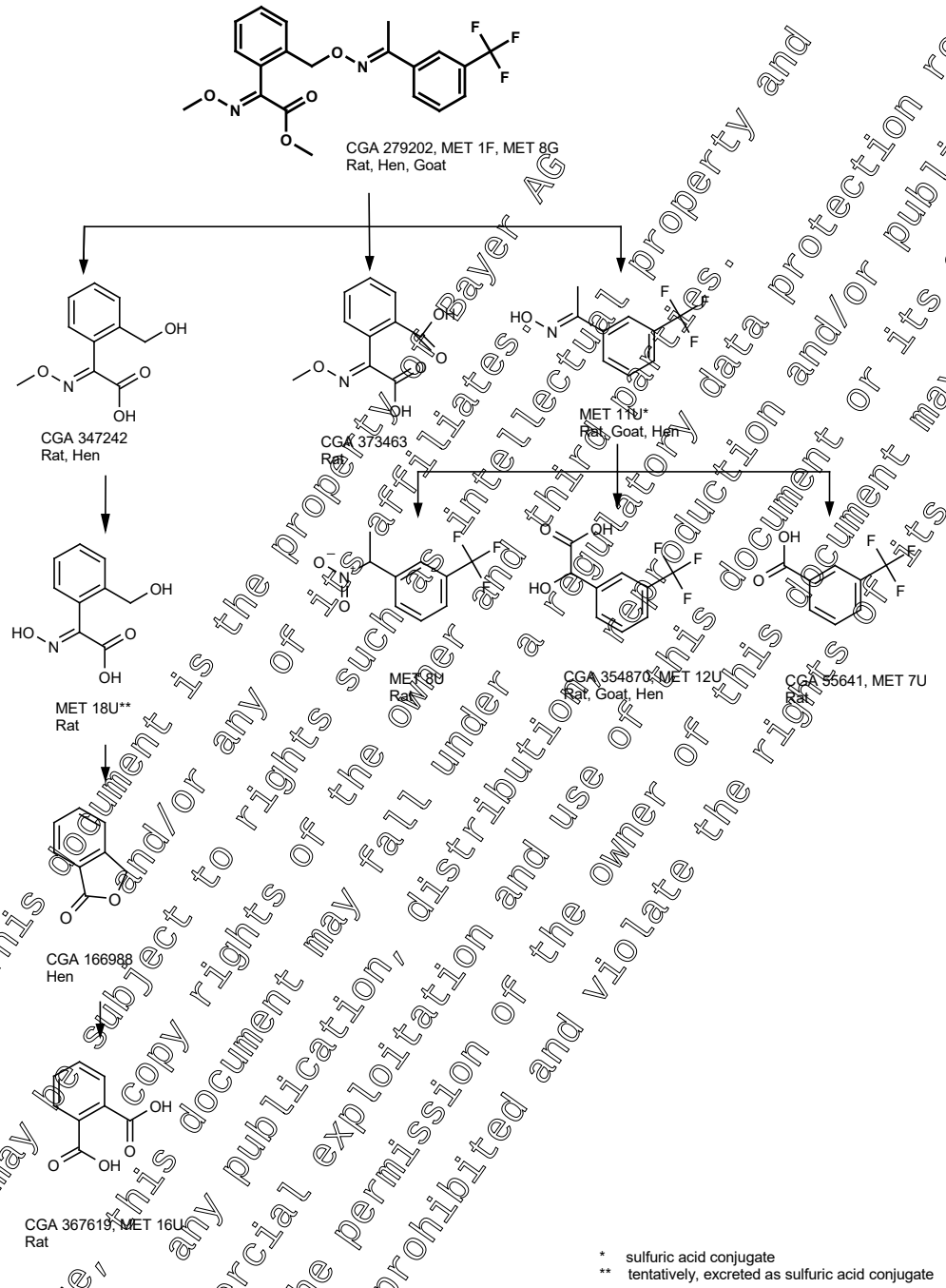


Figure 5.1.1- 1: Proposed metabolism of trifloxystrobin in rat, goat and hen (cleavage, contd.)



According to the data requirements published in the Commission Regulation (EU) No 283/2013 of 1-March-2013 a "comparative *in-vitro* metabolism study" should performed "on animal species to be used in pivotal studies and on human materials (microsomes or intact cell systems) in order to determine the relevance of the toxicological animal data …" However, no official test guideline of guidance exists at present.

In these cases, waiving of this particular data requirement is considered acceptable according to the "Guidance document for applicants on preparing dossiers for the approval of a chemical new active substance and the renewal of approval of the chemical active substance according to regulation (EU) No. 283/2013 and regulation (EU) No. 284/2013" (SANCO/10181/2013-rev.2 of 2-May-2013).

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

No studies on absorption, distribution, metabolism and excretion by other routes (e.g. intravenous or dermal) are available or needed from the toxicokinetics point of view.

CA 5.2 Acute toxicity

Table 5.2-1: Summary of acute toxicity, irritation and sensitization studies*

Route/Study	Species	Sex	Results	Reference
Oral	Rat	M F	LD_{50} >5000 mg/kg bw LD_{50} >5000 mg/kg bw	[redacted] (1994) M-039034-01-1
Oral	Mouse	M F	LD_{50} >5000 mg/kg bw LD_{50} >5000 mg/kg bw	[redacted] (1996) M-039046-02-1
Dermal	Rat	M F	LD_{50} >2000 mg/kg bw LD_{50} >2000 mg/kg bw	[redacted] (1995) M-040043-02-1
Dermal	Rabbit	M F	LD_{50} >2000 mg/kg bw LD_{50} >2000 mg/kg bw	[redacted] (1994) M-039075-01-1
Inhalation	Rat	M F	LC_{50} >4.6 mg/L	[redacted] (1995) M-040049-01-1
Skin irritation	Rabbit	M/F	slight irritant	[redacted] (1994) M-040053-01-1
Eye irritation	Rabbit	M/F	moderate irritant	[redacted] (1994) M-040060-01-1
Skin sensitization M&K method	Guinea pig	M/F	positive	[redacted] (1994) M-040063-01-1
Skin sensitization Buehler method	Guinea pig	M/F	negative	[redacted] (1994) M-040068-01-1
Skin sensitization Local lymph node assay	**Mouse**	**F**	**negative**	**[redacted] (2003) M-104762-01-1**
***In vitro* 3T3 NRU phototoxicity test**	**BALB/c 3T3 cells**		**negative**	**[redacted] (2013) M-463801-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold
M = male, F= female

Trifloxystrobin is of low acute toxicity via the oral, inhalational and dermal routes. It has a slight skin and moderate eye irritation potential but which does not warrant classification. A skin sensitization potential was demonstrated in a Maximization test but not in the modified Buehler test **and not in the modified local lymph node assay (LLNA) in mice up to the highest test concentration of 30%. Furthermore, trifloxystrobin does not show a phototoxic potential.**

CA 5.2.1 Oral

All necessary acute toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.2.2 Dermal

All necessary acute toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.2.3 Inhalation

All necessary acute toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.2.4 Skin irritation

All necessary acute toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.2.5 Eye irritation

All necessary acute toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.2.6 Skin sensitization

In addition to the two studies on skin sensitization already available in the Monograph and baseline dossier a new local lymph node assay was performed in 2003 in order to compare study results of the new testing method with the results of the existing studies.

Report:	KCA 5.2.6 /08; [redacted];2003;M-104762-01
Title:	CGA 279202 - Local lymph node assay in mice (LLNA/IMDS)
Report No:	AT00432
Document No:	M-104762-01-1
Guidelines:	**OECD 406; Guideline 96/54/EC, Method B.6; US-EPA 712-C-03-197, OPPTS 870.2600; OECD 429** **Deviation(s): none**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: CGA 279202

Description:	Beige brown powder
Lot/Batch no:	P405009
Purity:	95.9%
Stability of test compound:	guaranteed for study duration; expiry date: 2003-03-03

2. Vehicle: Dimethylacetamide (40%), acetone (30%), ethanol (30%) (= DEA 433)

3. Test animals

Species:		NMRI mice
Strain:		Hsd Win:NMRI
Age:		8 - 9 weeks
Weight at dosing:		25 g - 35 g
Source:		[redacted] GmbH, [redacted], Germany
Acclimatisation period:		at least seven days
Diet:		[redacted] SA 3883 maintenance diet for rats and mice ([redacted] SA, Kaiseraugst, Switzerland), *ad libitum*
Water:		tap water, *ad libitum*
Housing:	adaptation	up to 8 mice conventional in Makrolon type III cages
	study period	1 animal in type IIa cages bedding: low-dust wood granulate (J. Rettenmaier & Söhne Füllstoff-Fabriken, Germany)

B. Study design and methods

1. Animal assignment and treatment

Dose	0%-3%-10%-30%.
Application route:	epicutaneously onto the dorsal part of both ears
Application volume:	25 µL/ear
Duration:	three consecutive days (d1, d2, d3)
Group size:	6 females/group
Observations:	local lymph node weight and cell count determination, ear swelling, ear weight, body weight (at beginning and termination of study)

II. Results and discussion

A. Findings

The NMRI mice did not show any significant dose-dependent increase in the stimulation indices for the local lymph node weight or cell counts or ear swelling or ear weights.
The NMRI mice did not show an increase in the stimulation indices for cell counts or for the local lymph node weight. The "positive level" which is 1.3 for the cell count index was never reached or exceeded in any dose group.
The "positive level" of ear swelling which is $2x10^{-2}$ mm increase has also not been exceeded in any dose group. And no increases of the ear weights could be determined compared to control animals.

Body weights of the animals were not affected by treatment.

No activation of the cells of the immune system via dermal route was determined after application of up to and including 30% trifloxystrobin by the LLNA/IMDS method.

Figure 5.2.6-1: Stimulation indices of local lymph node weight and cell counts after application of 0, 3, 10, 30% trifloxystrobin for 3 consecutive days to the ears of mice

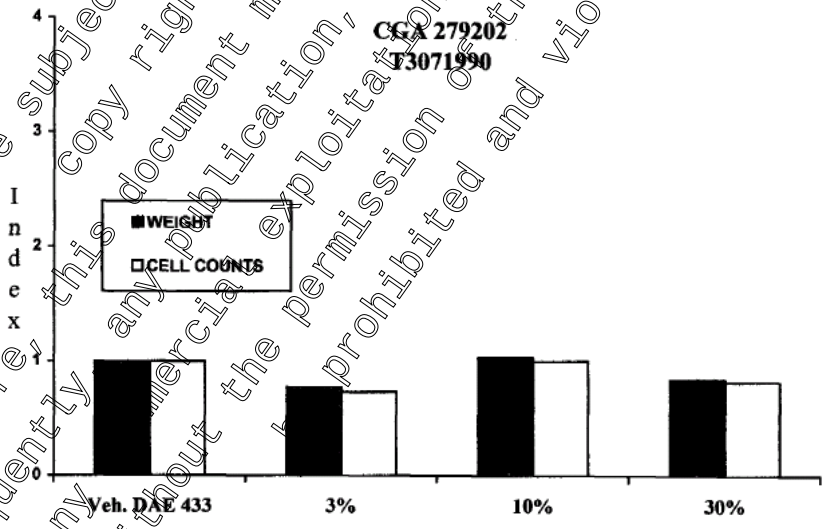


Table 5.2.6-1: Summary of LLNA results

Dose (%)	Direct LLNA Weight index (Mean ± SD in %)	Direct LLNA Cell count index (Mean ± SD in %)	Ear swelling Day 1 (Mean ± SD in %)	Ear swelling Day 4 (Mean ± SD in %)	Ear swelling Index day 4	Ear weight Day 4 (Mean ± SD in %)	Ear weight Index day 4
0*	1.00 ± 22.68	1.00 ± 30.25	17.83 ± 4.02	18.08 ± 6.86	1.00	11.13 ± 8.69	1.00
3	0.77 ± 13.73	0.73 ± 23.48	17.92 ± 3.73	18.08 ± 4.39	1.00	11.37 ± 5.30	1.02
10	1.03 ± 17.78	1.00 ± 21.52	17.25 ± 3.60	17.83 ± 4.68	0.99	11.33 ± 6.03	1.02
30	0.84 ± 38.14	0.82 ± 39.74	17.50 ± 3.85	18.33 ± 4.25	1.01	11.45 ± 10.19	1.03

* = vehicle control (DAE 433)

III. Conclusion

Trifloxystrobin shows no skin sensitizing potential in the local lymph node assay in mice. Moreover, no hint for a substance specific or non-specific activation of the cells of the immune system *via* dermal application was found in this study.

CA 5.2.7 Phototoxicity

According to the new data requirements (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013) (1), the conduct of a phototoxicity study is required under certain conditions.

The Circumstances in which a phototoxicity study according to the new data requirements is required is "*where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance is less than 10 L x mol-1 x cm-1, no toxicity testing is required.*"

As the Ultraviolet/visible molar extinction/absorption coefficient of the active substance exceeds the trigger of 10 L x mol-1 x cm-1 a cytotoxicity assay in vitro with BALB/c 3T3 cells has been performed.

Report: KCA 5.2.7 /01, [redacted];2013;M-463801-01
Title: Trifloxystrobin TC: Cytotoxicity assay in vitro with BALB/c 3T3 cells - Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No.: 1561100
Document No: M-463801-01-1
Guidelines: **Commission Regulation (EC) No. 440/2008 B 41; Committee for Proprietary Medicinal Products (CPMP), CPMP/SWP/398/01; OECD 432;**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	Trifloxystrobin TC
Synonyms:	CGA 279202; AE C642802
Description:	Light beige powder

Lot/Batch no:	EDFL006101
Purity:	99.1% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2014-11-12
2. Vehicle and or positive control:	Solvent control: Earle´s Balanced Salt Solution (EBSS) containing 1% (v/v) dimethylsulfoxide (DMSO). Positive control: chlorpropmazine (Sigma) dissolved in EBSS
3. Test system:	BALB/c 3T3 cell clone 31
Culture medium:	Dulbecco´s Minimal Essential Medium (DMEM) supplemented with 10% (v/v) NCS.
Cell cultures:	Thawed stock cultures were propagated at 37 ± 1.5 °C in 75 cm² plastic flasks. Seeding was done with about 1 x 10^6 cells per flask in 15 mL DMEM, supplemented with 10 % NCS. Cells were sub-cultured twice weekly. The cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

B. Study design and methods

1. Treatment

Dose:

Test item	+/– UV	Final concentrations in µg/mL*
TFS*	+/–	0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25
Positive control**	+	6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	–	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Solvent control	+/–	EBSS containing 1 % (v/v) DMSO

* trifloxystrobin, ** chlorpromazine

The test item trifloxystrobin was dissolved in DMSO. The final concentration of the solvent in EBSS was 1 % (v/v).

Seeding of cultures:	2 x 10^4 cells per well were seeded in 100 µL culture medium in two 96 well plates
Replicates:	2 (one for irradiation exposure, one for treatment in the dark)
Treatment & irradiation:	24 h after seeding the cultures were washed with EBSS. 100 µL solved test item added per well for pre-incubation of the plates for 1 hour in the dark. Afterwards one plate was irradiated at 2.7 mW/cm² (8.1 J/cm²) for 50 min ± 2 min at 20-31 °C, the other plate was stored for 50 min ± 2 min at 20-31 °C in the dark. Test item was removed and both plates were washed with EBSS. Fresh culture medium was added and the plates were incubated about 21.5 hours at 37 ± 1.5 °C and 7.5 ± 0.5 % CO_2.
Cytotoxicity determination:	For measurement of Neutral Red uptake the medium was removed and 0.1 mL serum-free medium containing 50 µg Neutral Red / mL were added to each well. The plates were incubated for another 3hours at 37°, before the medium was removed completely and the cells were washed with EBSS. For extraction of the dye 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approximately 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular

	Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.
Number of measurements:	Trifloxystrobin and positive control: each concentration was measured 6 times Solvent control: 12 times

2. Evaluation

The mean absorption (OD_{540}) value per concentration was calculated. The ED_{50}* values were determined by curve fitting by software. The Photo-irritancy factor (PIF), as well as the Mean Phototoxic effect (MPE) was calculated according to OECD guideline 432.

*ED_{50} = effective dose where only 50% of the cells survived

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

II. Results and discussion

In the range finding experiment (RFE) no cytotoxic effects were observed after exposure of the cells to the test item trifloxystrobin, neither in the presence nor in the absence of irradiation to artificial sunlight. Therefore, ED_{50}-values and PIF could not be calculated. The resulting MPE-value was 0.004.

In the main experiment (ME) the highest test item concentration of 31.25 µg/mL caused a cytotoxic effect in the presence and absence of light. The cell viabilities decreased slightly below the threshold for cytotoxicity of 70% (66.20% and 69.85%). Since the viabilities were not reduced below 50%, ED50-values or a PIF could not be calculated. The resulting MPE-value was -0.008.

MPE-values in all experiments were <0.1

The mean of solvent control values of the irradiated versus the non-irradiated group met the acceptance criteria. The positive control chlorpromazine induced phototoxicity in the expected range in the presence of irradiation.

The results are summarized in Table 5.2.7-1 and Table 5.2.7-2 below.

Table 5.2.7-1: OD_{540} values Neutral Red assay of the main experiment

Con-centration [µg/mL]	OD_{540} with artificial sunlight Mean	SD	% of solvent control	Con-centration [µg/mL]	OD_{540} without artificial sunlight Mean	SD	% of solvent control
Treatment with trifloxystrobin							
Solvent control	0.7210*	0.0431	100.00	**Solvent control**	0.8301*	0.0308	100.00
0.24	0.7828	0.0251	108.57	0.24	0.8334	0.0265	100.40
0.49	0.7589	0.0115	105.25	0.49	0.8282	0.0380	99.77
0.98	0.7569	0.0236	104.98	0.98	0.8326	0.0227	100.31
1.95	0.7288	0.0213	101.08	1.95	0.8174	0.0135	98.47
3.91	0.6624	0.0371	91.87	3.91	0.8138	0.0158	98.04
7.81	0.5842	0.0241	81.02	7.81	0.7253	0.0225	87.38
15.63	0.5105	0.0313	70.81	15.63	0.6318	0.0387	76.11
31.25	0.4773	0.0180	66.20	31.25	0.5798	0.0129	69.85
Treatment with positive control chlorpromazine							
Solvent control	0.7585*	0.0337	100.00	**Solvent control**	0.8954*	0.1066	100.00
0.125	0.6415	0.0260	84.57	6.25	0.9295	0.0879	103.80
0.250	0.3976	0.0362	52.41	12.50	0.5612	0.1113	62.67
0.500	0.0748	0.0138	9.87	25.00	0.0677	0.0058	7.56
0.750	0.0671	0.0064	8.85	37.50	0.0507	0.0022	5.66
1.000	0.0660	0.0013	8.70	50.00	0.0492	0.0037	5.49
1.500	0.0705	0.0079	9.29	75.00	0.0507	0.0025	5.66
2.000	0.0726	0.0213	9.57	100.00	0.0483	0.0016	5.40
4.000	0.0725	0.0094	9.56	200.00	0.0493	0.0016	5.50

* mean OD_{540} out of 12 wells

Table 5.2.7-2: Summary of results of the Neutral Red assay

	Substance	ED_{50} (+UV) [µg/mL]	ED_{50} (−UV) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated vs. non-irradiated plate
Range finding experiment	Trifloxystrobin	--	--	--	0.004	91.2
	Positive control	0.46	11.33	24.92	0.594	108.0
Main experiment	Trifloxystrobin	--	--	--	-0.008	86.9
	Positive control	0.25	13.38	54.40	0.740	84.7

ED_{50} = effective dose where only 50% of the cells survived
PIF = Photo-Irritation Factor
MPE = Mean Phototoxic effect

III. Conclusions

Trifloxystrobin is not phototoxic on BALB/c 3T3 cells.

CA 5.3 Short-term toxicity

Table 5.3-1: Summary of short-term toxicity studies

Study	Sex	NOAEL (mg/kg bw/d)	LOAEL	Main effects seen at LOAEL	Reference
Rat, 28-days feeding	M F	17 84	84 327	Clinical signs, reduced bodyweight gains, organ weight changes	[redacted] (1994) M-040074-01-1
Rat, 90-days feeding	M F	6.4 32.8	31 133	Reduced food consumption and bodyweight gain, increased liver and kidney weights	[redacted] (1997) M-040135-01-1
Dog, 28-days (capsule)	M F	20 50	50 50/500	Clinical findings, reduced food consumption and bodyweight effects	[redacted], 1994 M-040122-01-1
Dog, 90-days (capsule)	M F	30	150	Bodyweight loss, reduced food intake, liver effects (serum chemistry, hepatocyte hypertrophy)	[redacted] (1997) M-040184-01-1
Dog, 12-month (capsule)	M F	5	50	Reduced bodyweight gain and food consumption, liver effects (increased organ weight, serum chemistry, clotting times hepatocyte hypertrophy)	[redacted] (1997) M-040217-01-1
Mouse, 90-day feeding	M F	77 110*	315 425	Increased liver weight, liver hepatocyte necrosis, spleen extramedullary hematopoiesis	[redacted] (1997) M-040129-01-2
Rat, 28-days dermal	M F	100 >1000	1000	Increased liver and kidney weights	[redacted] (1997) M-040287-01-1

M = male, F = female, * = LOEL

Short-term toxicity of trifloxystrobin was assessed in two range finding 28-day studies in rats (dietary) and dogs (capsules), 90 day studies in rats (dietary), mice (dietary), and dog (capsules), and a one year dog study (capsules). A 28 day dermal toxicity study in the rat was also submitted.

All studies apart from the two range finding 28-day studies (non-guideline studies) and the 90 day mouse study met the essential requirements of their respective guidelines. The 90 day study in the mouse was of a limited design, since it was intended as a range finder for the carcinogenicity study.

In the rat there were reductions in bodyweight gains and food consumption at top dose levels. The main organs affected were liver, kidney and pancreas with increased organ weights or histopathological changes seen at higher doses. Major histopathological observations were hepatocellular hypertrophy, acute tubular lesions in kidney and atrophy in the endocrine pancreas. Changes were partly reversible following a recovery period. No signs of neurotoxicity could be detected in a functional observational battery, motor activity and neuropathological examinations, at any doses tested in the course of the 90-days feeding study. Following repeated dermal administration of trifloxystrobin to rats over 28 days the only indications of systemic toxicity were increased liver and kidney weights.

In mice dietary exposure to trifloxystrobin resulted in a reduction of bodyweight gain, slightly increased food consumption and a markedly increased water consumption (females) at top dose levels. There was evidence of effects on the liver and spleen with increased organ weights or histopathological changes seen at higher doses. Histopathological changes include hepatocellular hypertrophy and necrosis in liver as well as hemosiderosis and extramedullary hematopoiesis in spleen.

Effects on bodyweights and food consumption as well as vomiting and diarrhoea were noted in all the dog studies. Effects were seen on the liver (changes in blood chemistry, increased liver weights and hepatocellular hypertrophy), gall bladder (hyperplasia of gall bladder epithelium) and haematopoietic

system (including lowered erythrocyte, haemoglobin and haematocrit values and eosinopenia). There was some evidence of bone marrow toxicity.

CA 5.3.1 Oral 28-day study

All necessary toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.3.2 Oral 90-day study

All necessary toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.3.3 Other routes

All necessary toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.4 Genotoxicity testing

Table 5.4-1: Summary of genotoxicity testing

Study	Test system	Results		Reference
		activation	non-activation	
In-vitro				
Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537	negative	negative	[redacted] (1994) M-040308-01-1
Mammalian cell gene mutation test	Chinese hamster lung fibroblasts V79	negative	equivocal	[redacted] (1995) M-040439-01-1
Mammalian chromosome aberration test	Chinese hamster ovary cells CHO	negative	negative	[redacted] (1994) M-040332-01-1
Unscheduled DNA synthesis (UDS) assay	Primary rat hepatocytes		negative	[redacted] (1995) M-040338-01-1
In-vivo				
Micronucleus test	Mouse bone marrow		negative	[redacted] (1995) M-040451-02-1

When tested *in vitro*, trifloxystrobin was negative in an Ames test, a cytogenetic test in CHO cells and an UDS assay in rat hepatocytes. However an equivocal mutagenic effect was observed in a gene mutation assay in Chinese hamster V79 cells, although this occurred under extreme conditions. There was neither an evidence of chromosomal damage in an *in vivo* micronucleus study in the mouse nor an evidence of induction of DNA damage after in-vivo treatment of trifloxystrobin.

There was no evidence of chromosomal damage in an *in vivo* micronucleus study in the mouse. Trifloxystrobin and/or its metabolites did reach the bone marrow based on evidence from studies with radiolabelled trifloxystrobin although it is noted that different dosing vehicles were used in the ADME studies compared with the micronucleus study.

Overall it is concluded that trifloxystrobin demonstrates no genotoxic potential. It is also noted that there was no evidence of carcinogenicity in the long term studies.
Furthermore, there is no evidence of an effect on germ cells in other studies. Therefore, an *in vivo* study in germ cells was not regarded necessary.

Photomutagenicity

According to the new data requirements (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013) (1), the conduct of a photomutagenicity study may be indicated by the structure of the molecule.
If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance and its major metabolites is less than 1000 L × mol^{-1} × cm^{-1}, photomutagenicity testing is <u>not</u> required.

Trifloxystrobin does not have structural peculiarities, like presence of chromophors, particularly of substructures containing multiple interfering π-bonds allowing the construction of extended mesomeric structures which could indicate a potential to cause photochemical effects. In addition, for trifloxystrobin there is no evidence of a photo-reactivity potential (see chapter CA 5.2.7; KCA 5.2.7/01, M-463801-01-1). Thus, no photo-safety concern is expected and no further testing is required (for further details please refer to [redacted], 2013, M-467988-01-1).

CA 5.4.1 In vitro studies

Information presented and evaluated during the EU Annex I listing process. Please refer to Monograph and baseline dossier of trifloxystrobin.

CA 5.4.2 In vivo studies in somatic cells

Information presented and evaluated during the EU Annex I listing process. Please refer to Monograph and baseline dossier of trifloxystrobin.

CA 5.4.3 In vivo studies in germ cells

Overall it is concluded that trifloxystrobin did not show a genotoxic potential and no evidence of an effect on germ cells was seen in other studies. Therefore, an *in vivo* study in germ cells is not regarded necessary.

CA 5.5 Long-term toxicity and carcinogenicity

Table 5.5-1: Summary of short-term toxicity studies

Study	Sex	NOAEL (mg/kg bw/d)	LOAEL	Main effects seen at LOAEL	Reference
Rat, 2-year feeding	M F	9.8	29.7	Reduced bodyweight gain	[redacted] (1998) M-040512-02-1
Mouse, 18-month feeding	M F	35.7	124	Reduced bodyweight gain, increased liver weight and microscopic liver changes	[redacted] (1999) M-039533-03-1

M = male, F = female, * = LOEL

A combined 24-month toxicity/carcinogenicity study in rats, and an 18-month carcinogenicity study in mice have been submitted, both of which meet the essential requirements of their respective guidelines. Both studies achieved an MTD and there was no clear evidence for a carcinogenic potential in either species.

In the rat higher doses resulted in reduced body weight development and diminished food intake. Probably as a consequence of this survival was significantly improved in high dose animals. Although there were increases seen in absolute and/or relative liver, heart and kidney weights there was no clear correlated findings in serum chemistry parameters, nor histopathological observations. There were clear treatment related decreased incidences of some neoplastic and non-neoplastic findings, but no clear indication of any tumorigenic potential.

In mice reduced body weight development in both sexes at the top dose level, with slightly decreased food consumption in high dose females was observed. Liver toxicity was apparent with increases in absolute and relative weights and microscopic changes including single cell necrosis, necrosis, fatty change and hepatocellular hypertrophy. No treatment related effects were seen for neoplastic changes.

CA 5.6 Reproductive toxicity

Table 5.6-1: Summary of reproduction toxicity studies

Study	Sex	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Main effects seen at LOAEL	Reference
Rat, 2-gen feeding	M F	2.2-7.5 3.0-10.4	33-67 48-120	Reduced food consumption and body weight gain in parent F0 and F1 animals, retarded pup body weight gains during lactation	[redacted] (1997) M-039264-02-1
Rat, oral developmental	D Fet	100 100	1000 1000	Reduced maternal body weight gain and food consumption, fetal thymus enlarged	[redacted] (1999) M-039420-01-1
Rabbit, oral developmental	D Fet	50 50	250 250	Reduced maternal body weight gain and food consumption, slightly increased incidence of fused sternebrae	[redacted] (1999) M-039377-03-1

M = male, F= female, D = dam, Fet = fetus

Effects on reproduction were investigated in a 2-generation feeding study in rats. The teratogenic potential of trifloxystrobin was investigated in two studies, one in rats and one in rabbits, both by gavage dosing. All these studies met the essential requirements of their respective guidelines.

No toxic effects on reproduction were found in a rat 2-generation feeding study with trifloxystrobin at any dose level tested. The highest dose in parental animals of both sexes and the intermediate dose in female parents resulted in reduced food consumption as well as in a retarded bodyweight development. Both dose levels caused a reduced body weight gain in pups of both sexes. Target organs were liver and kidney.

There was no evidence of a teratogenic effect in either rats or rabbits.

Treatment of pregnant female rats with trifloxystrobin resulted in reduced bodyweight gain and lowered food consumption of the dams at the top dose. Reproductive parameters were not affected. Enlarged thymus was found in fetuses of the high dose group. In rabbits, maternal body weight gain and food consumption were reduced after oral treatment of trifloxystrobin at the two higher dose levels. No effects on reproduction parameters were seen. There was a slightly increased incidence of fused sternebrae in fetuses which obtained statistically significance at the top dose.

CA 5.6.1 Generational studies

All necessary toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.6.2 Developmental toxicity studies

All necessary toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.7 Neurotoxicity studies

Table 5.7- 1: Summary of neurotoxicity studies

Study	Sex	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Main effects seen at LOAEL	Reference
Acute oral neurotoxicity	M F	2000	---	No evidence for a neurotoxic effect	[redacted] (1999) M-039223-03-1
Rat, 90-days feeding*	M F	6.4 32.8	31 133	Reduced food consumption and bodyweight gain, increased liver and kidney weights No evidence for a neurotoxic effect	[redacted] (1997) M-040135-01-1

*90-day study with neurotoxicity evaluations

The neurotoxic potential of trifloxystrobin was investigated in rats in an acute neurotoxicity study and as part of a 90 day dietary study (CA 5.3, M-040135-01-1). There was no evidence of neurotoxicity in either study.

CA 5.7.1 Neurotoxicity studies in rodents

All necessary toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 5.7.2 Delayed polyneuropathy studies

Trifloxystrobin does not belong to a chemical class which is suspected to cause delayed neurotoxic effects (organophosphates, carbamates). Therefore, specific studies on delayed neurotoxicity are not necessary.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

Summaries of studies with metabolites

For the EU review process, the toxicological properties of several plant and/or soil metabolites (CGA 373466, CGA 357261, NOA 414412, NOA 413163 and NOA 413161) were investigated in acute oral toxicity studies and Ames tests. All metabolites were found to be of low acute oral toxicity to rats (LD50 >2000 mg/kg bw), and not mutagenic to bacteria.

The data base on metabolites has been supplemented as the parent compound trifloxystrobin shows an extensive metabolic behavior in rats and livestock and also in the majority of crops. Several plant metabolites were not detected as systemic metabolites in the rat ADME studies. Depending on the occurrence and the quantity of the metabolites to be addressed, a suitable approach has been chosen in order to meet the regulatory requirements and suffice the most recent scientific developments as addressed in the EFSA Scientific Opinion on evaluation of the toxicological relevance of pesticide metabolites for dietary risk assessment (EFSA Journal 2012;10(7):2799).
The toxicological profile and exposure assessment includes trifloxystrobin metabolites

(1) exceeding the trigger of 0.01 mg/kg in raw agricultural commodities relevant for human consumption
(2) exceeding the trigger of 0.05 mg/kg of raw animal fodder (e.g. cereal straw).

It has to be noted that individual metabolites occur in food items/feeding items and are predicted to reach groundwater in some scenarios.

For the detailed toxicological assessment the metabolites are grouped as follows:

- Photo-isomers of the parent compound trifloxystrobin
  CGA 357261 (ZE-isomer), CGA 331409 (EZ-isomer), CGA 357262 (ZZ-isomer)
- Rat metabolites originating from trifloxystrobin
  CGA 321113, NOA 413161, NOA 443152, NOA 414412, CGA 347242, CGA 367619
- Metabolites of the ZE-isomer of trifloxystrobin
  CGA 373466, NOA 413163, BO 172741
- Label specific metabolites formed by cleavage of the ethylideneaminooxy bridge between the phenyl rings
  CGA 367619 (also a rat metabolite), THW0115C, SA 04271

The detailed toxicological assessment of these metabolites can be found in the document M-469186-01-1 ("Trifloxystrobin - Toxicological profile and exposure assessment of the plant metabolites"). Based on commonality assessments, structure similarity considerations, evaluation of genotoxicity and further toxicological studies as well as exposure calculations, it is concluded that all plant metabolites are considered to be toxicologically adequately investigated and uncritical for human health.

A summary of the toxicological studies on several metabolites is provided below.

CGA 357261 (ZE-isomer)

The ZE-isomer CGA 357261 is non-toxic after acute oral exposure (LD_{50} >2000 mg/kg). There were no indications for a genotoxic effect in the bacterial reverse mutation assay and in the *in vitro* micronucleus test in human lymphocytes.

Table 5.8.1-1: Summary of studies with CGA 357261*

Study	Concentration range Dose level tested	Result	Author / Reference
Acute oral, rat	2000 mg/kg bw	LD_{50} >2000 mg/kg bw (no clinical signs)	[redacted] (1997) M-039079-02-1
Bacterial reverse mutation assay	312.5 - 5000 µg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1997) M-039138-01-1
***In vitro* Micronucleus test, human lymphocytes**	**8 - 100 µg/mL (-S9 mix) 63.8 - 300 µg/mL (+S9 mix)**	**Negative (+/- S9 mix)**	**[redacted] (2013) M-463623-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

CGA 331409 (EZ isomer)

The EZ-isomer CGA 331409 showed no genotoxic potential in the bacterial reverse mutation assay and in the *in vitro* micronucleus test in human lymphocytes.

Table 5.8.1-2: Summary of studies with CGA 331409*

Study	Concentration range Dose level tested	Result	Author / Reference
Bacterial reverse mutation assay	**3 - 5000 µg/plate (+/- S9 mix)**	**Negative (+/- S9 mix)**	**[redacted] (2011) M-414991-01-1**
***In vitro* Micronucleus test, human lymphocytes**	**7.8 - 62.5 µg/mL (-S9 mix) 12.5 - 200 µg/mL (+S9 mix)**	**Negative (+/- S9 mix)**	**[redacted] (2013) M-463619-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

CGA 357262 (ZZ isomer)

The ZE-isomer CGA 331409 showed no genotoxic potential in the bacterial reverse mutation assay and in the *in vitro* micronucleus test in human lymphocytes.

Table 5.8.1-3: Summary of studies with CGA 357262*

Study	Concentration range Dose level tested	Result	Author / Reference
Bacterial reverse mutation assay	**3 - 5000 µg/plate (+/- S9 mix)**	**Negative (+/- S9 mix)**	**[redacted] (2011) M-414989-01-1**
***In vitro* Micronucleus test, human lymphocytes**	**8 - 125 µg/mL (-S9 mix) 16 - 255.3 µg/mL (+ S9 mix)**	**Negative (+/- S9 mix)**	**[redacted] (2013) M-463639-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

Comparative in vitro studies

Effects on the respiratory chain of mammals of trifloxystrobin (TFS) in comparison to its isomers have been investigated in *in vitro* assays on rat liver mitochondria on the basis of a mode of action studies ([redacted], 1997, M-039240-01-1) conducted already for the first EU review process.

Potential effects on the respiratory chain of mammals have been investigated in rat liver mitochondria on trifloxystrobin (EE-isomer) in comparison to its photo-isomers and to some metabolites. The results demonstrate that trifloxystrobin is a potent inhibitor of mitochondrial respiration at nanomolar concentrations, whereas the photo-isomers (ZE, EZ, ZZ) are more than one order of magnitude less active related to the respiratory inhibition potential. Moreover, the study results confirmed the absence of a respiratory inhibition potential of the metabolites CGA 321113, CGA 373466, NOA 413161, and NOA 413163.

In the *in vitro* cytotoxicity test, trifloxystrobin shows clearly a higher cytotoxicity in rat hepatocytes in comparison to the photo-isomers (ZE, EZ). The ZE- and EZ-isomers are about 35 times less cytotoxic than trifloxystrobin. The ZZ-isomer and the metabolites CGA 321113, CGA 373466, NOA 413161, and NOA 413163 are even less cytotoxic than the EZ- and ZE-isomers.

Table 5.8.1-4: Summary of comparison studies with trifloxystrobin, isomers and metabolites*

Study	Concentration range Dose level tested	Result	Author / Reference
Interactions with mitochondrial respiration *in vitro*	**TFS: 0.001-0.03 µM** **photo-isomers** **CGA 331409: 1.563-50 µM** **CGA 357261: 0.313-10 µM** **CGA 357262: 0.313-10 µM** **metabolites** **CGA 321113: 10 µM** **CGA 373466: 10 µM** **NOA 413161: 10 µM** **NOA 413163: 10 µM**	• **TFS is a potent inhibitor of mitochondrial respiration at nanomolar concentrations** • **CGA331409, CGA 357261, CGA357 262 are more than one order of magnitude less active** • **Metabolites are at least more than two orders on magnitude less active**	**[redacted] (2013) M-463641-01-1**
***In vitro* cytotoxicity, rat hepatocytes**	**TFS: 0.1-30 µg/mL** **photo-isomers** **CGA 331409: 5-500 µg/mL** **CGA 357261: 5-500 µg/mL** **CGA 357262: 10-1000 µg/mL** **metabolites** **CGA 321113: 10-1000 µg/mL** **CGA 373466: 10-1000 µg/mL** **NOA 413161: 10-1000 µg/mL** **NOA 413163: 10-1000 µg/mL**	• **Very similar response for Alamar Blue reduction and LDH release** • **TFS is the most cytotoxic** • **CGA 331409, CGA 357261 are about 35 times less cytotoxic** • **CGA 357262 and all metabolites are even less cytotoxic than CGA 331409 and CGA 357261**	**[redacted] (2013) M-463388-01-2**

* New studies, i.e. studies that were not previously submitted, are written in bold

Overall, trifloxystrobin (EE-isomer) as well as the photo-isomers (ZE, EZ, ZZ) are considered uncritical with regard to genotoxicity. The ZE-isomer is non-toxic (LD_{50} >2000 mg/kg, no clinical signs) after acute oral exposure. The comparative *in vitro* tests showed a clear ranking of the photo-isomers in terms of cytotoxicity and their inhibiting potential on mitochondrial respiration. The photo-isomers in comparison to trifloxystrobin are at least 35 times less cytotoxic in rat hepatocytes. Trifloxystrobin is a potent inhibitor of mitochondrial respiration at nanomolar concentrations whereas the photo-isomers are more than one order of magnitude less active. Thus, the trifloxystrobin photo-isomers (ZE, EZ, ZZ) are considered to exhibit significantly lower toxicity.

CGA 321113 (EE-metabolite)

In order to fulfill SANCO/221/2000 - rev. 10, 25th February 2003 requirements, the genotoxicity potential of CGA 321113 has been investigated in a battery of *in vitro* and *in vivo* tests. CGA 321113 does not induce mutations in bacteria and in mammalian cell, both with and without metabolic activation. However, CGA 321113 shows a clastogenicity potential in the *in vitro* chromosome aberration assay but this response has not been confirmed in the micronucleus test *in vivo*. Furthermore, the *in vivo* unscheduled DNA synthesis assay also resulted negative.

It can be concluded that overall the metabolite CGA 321113 is considered to be non-genotoxic.

CGA 321113 is a major metabolite in animals. Therefore, the toxicity studies performed with the parent compound are sufficient for the toxicity assessment of the metabolite. In addition, mode of action studies investigated the toxicity potential of CGA 321113 in comparison to trifloxystrobin. The following extract is taken from the Addendum III to the DAR (December 2002):

““CGA 321113 is the mono-acid compound formed by cleavage of the ester moiety in the trifloxystrobin. This enzymatic reaction degrades the methoxyacrylate toxophor of the molecule. In further steps, CGA 321113 is metabolised by hydroxylation of the trifluoromethyl-phenyl-ring, oxidation of the methyl group next to the oxyimino group between the trifluoromethyl-phenyl and glyloxyl-phenyl moiety, and demethylation of the methoxyimino group. Thus, CGA 321113 can be regarded as a central intermediate in the metabolic degradation of trifloxystrobin. While CGA 321113 itself was detected in the rat metabolism study in relatively small amounts (1.6-7.5 % of administered dose, in bile), the applicant reports that the sum of metabolites which are formed via CGA 321113 amounted to about 10-20% of the administered dose. ....
In in-vitro tests with rat hepatocytes, CGA 321113 was at least 980 times less active than trifloxystrobin in inhibiting respiration by hepatic mitochondria. Since significant inhibition of cellular respiration is likely to have major toxicological consequences for mammals, it is to be expected that CGA 321113 would be significantly less toxic than the parent molecule. Indeed, CGA 321113 was found to be 10-30 times less toxic than trifloxystrobin to rat hepatocytes in vitro. ....
Since CGA 321113 is a major metabolite of trifloxystrobin in the rat its toxic potential should be covered by the toxicity data for the active ingredient. Notably, the ADI for trifloxystrobin was based on reduced body weight gain (in the 2-year rat study), an effect which conceivably could be related to inhibition of cellular respiration.”

It can be concluded that the metabolite CGA 321113 is less toxic than the parent compound and overall possesses no genotoxic potential.

Table 5.8.1-5: Summary of studies with CGA 321113*

Study	**Concentration range Dose level tested**	**Result**	**Author / Reference**
In vitro hepatotoxicity and inhibition of mitochondrial respiration	Cytotoxicity assessment: Trifloxystrobin: 5-100 µM CGA 321113: 5-100 µM Mitochondrial respiration: Trifloxystrobin: 10-600 nM CGA 321113: 1000-30000 nM	CGA 321113 is about 20 times less cytotoxic than TFS TFS inhibited rat liver mitochondrial respiration in vitro, CGA 321113 showed no inhibitory effect	[redacted] (1997) M-039240-01-1
Interactions with mitochondrial respiration *in vitro*	TFS: 10-600 nM CGA 321113: 5.9 µM CGA 373466: 5.5 µM NOA 413161: 5.8 µM NOA 413163: 5.9 µM	Compared to the parent compound trifloxystrobin, these metabolites are unlikely to contribute to toxicities mediated by mitochondrial complex III inhibition	[redacted] (2002) M-034840-02-1
In vitro hepatotoxicity, rat hepatocytes	TFS: 1-100 µg/mL CGA 321113: 1-100 µg/mL CGA 373466: 1-1000 µg/mL NOA 413161: 1-1000 µg/mL NOA 413163: 1-1000 µg/mL	TFS distictly higher hepatotoxic potential than CGA 321113 and CGA 373466. NOA 413161 and NOA 413163 revealed no hepatotoxic potential	[redacted] (2002) M-090653-02-1
Bacterial reverse mutation assay	**3 - 5000 µg/plate (+/- S9 mix)**	**Negative (+/- S9 mix)**	[redacted] **(2011) M-406346-01-1**
Mammalian cell gene mutation	**5 - 320 µg/mL (-S9 mix) 40 - 640 µg/mL (+ S9 mix)**	**Negative (+/- S9 mix)**	[redacted] **(2011) M-411413-01-1**
***In vitro* chromosome aberration**	**40 - 125 µg/mL (-S9 mix) 200 - 400 µg/mL (+ S9 mix)**	**Positive (+/- S9 mix)**	[redacted] **(2011) M-413745-01-1**
***In vivo* Micronucleus test**	**0-500-1000-2000 mg/kg bw (oral, gavage)**	**Negative**	[redacted] **(2013) M-463614-01-1**
***In vivo* UDS, rat hepatocytes**	**1000-2000 mg/kg bw (oral, gavage)**	**Negative**	[redacted] **(2013) M-458428-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

CGA 373466 (ZE-metabolite)

CGA 373466 was investigated for its genotoxicity potential. No evidence for a mutagenic or clastogenic effect in the bacterial reverse mutation assay, in the *in vitro* mammalian gene mutation assay and in the *in vitro* mammalian chromosome aberration assay were observed.

In addition, CGA 373466 is non-toxic (LD_{50} >2000 mg/kg bw) after acute oral exposure. Subacute dietary exposure showed retarded bodyweight development, effects on red blood cells and slight liver enzyme induction at 8000 ppm (903/928 mg/kg bw/day). The NOAEL is 2000 ppm (209/235 mg/kg bw/day in males/females). The study director established a NOEL of 500 ppm (47/61 mg/kg bw/day in males/females).

Comparative studies conducted with trifloxystrobin and metabolites revealed that the metabolite CGA 373466 has no inhibiting effect on mitochondrial respiration and that it is clearly less hepatotoxic in comparison to trifloxystrobin (see comparative studies [redacted], 2002, M-034840-02-1, [redacted], 2002, M-090653-02-1.)

Overall it can be concluded that the metabolite CGA 373466 is less toxic than the parent compound and possesses no genotoxic potential.

Table 5.8.1-6: Summary of studies with CGA 373466*

Study	Concentration range Dose level tested	Result	Author / Reference
Acute oral, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1997) M-039100-02-1
Bacterial reverse mutation assay	312.5 - 5000 mg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1997) M-039119-01-1
In vitro mammalian cell gene mutation	50 - 1200 mg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (2002) M-054116-01-1
In vitro mammalian chromosome aberration	125 - 600 µg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (2002) M-054928-01-1
Rat, 4 week feeding and 4 week recovery	**0-100-500-2000-8000 ppm males: 0-9.6-47-209-903 mg/kg bw/day females: 0-11-61-236-928 mg/kg bw/day**	**NOAEL 2000 ppm (209/236 mg/kg bw/d (m/f) based on retarded body weight development, effects on red blood cells, slight liver enzyme induction at 8000 (903/928 mg/kg bw/d) (m/f)**	[redacted], [redacted] **(2003) M-088404-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

NOA 413161

The metabolite NOA 413161 is non-toxic (LD_{50} >2000 mg/kg bw) after acute oral exposure. There were no indications for a mutagenic or clastogenic effect in the bacterial reverse mutation assay and in mammalian cells *in vitro*.
After four week oral exposure (gavage) the only treatment-related effect were increased urobilinogen levels in males. The NOAEL established by the study director is 1000 mg/kg bw/day in males/females. However, during the EU review process a NOAEL of 150 mg/kg bw/day in males and 1000 mg/kg bw/day in females was established.
Comparative studies conducted with trifloxystrobin and metabolites revealed that the metabolite NOA 413161 has no inhibiting effect on mitochondrial respiration and that it is clearly less hepatotoxic in comparison to trifloxystrobin (see comparative studies [redacted], 2002, M-034840-02-1, [redacted], 2002, M-090653-02-1.)

Overall it can be concluded that the metabolite NOA 413161 is less toxic than the parent compound and possesses no genotoxic potential.

Table 5.8.1-7: Summary of studies with NOA 413161*

Study	Concentration range Dose level tested	Result	Author / Reference
Acute oral, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1998) M-052694-01-1
Bacterial reverse mutation assay	312.5 - 5000 mg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1998) M-054210-01-1
In vitro mammalian cell gene mutation	375 - 3000 mg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (2000) M-054225-01-1

Study	Concentration range Dose level tested	Result	Author / Reference
In vitro mammalian chromosome aberration	625 - 2500 µg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1999) M-054214-01-1
Rat, 4 week oral (gavage), and 4 week recovery	0-15-50-150-1000 mg/kg bw/d	NOAEL 1000 mg/kg bw/d (NOEL 150/1000 mg/kg bw/d based on increased urobilinogen levels in males see Addendum III to the DAR)	[redacted] (2000) M-137124-01-1

* New studies, i.e. studies that were not previously submitted, are written in bold

NOA 413163

The metabolite NOA 413163 is non-toxic (LD_{50} >2000 mg/kg bw) after acute oral exposure. There were also no indications for a mutagenic effect in the bacterial reverse mutation assay.
Comparative studies conducted with trifloxystrobin and metabolites revealed that the metabolite NOA 413163 has no inhibiting effect on mitochondrial respiration and that it is clearly less hepatotoxic in comparison to trifloxystrobin (see comparative studies [redacted], 2002, M-034840-02-1, [redacted], 2002, M-090653-02-1.)

Table 5.8.1-8: Summary of studies with NOA 413163*

Study	Concentration range Dose level tested	Result	Author / Reference
Acute oral, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1998) M-052684-01-1
Bacterial reverse mutation assay	312.5 - 5000 mg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1998) M-052705-01-1

* New studies, i.e. studies that were not previously submitted, are written in bold

NOA 413161/NOA 413163

NOA 413161/NOA 413163 (mixture containing 48% NOA 413161 and 51% NOA 413163) showed no genotoxic potential in the *in vitro* mammalian cell gene mutation assay mutation assay and in the *in vitro* mammalian chromosome aberration test.
After four week oral exposure of 1000 mg/kg bw/day of NOA 413161/NOA 413163 slight changes of hematological and urinary parameters were observed but these findings were not considered adverse. The NOAEL is 1000 mg/kg bw/day in males/females.

Table 5.8.1-9: Summary of studies with NOA 413161/NOA 413163*

Study	Concentration range Dose level tested	Result	Author / Reference
In vitro mammalian cell gene mutation test HPRT, V79 cells	600 - 3000 µg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (2002) M-069760-01-1
In vitro mammalian chromosome aber-ration test, V79 cells	625 - 2500 µg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (2002) M-069747-01-1
Rat, 4-wk oral gavage and 4-wk recovery	**0-10-50-200-1000 mg/kg bw/day**	**NOAEL 1000 mg/kg bw/d (m/f)**	[redacted] **(2003) M-084123-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

Overall it can be concluded that the metabolite NOA 413163 is less toxic than the parent compound and possesses no genotoxic potential.

NOA 414412

The metabolite NOA 414412 is non-toxic (LD_{50} >2000 mg/kg bw) after acute oral exposure. There were no indications for a mutagenic effect in the bacterial reverse mutation assay *in vitro*.

Table 5.8.1-10: Summary of studies with NOA 414412*

Study	Concentration range Dose level tested	Result	Author / Reference
Acute oral, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1997) M-039147-01-1
Bacterial reverse mutation assay	312.5 - 5000 mg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1997) M-039158-01-1

* New studies, i.e. studies that were not previously submitted, are written in bold

Phthalic acid

The mutagenic and clastogenic potential of phthalic acid was investigated *in vitro* in bacteria and mammalian cells, as well as *in vivo* in mice. There was no mutagenic potential observed in the bacterial reverse mutation assay. Neither in CHO cells *in vitro*, nor in bone marrow of mice there was any indication for a clastogenic potential.
Thus, phthalic acid is considered to be non-genotoxic.

Table 5.8.1-11: Summary of studies with phthalic acid*

Study	Concentration range Dose level tested	Result	Author / Reference
Bacterial reverse mutation assay	**0 - 12500 µM (+/- S9 mix)**	**Negative (+/- S9 mix)**	**[redacted], 2007 M-462063-01-1**
***In vitro* mammalian chromosome aberration, CHO cells**	**20 - 12500 µM/mL (+/- S9 mix)**	**Negative (+/- S9 mix)**	
***In vivo* Micronucleus, mouse, ICR**	**20 - 12500 mM/kg**	**Negative**	

* New studies, i.e. studies that were not previously submitted, are written in bold

Comparative *in vitro* studies with trifloxystrobin, photo-isomers, metabolites

Report: KCA 5.8.1 /23; [redacted];2013;M-463641-01
Title: Trifloxystrobin (CGA 279202), Isomers and Metabolites - Studies on potential interactions with the mitochondrial respiration of freshly isolated rat liver mitochondria
Report No: AT06643
Document No: M-463641-01-1
Guidelines: **not applicable (mechanistic screening study)**
GLP/GEP: **no**

I. Materials and methods

A. Materials

1. Test materials:

Test substance 1:
Name: Trifloxystrobin (= EE isomer)
Synonyms: CGA 279202; AE C642802; **TFS**
Description: Light beige powder
Lot/Batch no: EDFL006101
Purity: 99.1% (w/w)
Stability of test compound: guaranteed for study duration; expiry date: 2013-01-14

Test substance 2:
Name: CGA 331409 (= EZ isomer)
Synonyms: CGA 279202-CGA 331409; AE 1344135; **EZi**
Description: Light beige powder
Lot/Batch no: SES 10355-2-1
Purity: 99.4% (w/w)
Stability of test compound: guaranteed for study duration; expiry date: 2013-05-19

Test substance 3:
Name: CGA 357261 (= ZE isomer)
Synonyms: CGA 279202-CGA 357261; AE 1393224; **ZEi**
Description: Light beige powder
Lot/Batch no: SES 10350-10-1
Purity: 95.7% (w/w)
Stability of test compound: guaranteed for study duration; expiry date: 2013-05-20

Test substance 4:
Name: CGA 357262 (= ZZ isomer)
Synonyms: CGA 279202-CGA 357262; AE 1344146; **ZZi**
Description: Colorless liquid
Lot/Batch no: SES 10487-2-1
Purity: 99.4% (w/w)
Stability of test compound: guaranteed for study duration; expiry date: 2013-05-19

Test substance 5:
Name: CGA 321113 (= EE metabolite)
Synonyms: CGA 279202-CGA 321113; AE 1344138; **EEm**
Description: White powder
Lot/Batch no: BCOO 6132-2-3
Purity: 98.6% (w/w)
Stability of test compound: guaranteed for study duration; expiry date: 2012-11-26

Test substance 6:

Name:	CGA 373466 (= ZE metabolite)
Synonyms:	CGA 279202-CGA 373466; AE 1344148; **ZEm**
Description:	White powder
Lot/Batch no:	M18457
Purity:	96.3% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2012-06-23

Test substance 7:

Name:	NOA 413161 (metabolite = M1)
Synonyms:	CGA 279202-NOA 413161; AE 1344143; **M1**
Description:	Off white powder
Lot/Batch no:	M19118
Purity:	91.8% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2016-07-04

Test substance 8:

Name:	NOA 413163 (metabolite = M2)
Synonyms:	CGA 279202-NOA 413163; AE 1344149; **M2**
Description:	Light pink powder
Lot/Batch no:	M18477
Purity:	99.2% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2016-06-30

2. Vehicle and or positive control: Vehicle: dimethylsulfoxide (DMSO)
Positive control: Menadione (C 8138, Sigma, Germany)

3. Test system: Rat liver mitochondria

Isolation and culture: Liver mitochondria were isolated from Wistar rats (250 to 274 g bodyweight; source: [redacted], The Netherlands) by *in situ* perfusion with ice-cold isolation medium. The perfused liver was homogenized in ice-cold isolation medium (10 mL medium per g liver) by means of a potter. Then, the homogenate was centrifuged at 380 g for 3 min under cooling. The sediment was discarded, whereas half of the supernatant (sufficient for a working day) was centrifuged at 8500 g for 10 min. The supernatant was again discarded, the sedimented mitochondria were re-suspended in 0.5 mL isolation medium and were then diluted further to achieve a ratio of 20 mL per g liver. After a further centrifugation step at 8500 g for 10 min, the sediment was finally re-suspended in isolation medium to achieve a ratio of 0.25 mL per g liver. The mitochondrial preparation was stored on ice until further used.

Isolation medium: 220 mM mannitol, 70 mM sucrose, 5 mM MOPS, 2 mM EGTA, pH 7.0.

Characterization of rat liver mitochondrial fractions

The protein content was assessed using the Comassie protein assay. Mitochondrial preparations were diluted 1:20, 1:25, 1:50, 1:75, 1:100, 1:150 and 1:200 with water. After mixing 20 µL of sample with 1 mL Coomassie reagent and standing at ambient temperature for 10 min, samples were measured at 595 nm in a spectrophotometer against a reagent blank. For quantitation, a calibration curve was established in parallel on each working day using BSA solutions in water in the concentration range of 0.125 to 1 mg/mL. All measurements were done in triplicate. The protein content of the mitochondrial preparation was calculated as the mean of the various dilutions.

The respiratory control ratio (RCR) as the ratio of oxygen consumption in the presence of stimulating ADP (state 3 respiration) and after exhaustion of ADP (state 4 respiration) was measured conventionally by means of a oxygen-sensitive electrode. Preparations with a RCR below 3 would be discarded. Mitochondria (corresponding to 2 mg protein) were added to respiration buffer (140 mM KCl, 20 mM MOPS, 5 mM potassium phosphate, 0.4 mM EGTA, pH 7.2) in a temperature-controlled incubation chamber at 30°C to yield a total volume of 1777 µL and recording of oxygen consumption by means of the oxygen partial pressure module OPPM type 697 (Hugo Sachs Elektronik) was started. Following a pre-incubation time of 60 sec, 18 µL respiration substrate (2500 mM succinate in water adjusted to pH 7.2 with KOH, final concentration 25 mM) was added. After 55 sec 0.5 µmol ADP in 5 µL water were added to stimulate respiration. Approximately after 180 sec of recording time, oxygen consumption rate starts to reach resting respiration conditions (ADP exhausted) again. To allow a proper assessment of state 4 respiration, oxygen consumption is recorded further for at least 60 sec. RCR was measured in at least five independent runs.

B. Study design and methods

1. Examinations

Test item concentrations

	Test item	Final concentrations in µM
Experiment 1	TFS	0.001, 0.0033, 0.0067, 0.010, 0.015, 0.030
	EZi	1.563, 3.125, 6.25, 12.5, 25, 50
	ZEi	0.313, 0.625, 1.25, 2.5, 5, 10
	ZZi	0.313, 0.625, 1.25, 2.5, 5, 10
	EEm	10
	ZEm	10
	M1	10
	M2	10
Experiment 2	TFS	0.0025, 0.005, 0.010, 0.015, 0.030, 0.060
	EZi	0.391, 0.781, 1.563, 3.125, 6.25, 12.5
	ZEi	0.313, 0.625, 1.25, 2.5, 5, 10
	ZZi	0.313, 0.625, 1.25, 2.5, 5, 10
	EEm	10
	ZEm	10
	M1	10
	M2	10
Experiment 3	TFS	0.0025, 0.005, 0.010, 0.015, 0.030, 0.060
	EZi	0.098, 0.195, 0.391, 1.563, 3.125, 6.25
	ZEi	0.313, 0.625, 1.25, 2.5, 5, 10
	ZZi	0.313, 0.625, 1.25, 2.5, 5, 10
	EEm	10
	ZEm	10
	M1	10
	M2	10

Concurrent vehicle controls (DMSO, no mitochondria) were run in each experiment.

Determination of mitochondrial respiration using a phosphorescent probe:

The assay was performed in a 96-well format in dark plates using a commercially available oxygen-sensitive probe. Incubations were done at 30°C; accordingly solutions and plate reader were temperature-controlled and adjusted to 30°C. Additions to the 96-well plates were made in the following order:

1. 1 mL DMSO (solvent control) or test substance in DMSO
2. 100 µL phosphorescent probe
3. 50 µL diluted mitochondrial preparation (1 mg protein / mL). (The final protein concentration in assay was 0.25 mg/mL)
4. 50 µL substrate solution containing ADP. The final concentration in assay was 1.65 mM ADP and also 25 mM succinate)
5. 100 µL mineral oil

Reactions were followed over a period of 30 min and phosphorescence was recorded every minute in a Tecan Ultra fluorescence reader (Tecan, Crailsheim, Germany)

Replicates:

In total 3 independent experiments were performed.

2. Evaluation

For each concentration of a test compound first the mean value of the recorded signals from triplicate incubations was calculated and plotted against the incubation time.
Since there is no linear relationship between the measured signal and the oxygen concentration, the data were linearised as follows: For each condition, $I_0/(I_t-I_0)$ was plotted against 1/t. [I_0 is the mean signal measured for the very first time point, whereas I_t is any of the following time points. For this approach, mainly data points around the inflection point can be used. (Especially data points decreasing over time indicating no activity or total inhibition of activity are not suitable.)] Then, linear curve was best-fitted through these data points. The slope of these linear curves represents the corresponding inverse slopes of oxygen consumption rates. Finally, slopes of oxygen consumption rates were calculated from inverted slopes, the slope of the corresponding control was set to 100%, slopes observed in the presence of test compounds were expressed as percentage of the corresponding control, and relative slopes were plotted against the log of the test compounds' concentrations to end up in conventional concentration response relationships.
IC_{50}-values were calculated from conventional concentration response relationships as follows: For trifloxystrobin through all data points obtained at ≥ 0.010 µM, a linear curve was best-fitted. The formula of this linear curve was then used to calculate the concentration resulting in 50% inhibition. For the other compounds, the approach was similar; however, data points showing 15% to 85% inhibition were included in the analysis.

II. Results and discussion

Trifloxystrobin concentration-dependently affected mitochondrial respiration. Corresponding IC_{50}-values were in the range 0.018 µM to 0.031 µM.
The trifloxystrobin isomers tested were less active than the parent compound. Corresponding IC_{50}-values were in the range 0.42 µM to 1.33 µM for CGA 331409 (EZ isomer), in the range 0.55 µM to 1.07 µM for CGA 357261 (ZE isomer), and in the range 1.06 µM to 2.03 µM for CGA 357262 (ZZ isomer) (see Table 5.8.1/23-1).

These findings confirm the known complex III-inhibitory properties of trifloxystrobin and furthermore demonstrate the sensitivity of the test system. Overall, IC_{50}-values for the individual compounds did not vary more than three times from one another, indicating the good reproducibility of the experimental system.
Comparison of the IC_{50}-values of trifloxystrobin and its isomers show that CGA331409 (EZ isomer), CGA357261 (ZE isomer), and CGA357262 (ZZ isomer) are at least 13.7-times (range 13.7-58.6), 27.7-times (range 27.7-47.1), and 48.5-times (range 48.5-89.4) less active than trifloxystrobin (see Table 5.8.1/23-1). The greater variability of this parameter is readily explained by the fact that it represents the ratio of two IC_{50}-values with their individual variability.

Table 5.8.1/23-1: Effect of trifloxystrobin, its isomers CGA331409, CGA357261, and CGA357262 and its metabolites, CGA321113, CGA373466, NOA413161, and NOA413163 on mitochondrial respiration – IC_{50}-values

Experiment No.	Plate No.	Compound	IC_{50}-Value	Times less active than trifloxystrobin*
1	1	Trifloxystrobin (TFS)	0.023 µM	-
2	1		0.031 µM	-
3	1		0.018 µM	-
1	1	CGA 331409 (EZ isomer, EZi)	1.33 µM	58.6
2	1		0.42 µM	13.7
3	1		0.45 µM	24.6
1	1	CGA 357261 (ZE isomer, ZEi)	1.07 µM	47.1
2	1		0.85 µM	27.7
3	1		0.55 µM	30.1
1	1	CGA 357262 (ZZ isomer, ZZi)	2.02 µM	89.4
2	1		1.49 µM	48.5
3	1		1.06 µM	57.9
1	2	Trifloxystrobin (TFS)	0.022 µM	-
2	2		0.030 µM	-
3	2		0.023 µM	-
1	2	CGA 321113 (EEm)	No IC_{50} achieved at 10 µM	> 454.5
2	2		No IC_{50} achieved at 10 µM	> 336.7
3	2		No IC_{50} achieved at 10 µM	> 440.5
1	2	CGA 373466 (ZEm)	No IC_{50} achieved at 10 µM	> 454.5
2	2		No IC_{50} achieved at 10 µM	> 336.7
3	2		No IC_{50} achieved at 10 µM	> 440.5
1	2	NOA 413161 (M1)	No IC_{50} achieved at 10 µM	> 454.5
2	2		No IC_{50} achieved at 10 µM	> 336.7
3	2		No IC_{50} achieved at 10 µM	> 440.5
1	2	NOA 413163 (M2)	No IC_{50} achieved at 10 µM	> 454.5
2	2		No IC_{50} achieved at 10 µM	> 336.7
3	2		No IC_{50} achieved at 10 µM	> 440.5

* Deviations may occur due to rounding

The trifloxystrobin metabolites CGA 321113, CGA 373466, NOA 413161, and NOA 413163 were only tested at 10 µM. These compounds showed only marginal effects on mitochondrial respiration rates. At 10 µM, respiratory rates corresponded to 66.8% to 91.1% of the control for CGA 321113, to 68.6% to 85.8% for CGA 373466, to 70.6% to 97.8% for NOA 413161, and to 88.6% to 96.9% for NOA 413163 (see Table 5.8.1/23-2). Whether these findings point towards a beginning inhibition remains open, as no concentration responses including higher concentrations were run. Importantly, none of these metabolites was able to inhibit respiration by 50% or more when tested at 10 µM. Accordingly, when comparing potencies of trifloxystrobin and its metabolites when tested under identical conditions, all metabolites were more than 336.7-times less active than trifloxystrobin (see Table 5.8.1/23-1). These results confirm earlier findings obtained in a similar experimental set up with measurement of mitochondrial respiration by means of an oxygen-sensitive electrode showing that these metabolites are more than 900-times less active than trifloxystrobin ([redacted] (2002), M-034840-02-1).

Table 5.8.1/23-2: Effect of trifloxystrobin and its metabolites on mitochondrial respiration

Experiment No.	Plate No.	Compound	IC_{50}-Value	Percent of control respiration rate at 10 µM
1	2	Trifloxystrobin (TFS)	0.023 µM	Not tested at 10 µM
2	2		0.031 µM	Not tested at 10 µM
3	2		0.018 µM	Not tested at 10 µM
1	2	CGA 321113 (EEm)	No IC_{50} achieved at 10 µM	66.8
2	2		No IC_{50} achieved at 10 µM	91.1
3	2		No IC_{50} achieved at 10 µM	68.3
1	2	CGA 373466 (ZEm)	No IC_{50} achieved at 10 µM	85.8
2	2		No IC_{50} achieved at 10 µM	83.8
3	2		No IC_{50} achieved at 10 µM	68.6
1	2	NOA 413161 (M1)	No IC_{50} achieved at 10 µM	85.3
2	2		No IC_{50} achieved at 10 µM	70.6
3	2		No IC_{50} achieved at 10 µM	97.8
1	2	NOA 413163 (M2)	No IC_{50} achieved at 10 µM	88.6
2	2		No IC_{50} achieved at 10 µM	94.7
3	2		No IC_{50} achieved at 10 µM	96.9

III. Conclusions

In conclusion, these findings indicate that in freshly isolated rat liver mitochondria supplemented with succinate as respiratory substrate and ADP to stimulate respiration trifloxystrobin potently inhibits respiratory respiration at nanomolar concentrations. In comparison, the trifloxystrobin isomers CGA 331409 (EZ isomer), CGA 357261 (ZE isomer), and CGA 357262 (ZZ isomer) are more than one order of magnitude, the metabolites CGA 321113, CGA 373466, NOA 413161, and NOA 413163 are at least more than two orders on magnitude less active in that respect.

Report: KCA 5.8.1/24; [redacted]2013/M-463388-01
Title: Trifloxystrobin, isomers and metabolites - Cytotoxicity in rat hepatocytes
Report No: AT06630
Document No: M-463388-01-2
Guidelines: **not applicable (mechanistic screening study)**
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test materials:

Test substance 1a:

Name:	Trifloxystrobin (= EE isomer)
Synonyms:	CGA 279202; AE C642802; **TFS**
Description:	Light beige powder
Lot/Batch no:	EDFL006101
Purity:	99.1% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2013-01-14

Test substance 2:

Name:	CGA 331409 (= EZ isomer)
Synonyms:	CGA 279202-CGA 331409; AE 1344135; **EZi**
Description:	Light beige powder
Lot/Batch no:	SES 10555-2-1
Purity:	99.4% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2013-05-19

Test substance 3:

Name:	CGA 357261 (= ZE isomer)
Synonyms:	CGA 279202-CGA 357261; AE 1393224; **ZEi**
Description:	Light beige powder
Lot/Batch no:	SES 10350-10-1
Purity:	95.7% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2013-05-20

Test substance 4:

Name:	CGA 357262 (= ZZ isomer)
Synonyms:	CGA 279202-CGA 357262; AE 1344146; **ZZi**
Description:	Colorless liquid
Lot/Batch no:	SES 10487-2-1
Purity:	99.4% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2013-05-19

Test substance 5:

Name:	CGA 321113 (= EE metabolite)
Synonyms:	CGA 279202-CGA 321113; AE 1344138; **EEm**
Description:	White powder
Lot/Batch no:	BCOO 6132-2-3
Purity:	98.6% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2012-11-26

Test substance 6:

Name:	CGA 373466 (= ZE metabolite)
Synonyms:	CGA 279202-CGA 373466; AE 1344148; **ZEm**
Description:	White powder
Lot/Batch no:	M18457
Purity:	96.3% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2012-06-23

Test substance 7:

Name:	NOA 413161 (metabolite = M1)
Synonyms:	CGA 279202-NOA 413161; AE 1344143; **M1**
Description:	Off white powder
Lot/Batch no:	M19118
Purity:	91.8% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2016-07-04

Test substance 8:

Name:	NOA 413163 (metabolite = M2)
Synonyms:	CGA 279202-NOA 413163; AE 1344149; **M2**
Description:	Light pink powder
Lot/Batch no:	M18477
Purity:	99.2% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2016-06-30

2. Vehicle and or positive control: Vehicle: dimethylsulfoxide (DMSO)
Positive control: Menadione (C 8138, Sigma, Germany)

3. Test system: Primary rat hepatocytes

Isolation and culture: Primary cells isolated from rat liver by collagenase perfusion followed by culture on collagen-coated plates. Cells were cultured without addition of specific growth factors, so that hepatocytes did not proliferate.

Culture medium: William´s Medium E without phenol red, containing 10% (v/v) FBS, 0.2% BSA, 0.01 mg/mL insulin and 1% Penicillin-Streptomycin.

Cell cultures: On day 0, isolated hepatocytes seeded on collagen-coated 24-well microtiter plates at 2 x 10^5 cells/well in 500 µL culture medium. Medium change to remove non-attached cells 3 hrs after planting.

B. Study design and methods

1. Treatment

Dose:

Test item	Final concentrations in µg/mL*
Trifloxystrobin (TFS)	0.1, 0.3, 1, 3, 10, 30
CGA 331409 (EZi)	5, 25, 50, 100, 250, 500
CGA 357261 (ZEi)	5, 25, 50, 100, 250, 500
CGA 357262 (ZZi)	10, 30, 100, 250, 500, 1000
CGA 321113 (EEm)	10, 30, 100, 250, 500, 1000
CGA 373466 (ZEm)	10, 30, 100, 250, 500, 1000
NOA 413161 (M1)	10, 30, 100, 250, 500, 1000
NOA 413163 (M2)	10, 30, 100, 250, 500, 1000
Menadione	1, 3.3, 10, 66, 100 µM
Vehicle control	1% DMSO

*except for positive control menadione and vehicle control

Stock solutions of the test substances were first diluted in DMSO to obtain 100-times solutions with respect to the final concentrations. These solutions were then diluted 50-fold in medium to achieve 2x dilutions with respect to the final concentrations (see Table above), and 500 µL of these 2x dilutions were added to the cells in 500 µL medium in 24-well plates.

Treatment: On day 1, ca. 20-22 h after seeding, cells were treated with increasing concentrations of the different compounds by adding 500 µL of a 2x concentrated dilution with respect to the final compound concentration. 1-2 cytotoxicity parameters were then assessed 24h, 48h and 72h after start of treatment. For the 72h treatment, the incubation medium was changed 48h after the first application.

Replicates: In total 3 independent tests were performed with different cell isolations

Treatment time: 24h, 48h, 72 h
For the 72h treatment, the incubation medium was changed 48h after the first application.

Cytotoxicity determination: Measurement of Alamar Blue reducing activity, used as an indicator for the reduction activity of the cells, which corresponds to the catabolic activity of living cells generating reduced NAD-nucleotides.

Measurement of Lactate dehydrogenase (LDH) release in cell supernants, as an indicator for membrane leakage.
For measurement of Alamar Blue reduction after 24 and 72 h of treatment, the medium with compounds or vehicle was removed, and the cells were incubated for 3h at 37°C with 1000 µL/well fresh medium containing 100 µL/well Alamar Blue solution. Then the fluorescence was measured with a spectrophotometer (SpectraFluorPlus, TECAN) at 540 nm/590 nm extinction/emission.
For measurement of LDH release after 24 and 48 h of treatment, 250 µL supernatant per well was transferred to a 24 well plate. 250 µL/well diluted LDH reagent was added and the plate was incubated for 15 minutes at room temperature protected from light. The LDH released from damaged cells was detected via its enzymatic activity in a coupled redox reaction using a spectrophotometer set at 490 nm absorbance. To also determine total cellular LDH content, a control sample of cells was lysed with 0.1% triton in medium.

2. Evaluation

Alamar Blue reducing activity: The % reduction of Alamar Blue reducing activity relative to vehicle-treated cells was used as a measure for cell viability. For calculation the raw data were blank corrected. The % Alamar Blue reducing activity per single replicate was calculated by dividing the fluorescence values of the treated wells through the mean fluorescence of the vehicle-treated cells. From these data, mean and standard deviation (SD) of the % Alamar Blue reducing activity per replicate group was derived. IC_{50} values were calculated using Analyzes Version 2.0.3 (in-house developed) software for dose-response analysis.

LDH activity increase: LDH activity increase is given as % relative to vehicle-treated samples. The "No effect concentration" (NOEC) for each compound was determined as that with LDH activity ≤ 120%, i.e. ≤ 20% increase of LDH activity. The latter is considered as normal variability.

Assay validity: For Menadione historical cytotoxicity data are available for rat primary hepatocytes. Therefore it was chosen as standard compound to judge the validity of the assays performed. The test for the endpoints hepatocytes are considered as valid if the determined IC_{50} value of menadione lies within a range that is defined by a lower control limit (LCL) and an upper control limit (UCL) of the specific assay. The control limits are derived from the last 10 IC_{50} values of menadione (historical data) by applying the 3-sigma rule known from process control. Calculation of control limits is performed by the SAS tool QC-CYTOTOX-IC50 version 1.1 developed and provided by Nonclinical Statistics.

II. Results and discussion

Alamar Blue reducing activity, representative of the percentage of viable, metabolically active cells after treatment with trifloxystrobin and derivatives, all relative to vehicle control (means of the 3 tests) are shown in Figure 5.8.1/24-1. As representative result the 72h time point is depicted Figure 5.8.1/24-1, all further data for both time points are available in Table 5.8.1/24-1 and Table 5.8.1/24-2 below.
The results of the Alamar Blue reductions indicate the following ranking of the compounds with respect to cytotoxicity induction:

Trifloxystrobin (TFS) >> CGA 331409 (EZi), CGA 357261 (ZEi) > CGA 321113 (EEm), CGA 373466 (ZEm) > NOA 413161 (M1) > CGA 357262 (ZZi), NOA 413163 (M2)

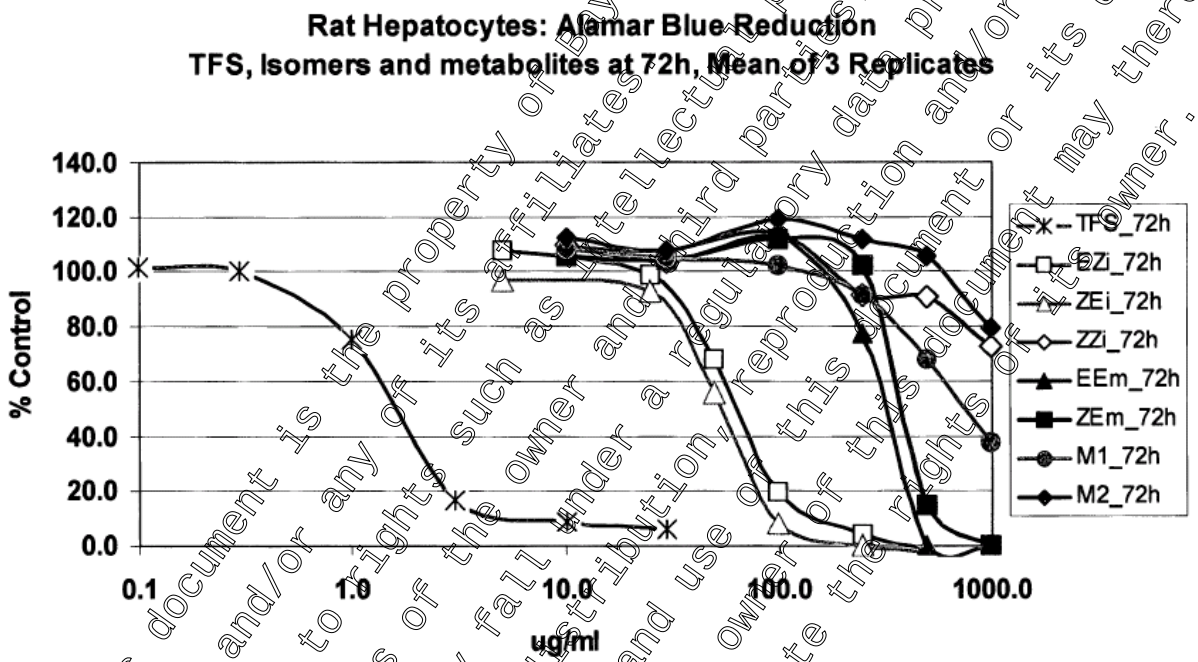


Figure 5.8.1/24-1: Alamar Blue reduction activity of compound-treated relative to vehicle-treated cells after 72 h of treatment, shown as mean of 3 replicates. Trifloxystrobin (TFS) is indicated with a ✱ symbol, isomers with non-filled, and metabolites with filled symbols.

IC_{50} values derived from mean % mean Alamar Blue reducing activity relative to vehicle control are summarized in the Table 5.8.1/24-1. According to these values, trifloxystrobin is at least 20 times or 35 times more cytotoxic after 24h and 72 h incubation, respectively, then the EZ and ZE isomer, which are the most cytotoxic among the trifloxystrobin isomers and metabolites.

Table 5.8.1/24-1: Summary of IC_{50}-values derived from Alamar Blue reducing activity

Test item Test item other name			IC_{50} after 24h (µg/mL)	IC_{50} after 72h (µg/mL)
Trifloxystrobin	TFS	EE isomer	3.20	1.63
CGA 331409	EZi	EZ isomer	70.00	59.67
CGA 357261	ZEi	ZE isomer	108.67	58.33
CGA 357262	ZZi	ZZ isomer	> 1000	> 1000
CGA 321113	EEm	EE metabolite	360.00	286.67
CGA 373466	ZEm	ZE metabolite	620.33	380.00
NOA 413161	M1	Metabolite M1	614.00	596.50
NOA 413163	M2	Metabolite M2	> 1000	> 1000

With Alamar Blue reducing activity as endpoint, IC_{50} values for the standard compound menadione mean were 16 µM and 20.7 µM after 24h and 72h of incubation, respectively.

Historical control values for menadione are available for 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl Tetrazolium Bromid (MTT)-reducing activity after 24h incubation of primary rat hepatocytes, an endpoint measuring the same parameter, i.e. metabolic reducing activity of the cells. For this parameter, the mean IC_{50} was 16.5 µM for 25 different hepatocyte isolates over a period of three years. Thus the IC_{50} values measured for the standard compound menadione were within the range of comparable historical values for the hepatocyte preparations used in this study.

LDH-release measurement showed a treatment-induced increase of LDH release relative to control cells. This indicates damage to cellular membranes and thus necrotic injury. Figure 5.8.1/24- 2 below, shows the results from the LDH-release after 24 hours as representative data. In Table 5.8.1/24-2 below the LDH release data of both time points for all test items are summarized. Based on the results for LDH release, the ranking of the compounds for cytotoxicity induction given below is highly comparable as derived from Alamar Blue reduction assessment:

Trifloxystrobin (TFS) >> CGA 331409 (EZi) > CGA 357261 (ZEi) > CGA 321113 (EEm), CGA 373466 (ZEm) > NOA 413161 (M1), CGA 357262 (ZZi), NOA 413163 (M2)

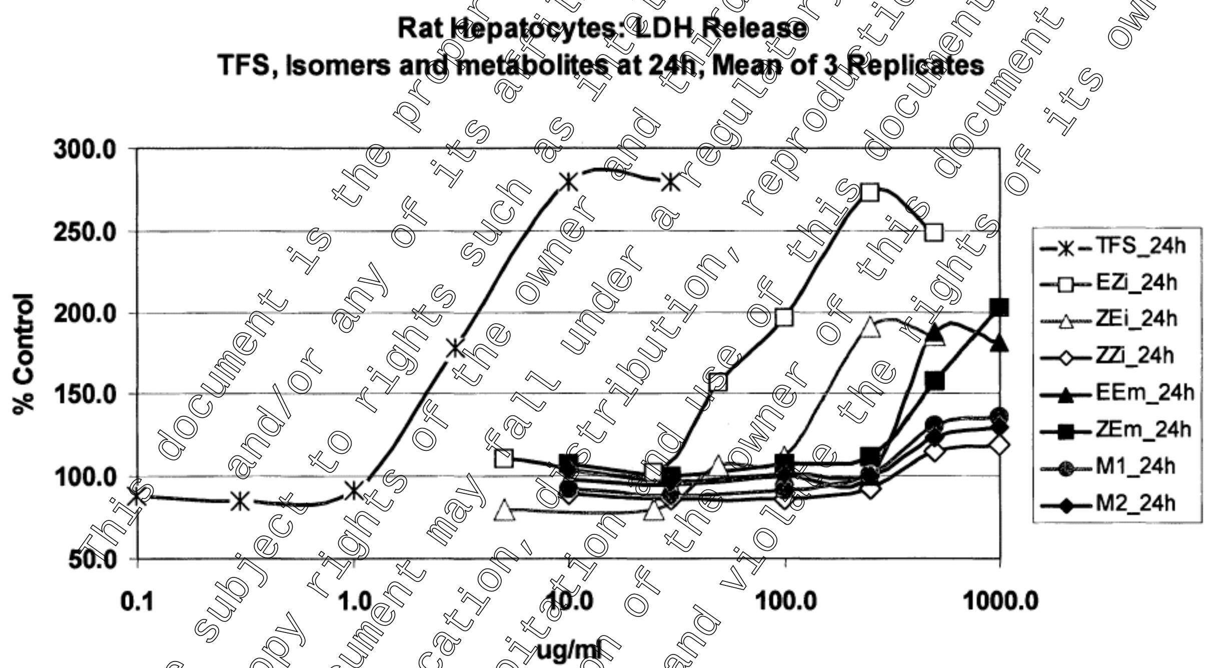


Figure 5.8.1/24- 2: LDH release into the culture supernatant of compound-treated relative to vehicle-treated cells after 24 hours of treatment, shown as mean of 3 replicates. Trifloxystrobin (TFS) is indicated with a ∗ symbol, isomers with non-filled symbols, and metabolites with filled symbols.

Table 5.8.1/24-2: Summary of Alamar Blue reduction activity and LDH release all time points*.

Test item	Con-centration µg/mL**	AB-reduction 24 h		AB-reduction 72 h		LDH-release 24 h		LDH-release 48 h	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
CGA 279202 (TFS)	0.1	95.5	1.5	101.9	1.0	88.8	3.9	94.3	6.2
	0.3	98.9	1.8	100.6	0.7	84.8	4.2	89.5	8.0
	1.0	90.2	7.6	75.1	28.3	91.3	13.7	93.9	6.8
	3.0	50.6	41.3	17.2	14.9	178.5	129.4	146.7	62.7
	10.0	4.6	2.5	8.7	4.2	279.4	96.2	225.1	27.4
	30.0	3.1	1.1	5.9	2.9	279.9	104.5	223.0	25.3
CGA 331409 (EZi)	5.0	102.7	14.9	107.7	2.0	110.5	24.2	97.2	5.1
	25.0	94.1	5.9	98.9	8.8	102.3	17.1	95.8	10.2
	50.0	73.7	14.4	67.8	19.1	157.7	77.0	132.7	33.7
	100.0	36.3	12.2	19.6	9.7	196.6	85.3	177.1	35.8
	250.0	15.3	5.7	4.3	4.3	273.0	148.4	217.7	31.2
	500.0	9.7	4.6	-1.3	3.5	248.6	106.1	217.3	26.1
CGA 357261 (ZEi)	5.0	94.4	5.4	97.0	8.8	79.8	21.6	79.9	22.2
	25.0	92.8	8.3	93.0	10.8	80.0	19.2	77.6	20.1
	50.0	66.3	31.0	56.0	47.3	106.4	21.4	95.1	14.3
	100.0	56.5	46.3	8.3	5.8	112.4	20.3	103.2	9.1
	250.0	3.0	3.8	0.0	2.1	190.8	51.1	159.3	33.8
	500.0	-0.7	2.1	-2.6	1.8	186.2	29.7	159.8	27.2
CGA 357262 (ZZi)	5.0	100.4	7.0	109.8	11.2	89.0	9.8	96.5	3.5
	25.0	101.5	5.6	103.4	7.4	86.8	4.0	87.6	0.9
	50.0	114.8	9.3	113.4	16.1	86.6	12.2	101.8	4.2
	100.0	107.8	4.1	91.3	15.5	93.0	2.7	100.2	5.2
	250.0	102.4	4.4	90.1	18.2	115.4	21.9	137.7	10.1
	500.0	89.6	6.0	72.6	12.9	119.1	14.8	141.2	15.7
CGA 321113 (EEm)	10.0	96.1	3.3	108.8	4.9	97.6	2.7	93.9	2.0
	30.0	96.6	3.5	107.9	2.8	95.1	4.7	92.9	1.3
	100.0	91.0	2.2	113.0	9.0	100.4	3.8	95.4	2.8
	250.0	75.4	6.8	77.6	4.7	100.7	7.0	110.0	5.0
	500.0	20.6	9.3	-0.2	0.0	188.3	25.3	191.1	11.3
	1000.0	0.6	0.6	0.1	0.2	181.8	22.2	156.0	14.4
CGA 373466 (ZEm)	10.0	98.3	8.1	105.3	5.0	107.5	7.7	101.7	0.4
	30.0	98.2	1.5	104.4	8.0	100.3	6.8	96.1	3.9
	100.0	96.4	6.3	111.8	6.2	107.2	8.4	100.8	4.4
	250.0	81.6	6.4	102.4	6.9	111.7	6.3	106.7	7.1
	500.0	52.7	9.4	14.8	14.9	158.0	23.0	170.7	20.9
	1000.0	-0.9	2.9	0.3	0.2	202.6	28.5	182.5	28.4
NOA 413161 (M1)	10.0	100.1	1.3	108.2	2.1	93.2	5.1	100.8	2.4
	30.0	99.0	3.3	105.2	0.6	88.2	6.3	94.0	4.0
	100.0	91.7	4.3	102.3	3.5	92.0	8.5	102.0	7.7
	250.0	75.6	11.4	91.6	5.8	101.5	10.2	112.4	11.6
	500.0	64.3	9.5	67.8	22.2	130.9	19.3	146.3	18.5
	1000.0	51.2	11.9	37.6	29.3	136.4	18.2	157.1	5.0
NOA 413163 (M2)	10.0	103.1	9.6	112.1	5.7	104.1	6.6	111.2	16.5
	30.0	103.3	1.4	107.6	4.5	97.1	6.2	65.2	5.0
	100.0	103.7	5.6	119.3	11.5	101.8	3.0	107.9	17.6
	250.0	96.9	6.8	111.9	7.7	100.5	3.7	98.6	8.0
	500.0	98.8	5.5	105.9	7.1	124.1	19.8	125.2	31.0
	1000.0	81.0	7.8	79.2	20.6	129.9	25.5	137.2	31.2
Menadione (positive control)	0.1	96.5	4.9	104.4	4.5	91.1	11.6	95.6	4.3
	0.3	98.7	3.3	104.2	2.7	87.7	8.7	94.0	5.3
	1.0	99.2	5.3	107.1	8.5	89.9	7.9	92.9	5.5
	3.0	16.2	27.1	-2.5	4.5	170.6	27.5	185.0	47.0
	10.0	0.6	0.1	-2.0	4.7	218.0	25.1	221.9	52.5
	30.0	0.7	0.2	-1.7	4.7	213.7	28.1	218.4	51.2

* Data are indicated as mean and SD % per time point vs. the matched vehicle control.
** Concentration in µg/mL (exception Menadione concentration in µM)

III. Conclusions

For both endpoints and all time points, trifloxystrobin was the most toxic of all compounds tested here. The isomers CGA 331409 (EZi) and CGA 357261 (ZEi) were about 35 times less cytotoxic than trifloxystrobin. CGA 357262 the ZZ isomer and all metabolites were even less cytotoxic than the EZ and ZE isomers. With respect to time-dependence, cytotoxicity only marginally increased between 24 and the later time points tested.

Overall, the ranking of the compounds with respect to cytotoxicity is very similar for Alamar Blue reduction and LDH release, except that the EZ isomer shows a somewhat greater influence on LDH release than on Alamar Blue reduction compared to the ZE isomer:

For Alamar Blue reduction after 24 or 72h:

Trifloxystrobin (TFS) >> CGA 331409 (EZi), CGA 357261 (ZEi) > CGA 321113 (EEm), CGA 373466 (ZEm) > NOA 413161 (M1) > CGA 357262 (ZZi), NOA 413163 (M2)

For LDH release after 24 or 48h:

Trifloxystrobin (TFS) >> CGA 331409 (EZi) > CGA 357261 (ZEi) > CGA 321113 (EEm), CGA 373466 (ZEm) > NOA 413161 (M1), CGA 357262 (ZZi), NOA 413163 (M2)

Overall the cytotoxicity correlates well with inhibition of mitochondrial activity.

CGA 357261 (ZE isomer)

Report: KCA 5.8.1 /25; [redacted];2013;M-463623-01
Title: CGA 279202-CGA 357261 - Micronucleus test in human lymphocytes in vitro
Report No: 1553700
Document No: M-463623-01-1
Guidelines: **OECD guideline 487 (2010); Commission Regulation 640/2012, B.49.**
Deviation(s): none
A series of in-house non-GLP validation experiments were performed to get distinct responses of statistical significance when using the specified positive controls. To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487.
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name: CGA 279202-CGA 357261
Description: Beige powder
Lot/Batch no: SES 10350-40-1
Content: 99.4 %
Stability of test compound: guaranteed for study duration; expiry date: 2014-08-09

2. Vehicle / positive control: Vehicle: DMSO
Positive controls:
without metabolic activation
Mitomycin C (MMC): 2 µg/mL (pulse treatment)
Demecolcin: 125.0 ng/mL (continuous treatment)
with metabolic activation
cyclophosphamide (CPA): 17.50 µg/mL (Exp. I); 12.5 µg/mL (Exp. II)

3. Test system: Human peripheral blood lymphocytes
After blood samples were drawn, human lymphocytes were stimulated for proliferation by the addition of phytohemeagglutinine (PHA) to the culture medium for a period of 48 hours

Culture conditions: Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 hours after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/ Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 2 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).
All incubations at 37 °C with 5.5 % CO_2 in humidified air

Metabolic activation: S9 mix

B. Study design and methods

Dose:

Experi-ment	Exposure period	S9 mix	Concentrations in µg/mL
IA	4 hrs	–	2.0, 4.0, **8.0**, **16.0**, **31.9**, 63.8, 127.6, 255.3, 510.5, 1021.0, 2042.0, 4048.0
IB	4 hrs	–	5.0, 10.0, 20.0, 30.0, 40.0, 50.0, **55.0**, **60.0**, **70.0**, **80.0**, 100.0, 200.0
IA	4 hrs	+	2.0, 4.0, 8.0, 16.0, 31.9, **63.8**, **127.6**, **255.3**, 510.5, 1021.0, 2042.0, **4048.0**
II	20 hrs	–	0.2, 0.4, 0.6, 1.1, 2.0, 3.5, 6.1, 10.7, **18.7**, **32.7**, **57.1**, **100.0**
II	4 hrs	+	25.0, 50.0, **100.0**, 150.0, 200.0, 225.0, **250.0**, **275.0**, **300.0**, 325.0, 350.0, 500.0

Concentrations in bold letters chosen for micronuclei analysis.
All concentrations used for cytotoxicity assessment.

Treatment duration: With (+) S9 mix: 4 hours
Without (-) S9 mix: 4 and 20 hours

Recovery: 16 hours after beginning of treatment for the experiments with 4 h treatment time; or after the 20 hour exposure

Cytochalasin B exposure: 20 hours

Preparation interval: 40 hours after beginning of treatment with test substance

Number of evaluated cells: 2000 binucleated cells (1000 cell / culture)

Replicates: 2 parallel cultures / dose

II. Results and discussion

A. Findings

No precipitation of the test item in the culture medium was observed. No relevant influence on osmolarity or pH value was observed.

Phase separation was observed microscopically at the end of treatment in Experiment IA at 63.8 µg/mL and above in the absence and presence of S9 mix, in Experiment IB at 60.0 µg/mL and above in the absence of S9 mix and in Experiment II at 100.0 µg/mL in the absence of S9 mix and at 150.0 µg/mL and above in the presence of S9 mix.

In Experiment IA in the absence of S9 mix, concentrations showing clear cytotoxic effects were not evaluable for cytogenetic damage. In Experiment IA and II in the presence of S9 mix and in Experiment IB and II in the absence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration.

In the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying micronuclei was observed (see Table 5.8.1/25-1 and Table 5.8.1/25-2). The micronucleus rates of the cells after treatment with the test item (0.15 - 1.35 % micronucleated cells) were within the range of the solvent control values (0.15 - 1.45 % micronucleated cells) and within the range of the laboratory historical control data.

Either Demecolcin (125.0 ng/mL), MMC (2.0 µg/mL) or CPA (12.5 or 17.5 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

Table 5.8.1/25-1: Summary of results of the *in vitro* micronucleus test in human lymphocytes with CGA 279202-CGA 357261 without metabolic activation

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs without S9 mix					
IA	40 hrs	Solvent control[1]	2.03		0.15
		Positive control[2]	1.17	83.2	6.40[S]
		8.0	2.07	n.c.	0.40
		16.0	1.98	4.8	0.35
		31.9	1.91	11.7	0.45
IB	40 hrs	Solvent control[1]	2.09		0.85
		Positive control[2]	1.55	49.2	7.00[S]
		55.0	1.83	23.8	0.75
		60.0[PS]	1.80	26.3	0.35
		70.0[PS]	1.57	47.7	0.25
		80.0[PS]	1.40	63.2	0.25
Exposure period 20 hrs without S9 mix					
II	40 hrs	Solvent control[1]	1.59		0.65
		Positive control[3]	1.25	57.5	4.30[S]
		32.7	1.52	11.6	0.70
		57.1	1.34	42.5	0.80
		100.0[PS]	1.22	62.5	0.55

Exp. = experiment
* For positive control groups and test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
PS Phase separation occurred microscopically at the end of treatment
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value
[1] DMSO 1.0 % (v/v), [2] MMC 2.0 µg/mL, [3] Demecolcin 125.0 ng/mL

Table 5.8.1/25-2: Summary of results of the *in vitro* micronucleus test in human lymphocytes with CGA 279202-CGA 357262 with metabolic activation

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs with S9 mix					
IA	40 hrs	Solvent control[1]	1.96		0.30
		Positive control[2]	2.01	n.c.	3.40[S]
		63.8[PS]	2.02	n.c.	0.35
		127.6[PS]	1.96	0.6	0.15
		255.3[PS]	1.61	36.5	0.20
		4084.0[PS]	1.42	56.8	0.30
II	40 hrs	Solvent control[1]	1.90		1.45
		Positive control[3]	1.51	43.8	5.95[S]
		100.0	1.99	n.c.	1.10
		250.0[PS]	1.71	21.8	1.35
		275.0[PS]	1.57	36.7	0.75
		300.0[PS]	1.29	67.4	0.50

Exp. = experiment
* For positive control groups and test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
PS Phase separation occurred microscopically at the end of treatment
S The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c. Not calculated as the CBPI is equal or higher than the solvent control value
[1] DMSO 1.0 % (v/v), [2] CPA 17.5 µg/mL, [3] CPA 12.5 µg/mL

III. Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.
Therefore, CGA 279202-CGA 357261 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic concentrations.

CGA 331409 (EZ isomer)

Report: KCA 5.8.1 /26; [redacted];2011;M-414991-01
Title: Salmonella typhimurium reverse mutation assay with CGA279202-CGA331409
Report No: 1429201
Document No: M-414991-01-1
Guidelines: **OECD 471; Commission Regulation (EC) 440/2008, B13/14; US-EPA OPPTS 870.5100 (1998); US-EPA 712-C-98-247;**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA 279202-CGA 331409
Article no:	Not stated
Description:	Light beige powder
Lot/Batch no:	SES 10555-2-1
Purity:	99.4% (w/w)
Stability of test compound:	guaranteed for study duration, expiry date: 2013-05-19

2. Vehicle and or positive control: Vehicle: DMSO
Positive controls without metabolic activation:
TA 1535, TA 100: Sodium azide (NaN_3) in deionised water
TA 1537, TA 98: 4-nitro-o-phenylene-diamine (4-NOPD) in DMSO
TA 102 : methyl methane sulfonate (MMS) in deionised water
With metabolic activation :
TA 1535, TA 1537, TA 98, TA 100, TA 102:
2-aminoanthracene (2-AA) in DMSO

3. Test system: *Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537

Metabolic activation: Mammalian Microsomal Fraction S9 Mix prepared from livers of 8-12 weeks old male Wistar rats treated with a mixture of 80 mg/kg bw phenobarbital i.p. and 80 mg/kg bw beta-naphthoflavone p.o.
The protein concentration was 25.7 mg/mL in the pre-experiment and 33.9 mg/mL in the main experiment.
Prior to the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath.

Pre-cultures: From the thawed ampoules of the strains 0.5 mL bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing 20 mL nutrient medium. A solution of 20 µL ampicillin (25 µg/mL) was added to the strains TA 98,

TA 100, TA 102. This nutrient medium contained 8 g/L Nutrient Broth (MERCK, Darmstadt) and 5 g/L NaCl (MERCK, Darmstadt)

B. Study design and methods

1. Treatment

Dose:

Test item concentrations:
Experiment I: 3-10-33-100-333-1000-2500-5000 µg/plate
Experiment II: 10-33-100-333-1000-2500-5000 µg/plate

Positive controls:

NaN_3:	10 µg/plate	(TA 1535, TA 100)
4-NOPD:	10 µg/plate	(TA 98)
	50 µg/plate	(TA1537)
MMS:	3 µL/plate	(TA102)
2-AA:	25 µg/plate + S9 mix	(TA 1535, TA 1537, TA 98, TA 100)
	10 µg/plate + S9	(TA 102)

For each test solution or control 3 plates were used.

Application volume: 100 µL(test solution)/plate
Incubation time / temperature: 48 hours, 37°C

II. Results and discussion

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5) occurred in the test groups with and without metabolic activation.
No substantial increase in revertant colony numbers of any of the five test strains was observed following treatment with CGA279202-CGA331409 at any concentration level, neither in the presence nor absence of metabolic activation.
There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
The positive controls showed a distinct increase in induced revertant colonies, and therefore confirmed the validity of the test system.
The results are summarized in the following Table 5.8.1/26-1 and Table 5.8.1/26-2.

Table 5.8.1/26-1: Result of the Pre-Experiment and Experiment 1 (mean values and SD n = 3 plates per test solution/control)

Compound	S9 mix	Concentration (µg/plate)	Revertants / plate				
			Base-pair substitution type			Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
DMSO	-		18 ± 3	117 ± 7	467 ± 24	18 ± 4	26 ± 5
Untreated	-		18 ± 7	120 ± 2	469 ± 7	14 ± 1	28 ± 2
CGA279202 - CGA331409	-	3	20 ± 5	117 ± 8	428 ± 12	15 ± 4	29 ± 5
	-	10	18 ± 4	115 ± 15	433 ± 21	16 ± 4	26 ± 3
	-	33	20 ± 5	118 ± 23	432 ± 13	15 ± 2	27 ± 5
	-	100	17 ± 4	125 ± 4	394 ± 15	12 ± 3	24 ± 9
	-	333	15 ± 1	125 ± 8	424 ± 21	12 ± 3	31 ± 6
	-	1000	13 ± 3 P	131 ± 5 P	403 ± 18 P	9 ± 3 P	32 ± 5 P
	-	2500	14 ± 1 P	129 ± 9 P	428 ± 28 P	12 ± 0 P	28 ± 8 P
	-	5000	13 ± 4 P	129 ± 8 P	403 ± 16 P	13 ± 2 P	25 ± 7 P
NaN_3	-	10	1682 ± 36	1974 ± 66			
4-NOPD	-	10					291 ± 14
	-	50				76 ± 7	
MMS	-	3			2894 ± 108		
DMSO	+		17 ± 3	155 ± 8	585 ± 44	19 ± 4	38 ± 8
Untreated	+		24 ± 5	169 ± 7	649 ± 34	19 ± 10	39 ± 3
CGA279202 - CGA331409	+	3	18 ± 5	159 ± 14	596 ± 33	21 ± 7	36 ± 8
	+	10	16 ± 1	131 ± 7	578 ± 27	18 ± 4	36 ± 1
	+	33	16 ± 4	138 ± 12	525 ± 20	21 ± 7	35 ± 7
	+	100	22 ± 7	164 ± 10	547 ± 42	20 ± 8	33 ± 2
	+	333	24 ± 3	148 ± 7	569 ± 12	18 ± 5	38 ± 5
	+	1000	17 ± 4 PM	151 ± 15 PM	576 ± 13 PM	15 ± 2 PM	25 ± 3 PM
	+	2500	12 ± 1 PM	132 ± 4 PM	566 ± 10 PM	15 ± 3 PM	25 ± 5 PM
	+	5000	13 ± 1 PM	121 ± 3 PM	550 ± 20 PM	12 ± 4 PM	30 ± 5 PM
2-AA		2.5	429 ± 20	3210 ± 62		427 ± 50	2201 ± 227
2-AA	+	10.0			2268 ± 120		

SD = standard deviation P = Precipitate M = Manual count

Table 5.8.1/26-2: Result of the Experiment 2 (mean values and SD n = 3 plates per test solution/control)

Compound	S9 mix	Concentration (µg/plate)	Revertants / plate				
			Base-pair substitution type			Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
DMSO	-		16 ± 6	116 ± 8	363 ± 14	14 ± 4	35 ± 2
Untreated	-		13 ± 1	150 ± 18	339 ± 25	22 ± 3	34 ± 4
CGA279202 - CGA331409	-	10	16 ± 2	116 ± 11	336 ± 28	13 ± 4	35 ± 7
	-	33	18 ± 5	104 ± 10	372 ± 19	15 ± 2	29 ± 3
	-	100	18 ± 8	116 ± 18	375 ± 32	13 ± 3	38 ± 4
	-	333	14 ± 6	93 ± 12	326 ± 8	14 ± 6	31 ± 5
	-	1000	15 ± 2 P	88 ± 11 P	327 ± 8 P	12 ± 2 P	32 ± 7 P
	-	2500	16 ± 6 P	89 ± 3 P	315 ± 45 P	14 ± 2 P	36 ± 7 P
	-	5000	12 ± 3 P	91 ± 15 P	315 ± 9 P	11 ± 1 P	28 ± 3 P
NaN_3	-	10	1656 ± 31	1825 ± 46			
4-NOPD	-	10					318 ± 17
	-	50				100 ± 20	
MMS	-	3			1513 ± 117		
DMSO	+		17 ± 2	101 ± 3	365 ± 18	13 ± 1	42 ± 12
Untreated	+		17 ± 6	130 ± 6	382 ± 48	23 ± 7	58 ± 13
CGA279202 - CGA331409	+	10	19 ± 6	110 ± 12	350 ± 27	14 ± 4	48 ± 1
	+	33	20 ± 4	106 ± 13	330 ± 24	15 ± 2	45 ± 5
	+	100	22 ± 2	110 ± 16	392 ± 21	16 ± 4	44 ± 11
	+	333	17 ± 4 P	83 ± 10 P	312 ± 30 P	15 ± 7 P	42 ± 1 P
	+	1000	19 ± 3 P	93 ± 25 P	308 ± 23 P	19 ± 5 P	43 ± 5 P
	+	2500	14 ± 2 PM	92 ± 9 PM	382 ± 18 PM	13 ± 3 P	37 ± 6 PM
	+	5000	14 ± 1 PM	85 ± 8 PM	288 ± 16 PM	13 ± 4 PM	32 ± 3 PM
2-AA	+	2.5	243 ± 10	1170 ± 16		158 ± 16	1144 ± 45
2-AA	+	10.0			1862 ± 76		

SD = standard deviation P = Precipitate M = Manual count

III. Conclusions

The test item CGA279202-CGA331409 did not induce gene mutations by base pair changes or frameshifts in the genome of *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537 neither in the presence nor absence of metabolic activation (S9 mix).
Therefore, CGA279202-CGA331409 is considered to be non-mutagenic in this assay.

Report:	KCA 5.8.1 /27; [redacted] ;2013;M-463619-01
Title:	CGA 279202-CGA 331409 - Micronucleus test in human lymphocytes in vitro
Report No:	1553600
Document No:	M-463619-01-1
Guidelines:	**OECD guideline 487 (2010); Commission Regulation 640/2012, B.49** **Deviation(s): none** A series of in-house non-GLP validation experiments were performed to get distinct responses of statistical significance when using the specified positive controls. To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487.
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA 279202-CGA 331409
Description:	White powder
Lot/Batch no:	SES 10555-1-3
Purity:	98 %
Stability of test compound:	guaranteed for study duration; expiry date: 2016-03-06
2. Vehicle / positive control:	Vehicle: DMSO Positive controls: without metabolic activation Mitomycin C (MMC): 2 µg/mL (pulse treatment) Demecolcin: 75.0 ng/mL (continuous treatment) with metabolic activation cyclophosphamide (CPA): 15.0 µg/mL (Exp. I); 17.5 µg/mL (Exp. II)
3. Test system:	Human peripheral blood lymphocytes After blood samples were drawn, human lymphocytes were stimulated for proliferation by the addition of phytohemeagglutinine (PHA) to the culture medium for a period of 48 hours
Culture conditions:	Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 hours after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/ Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL). All incubations at 37 °C with 5.5 % CO_2 in humidified air
Metabolic activation:	S9 mix

B. Study design and methods

Dose:

Experi-ment	Exposure period	S9 mix	Concentrations in µg/mL
I	4 h	−	8.9, **15.5**, **27.1**, **47.4**, 83.0, 145.2, 254.1, 444.6, 778.1, 1361.6, 2382.9, 4170.0
I	4 h	+	8.9, 15.5, **27.1**, **47.4**, 83.0, **145.2**, **254.1**, 444.6, 778.1, 1361.6, 2382.9, 4170.0
II	20 h	−	0.2, 0.5, 1.0, 2.0, 3.9, **7.8**, **15.6**, **31.3**, **62.5**, 125.0. 250.0, 500.0
II	4 h	+	**12.5**, **25.0**, 50.0, **100.0**, **150.0**, 175.0, **200.0**, 225.0, 250.0, 300.0, 500.0

Concentrations in bold letters chosen for micronuclei analysis.
All concentrations used for cytotoxicity assessment.

Treatment duration: With (+) S9 mix: 4 hours
Without (-) S9 mix: 4 and 20 hours

Recovery: 16 hours after beginning of treatment for the experiments with 4 h treatment time; or after the 20 hour exposure

Cytochalasin B exposure: 20 hours

Preparation interval: 40 hours after beginning of treatment with test substance

Number of evaluated cells: 2000 binucleated cells (1000 cell / culture)

Replicates: 2 parallel culture / dose

II. Results and discussion

A. Findings

In Experiment I, visible precipitation of the test item in the culture medium was observed microscopically at 27.1 µg/mL and above in the absence of S9 mix and at 47.4 µg/mL and above in the presence of S9 mix at the end of treatment. In addition, precipitation occurred in Experiment II at 15.6 µg/mL and above in the absence of S9 mix and at 25.0 µg/mL and above in the presence of S9 mix at the end of treatment.

No relevant influence on osmolarity or pH value was observed.

In Experiment I in the absence and presence of S9 mix and in Experiment II in the absence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration. In Experiment II in the presence of S9 mix concentrations showing clear cytotoxicity were not evaluable for cytogenetic damage.

In both experiments, in the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying micronuclei was observed (see Table 5.8.1/27-1 and Table 5.8.1/27-2). The micronucleus rates of the cells after treatment with the test item (0.10 - 0.75 % micronucleated cells) did not exceed the range of the solvent control values (0.20 - 1.10 % micronucleated cells) and were within the range of the laboratory historical control data.

Either Demecolcin (75.0 ng/mL), MMC (2.0 µg/mL) or CPA (15.0 or 17.5 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

Table 5.8.1/27-1: Summary of results of the *in vitro* micronucleus test in human lymphocytes with CGA 279202-CGA 331409 without metabolic activation

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs without S9 mix					
I	40 hrs	Solvent control[1]	1.84		0.30
		Positive control[2]	1.25	69.7	7.85[S]
		15.5	1.58	30.8	0.25
		27.1[P]	1.64	24.0	0.15
		47.4[P]	1.39	52.9	0.10
Exposure period 20 hrs without S9 mix					
II	40 hrs	Solvent control[1]	1.77		0.20
		Positive control[3]	1.42	45.5	3.00[S]
		7.8	1.70	9.1	0.25
		15.6[P]	1.66	13.6	0.30
		31.3[P]	1.53	30.9	0.20
		62.5[P]	1.33	56.5	0.10

Exp. = experiment
* For positive control groups and test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
P Precipitation occurred microscopically at the end of treatment
S The number of micronucleated cells is statistically significantly higher than corresponding control values
[1] DMSO 1.0 % (v/v), [2] MMC 2.0 µg/mL, [3] Demecolcin 75.0 ng/mL

Table 5.8.1/27-2: Summary of results of the *in vitro* micronucleus test in human lymphocytes with CGA 279202-CGA 331409 with metabolic activation

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs with S9 mix					
I	40 hrs	Solvent control[1]	2.13		0.45
		Positive control[2]	1.90	20.7	8.00
		27.1	2.12	1.2	0.70
		47.4[P]	2.08	4.7	0.60
		145.2[P]	1.69	39.2	0.15
		254.1[P]	1.35	69.5	0.55
II	40 hrs	Solvent control[1]	1.83		1.10
		Positive control[3]	1.63	24.0	4.15[S]
		12.5	1.84	n.c.	0.75
		25.0[P]	1.85	n.c.	0.55
		100.0[P]	1.71	15.1	0.35
		150.0[P]	1.58	30.1	0.65
		200.0[P]	1.49	41.5	0.20

Exp. = experiment
* For positive control groups and test item treatment groups the values are related to the solvent controls
** The number of micronucleated cells was determined in a sample of 2000 binucleated cells
P Precipitation occurred microscopically at the end of treatment
S The number of micronucleated cells is statistically significantly higher than corresponding control values
[1] DMSO 1.0 % (v/v), [2] CPA 15.0 µg/mL, [3] CPA 17.5 µg/mL

III. Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.
Therefore, CGA 279202-CGA 331409 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic and/or precipitating concentrations.

CGA 357262 (ZZ-isomer)

Report:	KCA 5.8.1 /28; [redacted];2011;M-414989-01
Title:	Salmonella typhimurium reverse mutation assay with CGA279202-CGA357262
Report No:	1429202
Document No:	M-414989-01-1
Guidelines:	**OECD 471; Commission Regulation (EC) 440/2008, B13/14; US-EPA OPPTS 870.5100 (1998), US-EPA 712-C-98-247; Deviations: none**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: CGA 279202-CGA 357262

Article no:	Not stated
Description:	Colorless liquid
Lot/Batch no:	SES 10487-2-1
Purity:	99.4% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2013-05-19

2. Vehicle and or positive control: Vehicle: DMSO

Positive controls without metabolic activation:
TA 1535, TA 100: Sodium azide (NaN_3) in deionised water
TA 1537, TA 98: 4-nitro-o-phenylene-diamine (4-NOPD) in DMSO
TA 102: methyl methane sulfonate (MMS) in deionised water

With metabolic activation
TA 1535, TA 1537, TA 98, TA 100, TA 102:
2-aminoanthracene (2-AA) in DMSO

3. Test system: *Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537

Metabolic activation: Mammalian Microsomal Fraction S9 Mix prepared from livers of 8–12 weeks old male Wistar rats treated with a mixture of 80 mg/kg bw phenobarbital i.p. and 80 mg/kg bw beta-naphthoflavone p.o.
The protein concentration was 25.7 mg/mL in the pre-experiment und 33.9 mg/mL in the main experiment.
Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath.

Pre-cultures: From the thawed ampoules of the strains 0.5 mL bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing 20 mL nutrient medium. A solution of 20 µL ampicillin (25 µg/mL) was added to the strains TA 98, TA 100, TA 102. This nutrient medium contained 8 g/L Nutrient Broth and 5 g/L NaCl (both MERCK, Darmstadt).

B. Study design and methods

1. Treatment

Dose	Test item concentrations: Experiment I: 3-10-33-100-333-1000-2500-5000 µg/plate Experiment II: 33-100-333-1000-2500-5000 µg/plate Positive controls: NaN_3: 10 µg/plate (TA 1535, TA 100) 4-NOPD : 10 µg/plate (TA 98) 50 µg/plate (TA1537) MMS : 3 µL/plate (TA 102) 2-AA: 2.5 µg/plate + S9 mix (TA 1535, TA 1537, TA 98, TA 100) 10 µg/plate + S9 (TA 102) For each test solution or control 3 plates were used.
Application volume:	100 µL (test solution)/plate
Incubation time / temperature:	48 hours, 37°C

II. Results and discussion

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5) occurred in the test groups with and without metabolic activation.
No substantial increase in revertant colony numbers of any of the five test strains was observed following treatment with CGA279202-CGA357262 at any concentration level, neither in the presence nor absence of metabolic activation (S9 mix).
There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.
The positive controls showed a distinct increase in induced revertant colonies, and therefore, confirmed the validity of the test system.
The results are summarized in the following Table 5.8.1/28-1 and Table 5.8.1/28-2.

Table 5.8.1/28-1: Result of the first experiment (mean values and SD of n = 3 plates per test solution/control)

Compound	S9 mix	Concentration (µg/plate)	Revertants / plate				
			Base-pair substitution type			Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
DMSO	-		15 ± 5	114 ± 10	402 ± 4	18 ± 4	27 ± 3
Untreated	-		17 ± 4	131 ± 9	403 ± 30	22 ± 3	28 ± 5
CGA279202 - CGA321113	-	3	13 ± 4	113 ± 2	388 ± 11	18 ± 3	30 ± 6
	-	10	16 ± 4	100 ± 21	412 ± 16	19 ± 2	31 ± 6
	-	33	13 ± 1	116 ± 12	405 ± 22	21 ± 1	26 ± 5
		100	14 ± 6	123 ± 10	445 ± 12	19 ± 2	24 ± 4
	-	333	13 ± 3	116 ± 13	392 ± 29	18 ± 4	29 ± 1
	-	1000	12 ± 3 P	108 ± 8 P	423 ± 46	17 ± 2 P	26 ± 10 P
	-	2500	10 ± 3 P	124 ± 11 P	400 ± 14 P	17 ± 2 P	25 ± 3 P
	-	5000	13 ± 2 P	124 ± 5 P	372 ± 12 P	17 ± 2 P	28 ± 7 P
NaN_3	-	10	1704 ± 50	1853 ± 63			
4-NOPD	-	10					267 ± 9
	-	50				80 ± 2	
MMS	-	3			4084 ± 77		
DMSO	+		21 ± 7	136 ± 2	570 ± 23	24 ± 2	40 ± 9
Untreated	+		21 ± 7	156 ± 19	570 ± 26	23 ± 6	40 ± 5
CGA279202 - CGA357262	+	3	20 ± 1	140 ± 11	547 ± 19	19 ± 4	47 ± 3
	+	10	21 ± 1	135 ± 13	508 ± 11	24 ± 6	34 ± 7
	+	33	19 ± 2	153 ± 7	571 ± 13	22 ± 1	32 ± 5
	+	100	21 ± 1	166 ± 3	628 ± 26	20 ± 8	40 ± 10
	+	333	20 ± 2	151 ± 6	516 ± 42	20 ± 3	42 ± 3
	+	1000	22 ± 6 P	141 ± 15 P	579 ± 31 P	25 ± 4 P	44 ± 6 P
	+	2500	17 ± 5 P	127 ± 7 P	467 ± 16 P	23 ± 5 P	30 ± 6 P
	+	5000	14 ± 2 PM	137 ± 11 PM	491 ± 20 PM	22 ± 2 P	26 ± 4 PM
2-AA	+	2.5	392 ± 13	2694 ± 105		386 ± 24	2109 ± 334
2-AA	+	10.0			2144 ± 426		

SD = standard deviation P = Precipitate M = Manual count

Table 5.8.1/28-2: Result of the second experiment (mean values and SD of n = 3 plates per test solution/control)

Compound	S9 mix	Concentr. (µg/plate)	Revertants / plate				
			Base-pair substitution type			Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
DMSO	-		12 ± 2	113 ± 8	340 ± 19	19 ± 2	33 ± 10
Untreated	-		16 ± 3	125 ± 6	312 ± 25	19 ± 4	34 ± 2
CGA279202	-	33	13 ± 4	104 ± 15	301 ± 13	16 ± 4	38 ± 5
-		100	12 ± 2	100 ± 16	322 ± 18	20 ± 6	34 ± 3
CGA357262	-	333	13 ± 4	110 ± 2	358 ± 22	18 ± 2	32 ± 2
		1000	12 ± 3	88 ± 8	295 ± 13	14 ± 2	34 ± 11
		2500	11 ± 3	97 ± 6	287 ± 18	17 ± 3	25 ± 4
	-	5000	17 ± 2	101 ± 18	283 ± 13	12 ± 3	33 ± 2
NaN_3	-	10	1512 ± 230	1619 ± 27			
4-NOPD	-	10					324 ± 9
	-	50				90 ± 8	
MMS	-	3			1661 ± 18		
DMSO	+		19 ± 3	105 ± 8	367 ± 28	24 ± 8	38 ± 2
Untreated	+		16 ± 4	131 ± 14	346 ± 21	26 ± 6	45 ± 2
CGA279202	+	33	18 ± 3	101 ± 17	322 ± 47	22 ± 2	37 ± 4
-	+	100	19 ± 3	113 ± 3	305 ± 33	21 ± 4	39 ± 4
CGA357262	+	333	18 ± 2	110 ± 11	358 ± 29	22 ± 5	44 ± 3
	+	1000	19 ± 2	100 ± 11	273 ± 11	24 ± 3	45 ± 9
	+	2500	17 ± 2^{P}	114 ± 17^{P}	253 ± 35^{P}	22 ± 3^{P}	35 ± 5^{P}
	+	5000	13 ± 0^{PM}	106 ± 10^{PM}	224 ± 106^{PM}	13 ± 4^{PM}	32 ± 3^{PM}
2-AA	+	2.5	239 ± 21	1022 ± 33		135 ± 22	1260 ± 72
2-AA	+	10.0			1644 ± 60		

SD = standard deviation P = Precipitate M = Manual count

III. Conclusions

The test item CGA279202-CGA357262 did not induce gene mutations by base pair changes or frameshifts in the genome of *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537 neither in the presence nor absence of metabolic activation (S9 mix).
Therefore, CGA279202-CGA357262 is considered to be non-mutagenic in this assay.

Report:	KCA 5.8.1 /29; [redacted];2013;M-463639-01
Title:	CGA 279202-CGA 357262 - Micronucleus test in human lymphocytes in vitro
Report No:	1553800
Document No:	M-463639-01-1
Guidelines:	**OECD guideline 487 (2010); Commission Regulation 640/2012, B.49** **Deviation(s): none** A series of in-house non-GLP validation experiments were performed to get distinct responses of statistical significance when using the specified positive controls. To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487.
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA 279202-CGA 357262
Description:	Sticky white crystals
Lot/Batch no:	SES 10487-1-2
Content:	96.7 %
Stability of test compound:	guaranteed for study duration; expiry date: 2016-02-06

2. Vehicle / positive control: Vehicle: DMSO
Positive controls:
without metabolic activation
mitomycin C (MMC): 2 µg/mL (pulse treatment)
Demecolcin: 15.0 ng/mL (continuous treatment)
with metabolic activation
cyclophosphamide (CPA): 15.6 µg/mL (Exp. I); 17.5 µg/mL (Exp. II)

3. Test system: Human peripheral blood lymphocytes
After blood samples were drawn, human lymphocytes were stimulated for proliferation by the addition of phytohemeagglutinine (PHA) to the culture medium for a period of 48 hours

Culture conditions: Blood cultures were established by preparing an 11 % mixture of whole blood in medium within 30 hours after blood collection. The culture medium was Dulbecco's Modified Eagles Medium/ Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/mL/100 µg/mL), the mitogen PHA (3 µg/mL), 10 % FBS (fetal bovine serum), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/mL).
All incubations done at 37 °C with 5.5 % CO_2 in humidified air

metabolic activation: S9 mix

B. Study design and methods

Dose:

Experi-ment	Exposure period	S9 mix	Concentrations in µg/mL
I	4 h	–	2.0, 4.0, **8.0**, 16.0, **31.9**, **63.8**, **127.6**, 255.3, 510.5, 1021.0, 2042.0, 4048.0
I	4 h	+	2.0, 4.0, 8.0, **16.0**, 31.9, **63.8**, **127.6**, **255.3**, 510.5, 1021.0, 2042.0, 4048.0
II	20 h	–	0.2, 0.5, 1.0, 2.0, 3.9, 7.8, 15.6, **31.3**, **62.5**, **125.0**, 250.0, 500.0
II	4 h	+	6.3, 12.5, 25.0, 50.0, 100.0, **150.0**, **200.0**, **225.0**, 250.0, 275.0, 300.0, 500.0

Concentrations in bold letters chosen for micronuclei analysis.
All concentrations used for cytotoxicity assessment.

Treatment duration: With (+) S9 mix: 4 hours
Without (-) S9 mix: 4 and 20 hours

Recovery: 16 hours after beginning of treatment for the experiments with 4 h treatment time; or after the 20 hour exposure

Cytochalasin B exposure: 20 hours

Preparation interval: 40 hours after beginning of treatment

Number of evaluated cells: 2000 binucleated cells (1000 cell / culture)

Replicates: 2 parallel culture / dose

II. Results and discussion

A. Findings

No precipitation of the test item in the culture medium was observed. No relevant influence on osmolarity or pH value was observed.

Phase separation was observed microscopically at the end of treatment in Experiment I at 16.0 µg/mL and above in the absence of S9 mix and at 31.9 µg/mL and above in the presence of S9 mix and in Experiment II at 125.0 µg/mL in the absence of S9 mix and at 50.0 µg/mL in the presence of S9 mix.

In the absence and presence of S9 mix, clear cytotoxic effects were observed at the highest evaluated concentration.

In the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying micronuclei was observed (see Table 5.8.1/29-1 and Table 5.8.1/29-2). The micronucleus rates of the cells after treatment with the test item (0.10 - 0.60 % micronucleated cells) did not exceed the range of the solvent control values (0.40 - 0.75 % micronucleated cells) and were within the range of the laboratory historical control data.

Either Demecolcin (75.0 ng/mL), MMC (2.0 µg/mL) or CPA (15.0 or 17.5 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

Table 5.8.1/29-1: Summary of results of the in vitro micronucleus test in human lymphocytes with CGA 279202-CGA 357262 without metabolic activation

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs without S9 mix					
I	40 hrs	Solvent control[1]	1.93		0.40
		Positive control[2]	1.32	65.7	8.90[S]
		8.0	1.88	4.6	0.30
		31.9[PS]	1.94	n.c.	0.25
		63.8[PS]	1.63	32.0	0.10
		127.6[PS]	1.25	73.5	0.30
Exposure period 20 hrs without S9 mix					
II	40 hrs	Solvent control[1]	1.93		0.45
		Positive control[3]	1.42	55.0	3.00[S]
		31.3	1.76	18.6	0.20
		62.5	1.63	31.8	0.35
		125.0[PS]	1.30	67.9	0.35

Exp. = experiment

* For the positive control groups and the test item treatment groups the values are related to the solvent controls

** The number of micronucleated cells was determined in a sample of 2000 binucleated cells

PS Phase separation occurred at the end of treatment

S The number of micronucleated cells is statistically significantly higher than corresponding control values

n.c. Not calculated as the CBPI is equal or higher than the solvent control value

[1] DMSO 1.0 % (v/v), [2] MMC 2.0 µg/mL, [3] Demecolcin 75.0 ng/mL

Table 5.8.1/29-2: Summary of results of the *in vitro* micronucleus test in human lymphocytes with CGA 279202-CGA 357262 with metabolic activation

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %*	Micronucleated cells in %**
Exposure period 4 hrs with S9 mix					
I	40 hrs	Solvent control[1]	1.99		0.40
		Positive control[2]	1.60	39.0	4.80[S]
		16.0	1.90	8.6	0.40
		63.8[PS]	1.93	6.2	0.60
		127.6[PS]	1.82	17.1	0.10
		255.3[PS]	1.45	54.8	0.30
II	40 hrs	Solvent control[1]	1.96		0.75
		Positive control[3]	1.63	34.2	4.15[S]
		25.0	1.95	1.6	0.40
		150.0[PS]	1.74	23.3	0.35
		200.0[PS]	1.55	42.5	0.20
		225.0[PS]	1.43	55.7	0.25

Exp. =experiment

* For the positive control groups and the test item treatment groups the values are related to the solvent controls

** The number of micronucleated cells was determined in a sample of 2000 binucleated cells

PS Phase separation occurred at the end of treatment

S The number of micronucleated cells is statistically significantly higher than corresponding control values

[1] DMSO 1.0 % (v/v), [2] CPA 15.0 µg/mL, [3] CPA 17.5 µg/mL

III. Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.

Therefore, CGA 279202-CGA 357262 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic concentrations.

CGA 321113 (EE-metabolite)

Report:	KCA 5.8.1 /30; [redacted];2011;M-406346-01
Title:	Salmonella typhimurium reverse mutation assay with CGA279202-CGA 321113
Report No:	1390501
Document No:	M-406346-01-1
Guidelines:	**Commission Regulation (EC) No 440/2008, B13/14; US-EPA 712-C-98-247, OPTTS 870.5100 (1998);** **Deviation(s): none**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA279202-CGA321113
Article no:	Not stated
Description:	White powder
Lot/Batch no:	BCOC96132-2-3
Purity:	98.6% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2012-11-26

2. Vehicle and or positive control: Vehicle: Tetrahydrofuran (THF)
Positive controls without metabolic activation
TA 1535, TA 100: Sodium azide (NaN_3) in deionized water
TA 1537, TA 98: 4-nitro-o-phenylene-diamine (4-NOPD) in DMSO
TA 102 : methyl methane sulfonate (MMS) in deionized water
With metabolic activation
TA 1535, TA 1537, TA 98, TA 100, TA 102: 2-aminoanthracene (2-AA) in DMSO

3. Test system: *Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537;

Metabolic activation: Mammalian Microsomal Fraction S9 Mix prepared from livers of 8–12 weeks old male Wistar rats treated with a mixture of 80 mg/kg bw phenobarbital i.p. and 80 mg/kg bw beta-naphthoflavone p.o.
The protein concentration was 25.7 mg/mL in the pre-experiment und 33.9 mg/mL in the main experiment.
Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath.

Pre-cultures: From the thawed ampoules of the strains 0.5 mL bacterial suspension was transferred into 250 mL Erlenmeyer flasks containing 20 mL nutrient medium. A solution of 20 µL ampicillin (25 µg/mL) was added to the strains TA 98,

TA 100, TA 102. This nutrient medium contained 8 g/L Nutrient Broth (MERCK, Darmstadt) and 5 g/L NaCl (MERCK, Darmstadt).

B. Study design and methods

1. Treatment

Dose

Test item concentrations:
Experiment I: 3-10-33-100-333-1000-2500-5000 µg/plate
Experiment II: 10-33-100-333-1000-2500-5000 µg/plate

Positive controls:

NaN_3:	10 µg/plate (TA 1535, TA 100)
4-NOPD:	10 µg/plate (TA 98)
	50 µg/plate (TA 1537)
MMS:	3 µL/plate (TA 102)
2-AA:	2.5 µg/plate + S9 mix (TA 1535, TA 1537, TA 98, TA 100)
	10 µg/plate + S9 (TA 102)

For each test solution or control 3 plates were used.

Application volume: 25 µL(test solution)/plate

Incubation time / temperature: 48 hours, 37 °C

II. Results and discussion

The plates incubated with the test item showed reduced background growth in all strains. Toxic effects, evident as a reduction in the number of revertants (i.e. below the factor of 0.5), were observed in all strains.

No substantial increase in revertant colony numbers of any of the five test strains was observed following treatment with CGA 279202-CGA 321113 at any concentration, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. Appropriate reference mutagens were used as positive controls. They showed a distinct increase in induced revertant colonies.

The results are summarized in the following Table 5.8.1/30-1 and Table 5.8.1/30-2.

Table 5.8.1/30-1: Result of the first experiment (mean values and SD of n = 3 plates per test solution/control)

Compound	S9 mix	Concentration (µg/plate)	Revertants / plate: Base-pair substitution type			Revertants / plate: Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
THF	-		18 ± 6	173 ± 10	500 ± 25	12 ± 2	29 ± 2
Untreated	-		22 ± 6	161 ± 13	494 ± 10	8 ± 2	33 ± 5
CGA279202 - CGA321113	-	3	16 ± 3	176 ± 20	482 ± 50	11 ± 2	30 ± 12
	-	10	20 ± 4	180 ± 15	496 ± 22	13 ± 1	25 ± 3
	-	33	17 ± 3	173 ± 10	498 ± 9	13 ± 2	31 ± 6
	-	100	17 ± 9	159 ± 14	493 ± 11	13 ± 1	29 ± 7
	-	333	16 ± 3	117 ± 5	473 ± 19	10 ± 2	29 ± 6
	-	1000	18 ± 5	100 ± 13	452 ± 14	7 ± 2	27 ± 7
	-	2500	17 ± 3	94 ± 12 R	420 ± 16	7 ± 2	22 ± 2
	-	5000	13 ± 5	79 ± 7 MR	378 ± 17	3 ± 2 R	19 ± 3
NaN_3	-	10	2225 ± 54	2292 ± 67			
4-NOPD	-	10					328 ± 33
	-	50				75 ± 11	
MMS	-	3		4645 ± 21			
THF	+		18 ± 4	195 ± 11	637 ± 15	13 ± 4	35 ± 9
Untreated	+		19 ± 7	172 ± 24	634 ± 17	13 ± 2	41 ± 3
CGA279202 - CGA321113	+	3	18 ± 4	189 ± 7	620 ± 19	15 ± 5	35 ± 2
	+	10	19 ± 4	209 ± 5	608 ± 37	13 ± 3	33 ± 4
	+	33	18 ± 8	195 ± 16	605 ± 39	14 ± 2	33 ± 4
	+	100	20 ± 3	196 ± 8	594 ± 31	14 ± 2	37 ± 7
	+	333	22 ± 5	182 ± 15	613 ± 50	15 ± 1	36 ± 10
	+	1000	19 ± 6	132 ± 16	569 ± 21	9 ± 3	32 ± 3
	+	2500	16 ± 6	120 ± 6	562 ± 25	7 ± 1	32 ± 5
	+	5000	14 ± 1	57 ± 6 MR	485 ± 22	5 ± 2	26 ± 9
2-AA	+	2.5	469 ± 27	2798 ± 111		368 ± 23	2709 ± 176
2-AA	+	10.0			3007 ± 71		

SD = standard deviation; R = Reduced background growth; M = Manual count

Table 5.8.1/30-2: Result of the second experiment (mean values and SD of n = 3 plates per test solution/control)

Compound	S9 mix	Concentration (µg/plate)	Revertants / plate				
			Base-pair substitution type			Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
THF	-		14 ± 1	152 ± 14	402 ± 4	11 ± 2	29 ± 1
Untreated	-		13 ± 3	169 ± 11	369 ± 27	13 ± 1	29 ± 9
CGA279202	-	10	15 ± 3	168 ± 10	388 ± 11	12 ± 2	27 ± 8
-	-	33	15 ± 6	167 ± 17	427 ± 44	13 ± 6	26 ± 9
CGA321113		100	16 ± 6	170 ± 9	445 ± 25	10 ± 2	29 ± 5
	-	333	14 ± 2	96 ± 22	372 ± 4	7 ± 1	24 ± 5
		1000	8 ± 1^{MR}	63 ± 12^{MR}	320 ± 8	5 ± 2 ^{MR}	17 ± 3
		2500	1 ± 1^{MR}	41 ± 9^{MR}	249 ± 12	1 ± 1 ^{MR}	13 ± 2^{R}
	-	5000	0 ± 0 ^{MR}	19 ± 5^{MR}	21 ± 6 ^{MR}	0 ± 1^{MR}	2 ± 3^{R}
NaN_3	-	10	1942 ± 39	1966 ± 184			
4-NOPD	-	10					466 ± 17
	-	50				82 ± 15	
MMS	-	3			3351 ± 275		
THF	+		16 ± 3	180 ± 15	567 ± 66	19 ± 2	34 ± 9
Untreated	+		21 ± 9	205 ± 3	532 ± 12	15 ± 2	41 ± 4
CGA279202	+	10	16 ± 4	191 ± 17	499 ± 29	17 ± 4	37 ± 5
-	+	33	17 ± 4	168 ± 6	497 ± 27	19 ± 0	35 ± 6
CGA321113	+	100	17 ± 4	191 ± 8	561 ± 17	17 ± 2	34 ± 6
	+	333	19 ± 2	116 ± 18	503 ± 34	21 ± 1	36 ± 8
	+	1000	12 ± 2 ^{R}	92 ± 0	431 ± 9	12 ± 2^{R}	31 ± 4
	+	2500	6 ± 2^{MR}	73 ± 5MR	440 ± 50	7 ± 2^{MR}	32 ± 2
	+	5000	2 ± 1	43 ± 9MR	264 ± 10^{R}	0 ± 1^{MR}	8 ± 2^{MR}
2-AA	+	2.5	362 ± 24	2219 ± 161		291 ± 40	1998 ± 326
2-AA	+	10.0			2561 ± 195		

SD = standard deviation; R = Reduced background growth; M = Manual count

III. Conclusions

The test item CGA279202-CGA321113 did not induce gene mutations by base pair changes or frameshifts in the genome of *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537 neither in the presence nor absence of metabolic activation (S9 mix).
Therefore, CGA279202-CGA321113 is considered to be non-mutagenic in this assay.

Report: KCA 5.8.1 /31; [redacted];2011;M-411413-01
Title: CGA 279202-CGA 321113 - Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT)
Report No: 1390503
Document No: M-411413-01-1
Guidelines: **OECD 476; Commission Regulation (EC) No. 440/2008, B.17; US-EP 712-C-98-221, OPPTS 870.5300 (1998)**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA279202+CGA321113
Article no:	Not stated
Description:	White powder
Lot/Batch no:	BCOO6132-2-3
Purity:	98.6% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2012-11-26

2. Vehicle and or positive control: Vehicle: tetrahydrofuran (THF).
Positive controls without metabolic activation:
0.15 mg/mL ethylmethane sulfonate (EMS) in nutrient medium
With metabolic activation:
1.1 µg/mL 7,12-dimethylbenz(a)anthracene (DMBA) in DMSO

3. Test system: Chinese hamster V79 cells
Medium: MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine.
Metabolic activation: Mammalian Microsomal Fraction S9 Mix prepared from livers of 8-12 weeks old male Wistar rats treated with a mixture of 80 mg/kg phenobarbital i.p. and 80 mg/kg beta-naphthoflavone p.o.
Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix.
Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath.

B. Study design and methods

1. Treatment

Dose

Exposure period	S9 mix	Concentrations in µg/mL
Experiment I		
4 h	−	**5.0, 10.0, 20.0, 40.0, 80.0**, 160.0, 240.0, 320.0
4 h	+	**40.0, 80.0, 160.0, 320.0, 480.0, 640.0**
Experiment II		
24 h	−	5.0, 10.0, 20.0, **40.0, 80.0, 160.0, 240.0, 320.0**
4 h	+	40.0, **80.0, 160.0, 240.0, 360.0, 480.0**

Concentrations in bold letters chosen for mutation rate analysis; all concentrations used for toxicity assessment; for each test solution or control two parallel cultures used.

Incubation time / temperature: 4 or 24 hours, 37°C

2. Statistical analysis

A linear regression (least squares) was performed to assess a possible dose-dependent increase of mutant frequencies. The number of mutant colonies obtained for the groups treated with the test item was compared to the solvent control groups. A trend was judged as significant whenever the p-value (probability value) is below 0.05.

II. Results and discussion

In the first experiment cytotoxicity was observed at 80 µg/mL and above without metabolic activation, and at 320 µg/mL without metabolic activation. In the second experiment cytotoxicity was evident at 320 and 480 µg/mL without and with metabolic activation, respectively.
No relevant and reproducible increase mutant frequency was observed in any of the experiments with and without metabolic activation.
An isolated increase of the mutation frequency exceeding the threshold of three times the mutation frequency of the corresponding solvent control was observed in the first culture of the first experiment without metabolic activation at the maximum concentration of 80 µg/mL. However, the data set was not considered valid since cytotoxicity was exceedingly severe at this concentration and both parameters of toxicity (survival and relative cell density) were far below the limit of 10% (1.4% and 7.6%). No comparable increase was noted in the parallel culture where the relative survival met the 10% limit (9.7%).

The positive controls EMS (0.15 mg/mL) and DMBA (1.1 µg/mL) showed a distinct increase in induced mutant colonies, and therefore demonstrated the validity of the test system.

The results are summarized in the following Table 5.8.1/31-1.

Table 5.8.1/31-1: Summary of results

	Conc. In µg/mL	S9 mix	Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Experiment I / 4 h treatment			**Culture I**		**Culture II**	
THF		−	18.9	1.0	34.5	1.0
EMS	150.0	−	141.4	7.5	136.1	3.9
CGA279202-CGA321113	5.0	−	22.6	1.2	4.8	0.1
	10.0	−	18.1	1.0	26.5	0.8
	20.0	−	20.1	1.1	16.0	0.5
	40.0	−	9.1	0.5	28.2	0.8
	80.0	−	156.6	8.3	12.0	0.3
	160.0	−	Cultures were not continued[#]			
	260.0	−				
	320.0	−				
THF		+	19.9	1.0	19.0	1.0
DMBA	1.1	+	758.3	38.2	823.8	43.3
CGA279202-CGA321113	40.0	+	15.3	0.8	16.1	0.8
	80.0	+	25.4	1.3	19.9	1.0
	160.0	+	23.7	1.2	16.1	0.8
	320.0	+	11.7	0.6	32.0	1.7
	480.0	+	17.9	0.9	30.9	1.6
	640.0	+	Cultures were not continued[#]			
Experiment II / 24 h treatment			**Culture I**		**Culture II**	
THF		−	12.7	1.0	29.5	1.0
EMS	150.0	−	467.7	36.9	392.0	13.3
CGA279202-CGA321113	5.0	−	Cultures were not continued[##]			
	10.0	−				
	20.0	−				
	40.0	−	13.1	1.0	12.1	0.4
	80.0	−	6.1	0.5	12.8	0.4
	160.0	−	5.0	0.4	9.5	0.3
	260.0	−	18.6	1.5	15.3	0.5
	320.0	−	9.3	0.7	8.7	0.3
Experiment II / 4 h treatment			**Culture I**		**Culture II**	
THF		+	13.5	1.0	8.4	1.0
DMBA	1.1	+	868.4	56.0	626.9	74.2
CGA279202-CGA321113	40.0	+	Cultures were not continued[##]			
	80.0	+	10.6	0.7	26.3	3.1
	160.0	+	3.9	0.3	21.9	2.6
	240.0	+	9.7	0.6	9.5	1.1
	360.0	+	26.4	1.7	17.4	2.1
	480.0	+	12.4	0.8	12.8	1.5

[#] Culture was not continued due to exceedingly severe cytotoxic effects

[##] Culture was not continued since a minimum of only four analyzable concentrations is required

III. Conclusions

The test item CGA 279202-CGA 321113 did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CGA 279202-CGA 321113 is considered to be non-mutagenic in this HPRT assay.

Report: KCA 5.8.1 /32; [redacted];2011;M-413745-01
Title: CGA 279202-CGA 321113 - In vitro chromosome aberration test in Chinese hamster V79 cells
Report No: 1390502
Document No: M-413745-01-1
Guidelines: **OECD 473 (1997); Commission Regulation (EC) No. 440/2008, Method B.10; US-EPA 712-C-98-223, OPPTS 870.5375 (1998);**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA279202+CGA321113
Article no:	Not stated
Description:	White powder
Lot/Batch no:	BCOO6132-2-3
Purity:	98.6% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2012-11-26

2. Vehicle and or positive control: Vehicle: tetrahydrofuran (THF).
Positive controls without metabolic activation:
1000 µg/mL ethylmethane sulfonate (EMS) in nutrient medium
With metabolic activation:
1.4 µg/mL cyclophosphamide (CPA) in saline

3. Test system: Chinese hamster V79 cells

Medium: MEM (minimal essential medium) containing Hank's salts, neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine.

Metabolic activation: Mammalian Microsomal Fraction S9 Mix prepared from livers of 8-12 weeks old male Wistar rats treated with a mixture of 80 mg/kg phenobarbital i.p. and 80 mg/kg beta-naphthoflavone p.o.
Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix.
Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM Glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath.

B. Study design and methods

1. Treatment

Dose:

Exposure period	S9 mix	Concentrations in µg/mL
4 h*	–	12.5, 25.0, 50.0, 75.0, 100.0, 125.0, 150.0, 200.0, 300.0
4 h	–	5.0, 10.0, 20.0, **40.0**, 60.0, **80.0**, **100.0**, **125.0**, 150.0
4 h**	+	12.5, 25.0, 50.0, 100.0, 150.0, 200.0, 250.0, 300.0, 400.0
4 h	+	25.0, 50.0, 100.0, **200.0**, 250.0, **300.0**, **350.0**, **400.0**, 600.0

* was repeated due to invalid solvent control

** was repeated due to requested range of cytotoxicity was not achieved

Concentrations in bold letters chosen for the mutation rate analysis, all concentrations used for cytotoxicity assessment; for each test solution or control two parallel cultures used.

Incubation time / temperature: 4 hours, 37°C

2. Evaluation

100 metaphases per culture were evaluated for structural chromosome aberrations, except for the positive control without S9 mix and test substance at 400.0 µg/mL in the presence of metabolic activation, where only 50 metaphases were evaluated.

II. Results and discussion

A. Cytotoxicity

After 4h exposure in the absence of S9 mix cytotoxicity was observed at 125 and 150 µg/mL (54.4% and 38.4 % of solvent control). In addition, the mitotic indices were reduced after treatment with test item concentrations of 125 and 150 µg/mL (65.2 % and 35.2 % of control).

With metabolic activation treatment with test item concentrations of 400 and 600 µg/mL for 4 hours caused reduced mitotic indices (28.0 % and 0.0 % of control). Cytotoxicity was evident at 600.0 µg/mL (30.1 % of control).

B. Clastogenicity

Clastogenicity was observed at the two highest evaluated concentrations with and without metabolic activation.

At concentrations 100.0 and 125.0 µg/mL without S9 mix, 6.0 % aberrant cells exceeding the laboratory's historical solvent control data (0.0-4.0% aberrant cells) were observed. After treatment with 40.0, 80.0, 100.0 and 125.0 µg/mL without S9 mix the aberrant cells excluding gaps increased from 3.5 % up to 6.0 % as compared to the corresponding controls (1.5 %).

In the presence of S9 mix the percentage of aberrant cells excluding gaps increased from 2.0 % after treatment with 200.0 µg/mL up to 43.0 % after treatment with 400.0 µg/mL. The number of cells carrying exchanges observed in these two concentration groups (6.5 % and 21.0 % as compared to the solvent controls 1.0 %) provides an additional evidence for the clastogenic potential of the test item.

However, it needs to be taken into consideration the cytotoxicity following treatment with 400.0 µg/mL strongly exceeded the range of cytotoxicity required by the guideline.

No relevant evidence of an increase in polyploid metaphases or endomitotic cells was noticed after treatment with the test item as compared to the controls.

The positive controls EMS (without S9 mix) and CPA (with S9 mix) induced statistically significant increases ($p < 0.05$) of cells with structural chromosome aberrations.
The results are summarized in the following Table 5.8.1/32-1.

Table 5.8.1/32-1: Summary of the results

Exp.	Test item concentration in µg/mL	Polyploid cells in %	Endomitotic cells in %	Cell numbers in % of control	Mitotic indices in % of control	Aberrant cells in % incl. gaps*	Aberrant cells in % excl. gaps*	Aberrant cells in % with exchanges
Exposure period 4 h without S9 mix								
I	Solvent control[1]	4.1	0.0	100.0	100.0	1.5	1.5	1.0
	Positive control[2#]	n.d.	0.0	n.t.	101.1	33.0	31.0[S]	18.0
	40.0	4.0	0.0	93.0	89.9	3.5	3.5	2.0
	80.0	4.0	0.1	115.2	79.8	4.5	4.0	3.0
	100.0	3.0	0.0	91.4	94.8	7.0	6.0[S]	4.5
	125.0	2.8	0.0	54.4	65.2	7.5	6.0[S]	1.0
Exposure period 4 h with S9 mix								
I	Solvent control[1]	2.0	0.0	100	100.0	3.5	3.0	1.0
	Positive control[3]	n.d.	0.0	n.t.	83.1	14.5	14.0S	6.0
	200.0	3.1	0.0	90.2	110.8	2.5	2.0	0.5
	300.0	2.9	0.0	86.4	111.7	6.0	5.0	1.5
	350.0	3.5	0.0	74.5	92.9	16.5	16.5S	6.5
	400.0[#]	2.6	0.0	76.9	28.0	45.0	43.0S	21.0

* Inclusive cells carrying exchanges
[#] Evaluation of 50 metaphases per culture
n.d. Not determined
n.t. Not tested
[S] Aberration frequency statistically significant higher than corresponding control values ($p < 0.05$)
[1] THF 0.5 % (v/v), [2] EMS 1000.0 µg/mL, [3] CPA 1.4 µg/mL

III. Conclusions

In conclusion, it can be stated that under the experimental conditions reported, the test item CGA 279202-CGA 321113 induced structural chromosome aberrations in *V79* cells (Chinese hamster cell line), when tested up to cytotoxic concentrations.

Report: KCA 5.8.1/33; [redacted];2013;M-463614-01
Title: CGA 279202-CGA 321113 - Micronucleus test in bone marrow cells of the mouse
Report No: 1578200
Document No: M-463614-01-1
Guidelines: **OECD 474; Commission Regulation (EC) 440/2008, B12; US-EPA 712-C-98-226, OPPTS 870.5395;**
Deviation(s): none
GLP/GEP: yes

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA 279202-CGA 321113
Synonyms:	AE 1344138, technical
Description:	White powder
Lot/Batch no:	BCOO 6132-3-9
Purity:	98.4 % (w/w) (HPLC)
Stability of test compound:	guaranteed for study duration; expiry date: 2014-10-17
2. Vehicle / positive control:	Vehicle: Methylcellulose (MC), 0.5% positive control: cyclophosphamide (CPA) in sterile water

3. Test animals

Species:	mouse
Strain:	NMRI
Age:	8 - 11 weeks
Weight at dosing:	Males: 34.8 ± 1.6 g
Source:	[redacted], [redacted], Germany
Acclimatisation period:	at least five days
Diet:	Pelleted, standard died (certified), *ad libitum*
Water:	tap water, *ad libitum*
Housing:	Individually in Makrolon Type III cages with wire mesh top

B. Study design and methods

1. Animal assignment and treatment

Dose:	0-500-1000-2000 mg/kg bw positive control CPA: 40 mg/kg bw
Application route:	Oral, gavage
Application volume:	10 mL/kg bw
Group size:	7 males / dose group
Observations:	mortality, clinical signs, body weights
Sacrifice/bone marrow preparation:	High-dose: 24 and 48 h after treatment Negative and positive control, low- and intermediate dose: 24 h after treatment
No. of cells scored:	2000 polychromatic erythrocytes (PCE) per animal

2. Evaluation

Scoring:	Per animal 2000 polychromatic erythrocytes (PCE) were analysed for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic erythrocytes (NCE) was determined in the same sample and expressed in polychromatic erythrocytes per 2000 erythrocytes. The analysis was performed with coded slides.
Statistics:	The significance of differences was assessed nonparametric Mann-Whitney test.

II. Results and discussion

A. Clinical observations

There were no clinical symptoms observed in the vehicle control and low-dose group (500 mg/kg bw). The highest dose (2000 mg/kg; maximum guideline-recommended dose) was estimated by a pre-experiment to be suitable. However in the main study 2 males died unexpectedly after treatment with this dose.

Clinical symptoms included ruffled fur and reduced spontaneous activity in the mid dose group. Animals of the high dose group exhibited ruffled fur, reduced spontaneous activity and eyelid closure. Symptoms observed in the medium and high dose are summarized in the following Table 5.8.1/33-1.

Table 5.8.1/33-1: Summary of clinical symptoms observed

Clinical symptom	Symptoms observed in males at hours post-treatment*				
	1	2-4	6	24	48
High dose: 2000 mg/kg bw					
mortality	0/14	0/14	0/14	1/14	1/7
ruffled fur	14/14	14/14	14/14	12/14	2/7
reduction of spontaneous activity	9/14	11/14	11/14	0/14	0/7
abdominal position	0/14	1/14	1/14	0/14	0/7
eyelid closure	7/14	5/14	5/14	0/14	0/7
death	0/14	0/14	0/14	1/14	0/7
Medium dose: 1000 mg/kg bw					
ruffled fur	3/7	3/7	3/7	0/7	--
reduction of spontaneous activity	3/7	3/7	3/7	0/7	--

* x/y = number of animals affected / total number of animals

B. Microscopic Evaluation

The mean number of polychromatic erythrocytes was not decreased after treatment with the test item as compared to the mean value of PCEs of the vehicle control indicating that CGA 279202-CGA 321113 did not have any cytotoxic properties in the bone marrow.

In comparison to the corresponding vehicle control there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval and any dose level. The mean values of micronuclei observed after treatment with CGA 279202-CGA 321113 below or near to the value of the respective vehicle control group and within the historical vehicle control range. Additionally no dose dependence in micronucleus response was observed.

40 mg/kg b.w. cyclophosphamide administered orally was used as positive control which showed a statistically significant increase of induced micronucleus frequency.

The results are summarized in the following Table 5.8.1/33-2.

Table 5.8.1/33-2: Summary of results

Experimental groups	Sacrifice after treatment [hours]	PCEs with miconuclei [%]	Range [number]	PCE per 2000 erythrocytes [number]
Negative control 0.5% MC	24	0.150	0 - 7	1209
500 mg/kg bw	24	0.086	0 – 4	1159
1000 mg/kg bw	24	0.229	2 – 7	1192
2000 mg/kg bw	24	0.092	0 – 5	1195
	48	0.108	1 – 3	1195
Positive control (CPA) 40 mg/kg bw	24	2.179*	17 – 67	1175

* Statistically significant different from control p <= 0.05

III. Conclusions

In conclusion, it can be stated that during the study described and under the experimental conditions reported, the test item CGA 279202-CGA 321113 did not induce micronuclei as determined by the micronucleus test in the bone marrow cells of the mouse *in vivo*.

Report: KCA 5.8.1 /34; [redacted];2013;M-458428-01
Title: In vivo unscheduled DNA synthesis in rat hepatocytes with trifloxystrobin-CGA 321113
Report No: 1504401
Document No: M-458428-01-1
Guidelines: **OECD 486; EEC Directive 2000/32, B.39; US-EPA 712-C-98-230, OPPTS 870.5550;**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name: Trifloxystrobin-CGA 321113
Description: White powder
Lot/Batch no: BCOO 6132-3-9
Purity: 98.4% (w/w) (Dose calculation was not adjusted to purity)
Stability of test compound: guaranteed for study duration; expiry date: 2014-10-17

2. Vehicle and positive control: Vehicle: 0.5% MC.
Positive control:
4 h preparation interval:
80 mg/kg bw N,N´-dimethylhydrazinedihydrochloride (sym., DMH) in saline
16 h preparation interval:
100 mg/kg b.w. 2-acetylaminofluorene (2-AAF) in DMSO/polyethylene glycol 400 (1 + 9)

3. Test animals

Species: Rat
Strain: Wistar
Age: 9-10 weeks (pre-experiment)

	8-9 weeks (main experiment)
Sex:	Males
Weight at dosing:	267.7 ± 10.8 g
Source:	[redacted], [redacted], Germany
Acclimatisation period:	At least 5 days
Diet:	Pelleted standard diet ([redacted], Netherlands), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	In groups in Makrolon® cages Type IIII/IV with a wire mesh top. Granulated soft wood bedding.

B. Study design and methods

1. Animal assignment and treatment

Dose	4 and 16 hours preparation intervals: 1000 and 2000 mg/kg bw
Positive control	4 h preparation interval: 80 mg/kg bw DMH 16 hours preparation: 100 mg/kg bw 2-AAF
Application route:	Oral, gavage
Application volume:	10 mL/kg
Group size:	4 males /dose group
Observations:	Mortality, clinical signs, body weight, gross necropsy

2. Primary Hepatocytes

Culture conditions:	Williams medium E supplemented with:
	Hepes: 2.38 mg/mL
	Penicillin: 100 units/mL
	Streptomycin: 0.10 mg/mL
	L-Glutamine: 0.29 mg/mL
	Insulin: 0.50 µg/mL
	Fetal calf serum(FCS): 100 µl/mL
	This complete medium was adjusted to pH 7.6.
Evaluation:	At least three cultures were established from each animal.
	At least two slides per animal and 50 cells per slide were evaluated.

Methodology

The test item was suspended in 0.5% MC, which was used as vehicle control. The volume administered orally by gavage was 10 mL/kg body weight. After a single oral treatment and a post-treatment period of 4 and 16 hours, respectively, the animals were anaesthetised and sacrificed by liver perfusion. Primary hepatocyte cultures were established and exposed for 4 hours to ^{3}HTdR (methyl-^{3}H-thymidine), which is incorporated if UDS occurs. Hepatocytes of four males for each experimental group including the controls were assessed for the occurrence of UDS.

II Results and discussion

A. Mortality

No unscheduled deaths occurred during the study

B. Clinical signs

Abdominal posture was observed as clinical sign for one male of the high dose group (4 hours treatment) at one hour after application of the test item.

C. Primary hepatocytes

The viability of the hepatocytes was not substantially affected by the *in vivo*-treatment with the test item.

None of the tested dose levels revealed UDS induction in the hepatocytes of the treated animals as compared to the corresponding vehicle controls.

Treatment with the positive control substances (DMH and 2-AAF) revealed distinct increases in the number of nuclear and net grain counts

The results are summarized in the following Table 5.8.1/34-1.

Table 5.8.1/34-1: Group mean nucleus, cytoplasmic area, and net grains

Test group	Nuclear grain count		Cytoplasmic grain count		Net grain counts		Nuclear grain count of cells in repair		Cells in repair
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	%
4 h preparation interval									
Vehicle control (0.5% MC)	24.96	9.84	36.89	12.45	−11.93	10.42	8.68	2.62	5.5
1000 mg/kg b.w. Trifloxystrobin-CGA 321113	19.99	7.48	28.21	8.96	−8.23	8.58	7.20	3.72	6.25
2000 mg/kg b.w. Trifloxystrobin-CGA 321113	21.95	8.63	31.37	11.07	−9.42	9.57	7.68	1.96	6.5
Positive control (DMH)	68.49	18.30	29.31	10.46	39.18	17.79	40.38	16.94	95.75
16 h preparation interval									
Vehicle control (0.5% MC)	27.02	11.20	45.15	14.42	−18.13	11.77	7.83	0.47	1.5
1000 mg/kg b.w. Trifloxystrobin-CGA 321113	21.74	8.89	28.88	10.44	−7.14	9.17	8.50	3.94	12
2000 mg/kg b.w. Trifloxystrobin-CGA 321113	22.79	8.95	32.39	11.30	−9.60	9.54	7.11	2.55	6.25
Positive control (2-AAF)	52.59	16.72	35.85	12.42	16.74	13.00	20.92	10.20	78

SD = standard deviation

III Conclusion

The test item Trifloxystrobin-CGA 321113 did not induce DNA-damage leading to increased repair synthesis in the hepatocytes of the treated rats.
Therefore, Trifloxystrobin-CGA 321113 is considered to be non-genotoxic in this assay.

CGA 373466 (ZE metabolite)

Report:	KCA 5.8.1 /35; [redacted]; [redacted];2003;M-088404-01-1
Title:	CGA 279202-CGA 373466 - Study for subacute oral toxicity in rats (feeding study for 4 weeks and 4 weeks recovery period)
Report No:	AT00343
Document No:	M-088404-01-1
Guidelines:	**Directive 96/54/EEC Guideline B.7, OECD 407 (1995)**
	Deviation(s): none
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA 279202 CGA 373466
Article no:	Not reported
Description:	White powder
Lot/Batch no:	KTS9813-7-2
Purity:	99.44%
Stability of test compound:	guaranteed for study duration; expiry date: 2002-11-27

2. Vehicle: Diet

3. Test animals

Species:	Rat
Strain:	Wistar Hsd cpd:WU
Sex:	Male and female
Age:	About 6 weeks (males), about 7 weeks (females)
Weight at dosing:	Males: 122 - 137 g (group means) Females: 132 - 138 g (group means)
Source:	[redacted], [redacted], Germany
Acclimatisation period:	At least 5 days
Diet:	[redacted] R 3883.9.25'' ([redacted] SA, Kaiseraugst, Switzerland), *ad libitum*
Water:	Tap water (drinking bottles), *ad libitum*
Housing adaptation period:	5 or 6 per sex in Polycarbonate cages Type III
study period:	individually in cages type II on low-dust wood granulate (Sniff Spezialdiäten GmbH, Soest, Germany)

B. Study design and methods

1. Animal assignment and treatment

Dose:	0 - 100 - 500 - 2000 - 8000 ppm males: 0 - 9.6 - 47 - 209 - 903 mg/kg bw/day females: 0 - 11 - 61 - 236 - 928 mg/kg bw/day
Duration:	Treatment: 4 weeks Recovery: 4 weeks
Application route:	Oral, via diet
Group size:	5/sex (except male recovery high-dose group: 4)
Observations:	Mortality, clinical signs, body weight, food and water intake, haematology, clinical chemistry (incl. thyroid hormones and liver enzymes), urinalysis, gross necropsy, organ weight, histopathology, neurotoxicity (incl. FOB and motor activity (MA))

II. Results and discussion

A. Mortality

There were no treatment-related mortalities.
One female of the low mid dose (500 ppm) group was sacrificed in moribund condition. Necropsy revealed severe changes in the kidney which were not considered to be treatment-related.

B. Clinical signs

Treatment-related findings observed in 2 high-dose (8000 ppm) males were piloerection and increased faeces. In addition, high-dose animals of both sexes exhibited discoloured faeces. This effect is caused most likely by non-resorbed test substance and is therefore considered to be of no toxicological relevance.

C. Body weight

There were no dose-related effects on body weight or body weight gain at doses up to and including 2000 ppm.
At 8000 ppm a marked retardation of body weight development was observed after the first week of treatment in both sexes. Thus, the body weights were lower at 8000 ppm in both sexes. Compared to the controls these differences became statistically significant for the recovery groups.
The total body weight gain during the study was in the main groups 156-155-156-147-136 g for males and 53-54-53-46-37 g for females, and in the recovery groups 251-210 g for males and 97-76 g for females (in increasing dose levels).
The results are summarized in the following Table 5.8.1/35-1 and Table 5.8.1/35-2.

Table 5.8.1/35-1: Summary of body weight development of main groups

Dose (ppm)	Mean body weight (g) Day 1	Day 8	Day 15	Day 22	Day 29	Day 1	Day 8	Day 15	Day 22	Day 29
	Males					Females				
0	122	170	221	263	279	132	153	169	185	184
100	126	177	226	269	281	138	165	179	189	192
500	137++	190+	238	276	293	138	162	175	189	194
2000	126	169	215	254	274	137	158	169	179	184
8000	124	155	202	245	260	136	147	161	169	173
Dose (ppm)	**Mean body weight gain (g) Days 1-8**	**Days 8-15**	**Days 15-22**	**Days 22-29**		**Days 1-8**	**Days 8-15**	**Days 15-22**	**Days 22-29**	
	Males					Females				
0	48	51	42	15		21	16	16	-0	
100	51	49	43	12		27	14	10	3	
500	53	48	38	17		24	13	14	2	
2000	43	46	39	19		21	11	9	5	
8000	31+	47	43	15		11+	14	8	4	

+ = statistically significantly different at $p \leq 0.05$
++ = statistically significantly different at $p \leq 0.01$

Table 5.8.1/35-2: Summary of body weight development of recovery groups

Dose (ppm)	Mean body weight (g)								
	Day 1	Day 8	Day 15	Day 22	Day 29	Day 36	Day 43	Day 50	Day 57
	Males								
0	128	177	229	269	295	327	350	368	377
8000	127	159^{++}	205^{++}	243^{+}	260^{+}	289^{++}	313^{+}	329^{+}	337^{+}
	Females								
0	144	172	189	206	215	229	236	242	242
8000	136	150^{+}	167+	177^{+}	187^{+}	193^{+}	205^{+}	208^{+}	211^{+}

Dose (ppm)	Mean body weight gain (g)							
	Days 1-8	Days 8-15	Days 15-22	Days 22-29	Days 29-36	Days 36-43	Days 43-50	Days 50-57
	Males							
0	49	52	41	26	32	24	18	9
8000	31^{++}	47	38	16^{+}	30	24	16	8
	Females							
0	28	17	17	8	13	6	7	4
8000	13^{++}	18	10	10	6^{+}	13	3	3

$^{+}$ = statistically significantly different at $p \leq 0.05$
$^{++}$ = statistically significantly different at $p \leq 0.01$

D. Food and water consumption

No relevant effects were observed on the food intake per animal per day and per kg body weight per day during the treatment period in the main groups. The female recovery group (8000 ppm) showed at the end of the treatment and during the recovery period increased food intake per kg body weight per day.

Table 5.8.1/35-3: Mean food intake – main groups

Dose (ppm)	Mean food intake (g/animal/day)				Mean food intake (g/kg bw/day)			
	Day 8	Day 15	Day 22	Day 29	Day 8	Day 15	Day 22	Day 29
	Males							
0	21	21	22	22	122	97	85	80
100	21	22	23	21	122	99	87	75
500	22	23	24	22	115	97	87	74
2000	23	24	23	22	136	112	92	79
8000	20	24	26	25	132	119	105^{+}	96
	Females							
0	18	19	19	16	115	113	104	88
100	20	21	18	17	123	118	96	91
500	22	22	22	21^{nc}	137	125	115	110^{nc}
2000	20	23	19	18	127	136	109	99
8000	16	19	20	21	111	118	117	119

$^{+}$ = statistically significantly different at $p \leq 0.05$
^{nc} = no statistic evaluation performed due to low number of values

Table 5.8.1/35-4: Mean food intake – recovery groups

Dose (ppm)	Mean food intake (g/animal/day)							
	Day 8	Day 15	Day 22	Day 29	Day 36	Day 43	Day 49	Day 57
Males								
0	23	22	23	24	25	24	25	22
8000	20 nc	24 nc	25 nc	24 nc	26 nc	24 nc	25 nc	21 nc
Females								
0	19	18	18	19	19	18	17	15
8000	17	18	19	23	26	22	22+	18
Mean food intake (g/kg bw/day)								
Males								
0	131	98	86	82	76	69	67	59
8000	127nc	119nc	101 nc	91 nc	88nc	75 nc	77 nc	64nc
Females								
0	109	95	90	88	80	75	71	61
8000	115	109	110	127+	139+	110+	107++	84

+ = statistically significantly different at $p \leq 0.05$
++ = statistically significantly different at $p \leq 0.01$
nc = no statistic evaluation performed due to low number of values

Water consumption

There was no effect on water intake at doses up to and including 2000 ppm. At 8000 ppm water intake was increased during treatment. During recovery no effects on water intake were observed.

Table 5.8.1/35-5: Summary of water consumption

Dose (ppm)	Mean water intake (g/kg bw/day)							
	Day 8	Day 15	Day 22	Day 29	Day 8	Day 15	Day 22	Day 29
	Males				Females			
0	138	115	103	97	121	114	107	109
100	140	118	106	94	118	112	112	109
500	148	123	114	105	137	144	126	108nc
2000	145	123	112	106	131	113	116	115
8000	169	134	132	120	151	151+	138+	140

+ = statistically significantly different at $p \leq 0.05$
nc = no statistic evaluation performed due to low number of values

E. Haematology

The concentrations of haemoglobin (Hb) and of the mean corpuscular haemoglobin concentration (MCHC) were decreased at 8000 ppm in both sexes (see Table 5.8.1/35-6). However, the values were clearly within the 3-s-range of historical control data for males and females, respectively (see Table 5.8.1/35-7).

A higher number of males at 2000 ppm and above and females at 8000 ppm showed increased incidences of hypochromasia. However, the finding occurred in males in all dose groups. Corresponding decreases in Hb and MCHC concentrations and increased bilirubin concentration were observed at 8000 ppm. At the end of the recovery period hypochromasia did not occur. Therefore, the finding at 2000 ppm in males is considered incidental rather than an adverse finding.

No effects were observed on white blood cells in the main groups. At the end of the recovery period, the counts of basophils (BASO) were decreased (statistically significant in females). Coagulation parameters were not affected in any dose group.

Table 5.8.1/35-6: Summary of treatment-related haematological effects

Dose (ppm)	Males			Females		
	Hb (g/L)	MCHC (g/L)	BASO (10^9/L)	Hb (g/L)	MCHC (g/L)	BASO (10^9/L)
Main groups						
0	147	302	0.10	145	306	0.08
100	147	302	0.11	151	305	0.08
500	144	302	0.12	146	306	0.06[nc]
2000	144	299	0.12	142	304	0.06
8000	137[+]	294[++]	0.06	138[+]	299[+]	0.06
Recovery groups						
	Hb (g/L)	MCHC (g/L)	BASO (10^9/L)	Hb (g/L)	MCHC (g/L)	BASO (10^9/L)
0	141	310	0.08	141	310	0.04
8000	143	309	0.04[nc]	139	306	0.02[+]

BASO = basophils
[+] = statistically significantly different at $p \leq 0.05$
[++] = statistically significantly different at $p \leq 0.01$
[nc] = no statistic evaluation performed due to low number of values

Table 5.8.1/35-7: Haematology – historical control values of Hsd Cpb:WU rats

Parameter	Unit	N	Mean	SD	Range -2s	Range +2s	Range -3s	Range +3s
Males 8-11 weeks								
HB	g/L	72	147	4.8	138	157	133	161
MCHC	g/L ERY		312	7.6	296	327	289	334
BASO	10^9/L		0.03	0.015	0.01[5P]	0.06	Up to	0.07
Females 8-11 weeks								
HB	g/L	65	147	5.4	136	158	130	163
MCHC	g/L ERY		317	8.5	300	334	292	343
BASO	10^9/L		0.02	0.013	0.00[5P]	0.05	Up to	0.06
Males 12-25 weeks								
BASO	10^9/L	283	0.03	0.016	0.01[5P]	0.06	Up to	0.08
Females 12-25 weeks								
BASO	10^9/L	270	0.02	0.014	0.00[5P]	0.05	Up to	0.06

[5P] = 5th percentile;

F. Clinical chemistry

Clinical laboratory investigations showed a decreased concentration of triglycerides (TRGL), and increased concentrations of bilirubin (BILI) and urea at the end of the treatment period at 8000 ppm in both sexes. Additionally, the concentration of albumin (Alb) was decreased and the concentration of chloride (Cl) increased at 8000 ppm in males. Except the concentration of triglycerides in females, these effects were reversible during the recovery period. However, all significantly different values are within the 2-s range (urea, chloride, bilirubin (males), triglycerides (females)) or 3-s range (albumin, bilirubin (females), triglycerides (males)) of historical control data (see Table 5.8.1/35-8 and Table 5.8.1/35-9).

There were no treatment-related effects noted on liver enzyme activities (i.e. ALAT, ASAT, alkaline phosphatase, gamma-glutamyltransferase, glutamat dehydrogenase).

Table 5.8.1/35-8: Summary of treatment-related clinical chemistry effects

Dose	Males					Females				
	TRGL	Urea	Cl	BILI-t	Alb	TRGL	Urea	Cl	BILI-t	Alb
(ppm)	(mmol/L)			(µmol/L)	(g/L)	(mmol/L)			(µmol/L)	(g/L)
	Main groups					Main groups				
0	1.69	5.55	97	0.8	37.8	1.25	6.22	98	1.0	36.0
100	2.06	6.27	97	0.6	36.7	1.63	5.98	96	0.8	37.1
500	2.43	5.96	97	0.6^{+}	37.0	1.01	6.18	98	1.0^{nc}	36.7
2000	1.80	6.36	96	0.8	37.1	1.57	5.92	97	1.1	37.1
8000	0.37^{+}	7.07^{++}	100^{+}	1.4^{+}	34.3^{+}	0.85	7.53^{+}	99	1.6^{+}	35.9
	Recovery groups					Recovery groups				
0	2.07	7.00	98	1.0	35.3	2.11	6.55	101	1.3	37.8
8000	1.87	7.08	99	1.0^{nc}	36.4	0.89	7.29	101	1.3	38.5

TRGL = triglycerides; Cl = chloride, BILI-t = total bilirubin, Alb = albumin
$^{+}$ = statistically significant different at $p \leq 0.05$
$^{++}$ = statistically significant different at $p \leq 0.01$
^{nc} = no statistic evaluation performed due to low number of values

Table 5.8.1/35-9: Clinical chemistry – historical control values of Hsd Cpb:WU rats

Parameter	Unit	N	Mean	SD	Range -2s	Range +2s	Range -3s	Range +3s
Males 8-11 weeks								
TRGL	mmol/L	222	1.48	0.467	0.79^{5P}	2.42	0.08	2.89
Urea	mmol/L	212	6.96	0.853	5.26	8.67	4.40	9.52
Cl	mmol/L	212	98	1.8	95	102	93	107
Alb	g/L	135	29.2	1.32	26.6	31.9	25.3	33.2
BILI-t	µmol/L	213	1.2	0.37	0.5	2.0	0.1	1.7
Females 8-11 weeks								
TRGL	mmol/L	133	1.17	0.429	0.56^{5P}	2.03	Up to	2.45
Urea	mmol/L	124	7.23	0.993	5.24	9.22	4.25	10.21
Cl	mmol/L	124	100	2.3	95	104	93	107
Alb	g/L	45	31.2	2.37	26.5	36.0	24.1	38.3
BILI-t	µmol/L	132	1.0	0.22	0.6	1.5	0.4	1.7

Thyroid hormones

There were no treatment-related effects observed in T3, T4 and TSH-values in male rats in any dose group. In females T4-values were significantly decreased at 2000 ppm and above during treatment (49 and 37 nmol/L versus 64 nmol/L in controls). However, the values were within the 2-s-range of historical controls (i.e. 27 - 83 nmol/L) and therefore considered not to be toxicologically relevant in the absence of histopathological findings.

G. Determination of enzymes in liver tissue

There were treatment-related increases of cytochrome P-450 activities (P-450) in both sexes at 8000 ppm. N-demethylase activities (N-DEM) were increased in high-dose animals at the end of the treatment period. Both effects were reversible at the end of the recovery period. In addition, the measured values for N-demethylase and P-450 in the high-dose animals were within the 2-s-range of historical controls (see Table 5.8.1/35-10 and Table 5.8.1/35-11).

O-demethylase activities (O-DEM) were not affected by treatment in any dose group.

Table 5.8.1/35-10: Summary of enzyme activities determined in liver tissues

Dose (ppm)	Males N-DEM (mU/g)	Males O-DEM (mU/g)	Males P-450 (nmol/g)	Females N-DEM (mU/g)	Females O-DEM (mU/g)	Females P-450 (nmol/g)
Main groups						
0	157.7	12.3	42.0	64.9	10.6	33.6
100	142.6 nc	11.9 nc	40.9 nc	58.0	8.4	32.9
500	139.8 nc	14.6 nc	41.4 nc	52.5 nc	8.5 nc	31.7 nc
2000	167.3 nc	14.0 nc	42.8 nc	58.7	8.7	34.6
8000	194.3 nc	14.6 nc	49.7 nc	85.5	9.8	40.5 ++
Recovery groups						
	N-DEM (mU/g)	O-DEM (mU/g)	P-450 (nmol/g)	N-DEM (mU/g)	O-DEM (mU/g)	P-450 (nmol/g)
0	153.4	12.8	38.9	54.6	9.9	32.9
8000	141.3 nc	12.4 nc	38.0 nc	57.1	10.3 +	32.2

+ = statistically significantly different at $p \leq 0.05$
++ = statistically significantly different at $p \leq 0.01$
nc = no statistic evaluation performed due to low number of values

Table 5.8.1/35-11: Enzyme activities in liver tissue – histolorical control values of Hsd Cpb:WU rats

Parameter	Unit	N	Mean	SD	Range -2s	Range +2s	Range -3s	Range +3s
Males 8-11 weeks								
N-DEM	mU/g	159	143.8	32.25	79.1	208.5	46.7	240.8
P-450	nmol/g	260	42.5	4.49	33.5	51.5	29.0	56.0
Females 8-11 weeks								
N-DEM	mU/g	158	66.4	11.26	43.9	89.0	32.6	100.2
P-450	nmol/g	261	36.6	4.19	28.2	45.0	24.0	49.2

H. Urinanalysis

In high-dose males there was an increased incidence of ketone bodies. This effect was reversible at the end of the recovery period. None of the other urine analysis parameters was affected in any dose group.

I. Gross necropsy

Organ weights

At 8000 ppm increased (+13%) relative liver weight was observed in main group males. This finding was considered to be treatment-related.

Gross pathology

At the end of the treatment period the liver was swollen and/or enlarged in 1/5 males at 2000 ppm, and in 2/5 males at 8000 ppm. Although a histopathological correlate for the observed liver changes was absent, the finding is considered as most probably due to a slight liver enzyme induction.

J. Histopathology

There were no treatment-related microscopic findings observed at any dose level tested. Moreover, there were no correlations to the gross pathological findings of swollen and / or enlarged livers.

K. Neurotoxicity evaluation

Functional observational battery (FOB)

The functional observation battery, reflex testing, and grip strength measurement, showed no relevant signs or symptoms indicating evidence for a neurotoxic potential.

Motor activity (MA)
There were no treatment-related effects observed for motor and locomotor activity in any dose group.
There was no indication for a neurotoxic potential.

III. Conclusion

Based on the study results the NOAEL is 2000 ppm, equivalent to 209/236 mg/kg bw/day in males/females based on retarded body weight development, effects on red blood cells and slight liver enzyme induction at 8000 ppm (903/928 mg/kg bw/d in males/females)
The study director established a NOEL at 500 ppm (47/61 mg/kg bw/day in males/females).

NOA 413161 / NOA 413163

Report:	KCA 5.8.1 /36; [redacted]; [redacted]; 2003; M-084123-01-1
Title:	CGA 279202-NOA 413161/413163: Study for subacute oral toxicity in rats (4 week application by gavage and 4 weeks recovery period)
Report No:	AT00342
Document No:	M-084123-01-1
Guidelines:	**Directive 96/54/EEC Guideline B.7, OECD 407 (1995)** **Deviation(s): none**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CGA 279202 – NOA 413161 / 413161
Article no:	Not reported
Description:	White solid
Lot/Batch no:	NLI 7184
Purity:	99.6% (NOA 413161: 48.26%, NOA 413163: 51.43%)
Stability of test compound:	guaranteed for study duration; expiry date: 2003-04-22

2. Vehicle: Polyethylene glycol 400

3. Test animals

Species:		Rat
Strain:		Wistar HsdCpd:WU
Sex:		Male and female.
Age:		About 7 weeks (males), about 8 weeks (females)
Weight at dosing:		Males: 135 - 139 g (mean) Females: 139 - 148 g (mean)
Source:		[redacted], [redacted], Germany
Acclimatisation period:		At least 5 days
Diet:		[redacted] ® 3883.0.15'' ([redacted] SA, Kaiseraugst, Switzerland), *ad libitum*
Water:		Tap water (drinking bottles), *ad libitum*
Housing:	adaptation	In groups of 5 or 6 per sex in polycarbonate cages Type III.
	study period	Individually in cages type II on low-dust wood granulate (Sniff Spezialdiäten GmbH, Soest, Germany).

B. Study design and methods

1. Animal assignment and treatment

Dose	0, 10, 50, 200 and 1000 mg/kg bw/day
Duration:	Treatment: 4 weeks Recovery: 4 weeks
Application route:	Oral, gavage
Group size:	5/sex
Observations:	Mortality, clinical signs, open field observation (OFO), body weight, food and water intake, haematology, clinical chemistry (incl. thyroid hormones and liver enzymes), urinalysis, gross necropsy, organ weight, histopathology, neurotoxicity (incl. FOB and motor activity (MA))

II. Results and discussion

A. Mortality

There were no treatment-related mortalities.

B. Clinical signs

Increased incidences of piloerection was observed in males at 200 mg/kg bw/day and above from week 2 on (0-1-2-4-5 incidences at 0-10-50-200 and 1000 mg/kg bw/day). This finding was not observed during FOB investigations. Therefore, this finding was considered to be not related to treatment.

C. Body weight

There were no treatment-related effects on body weight or body weight gain observed in any dose group during treatment and recovery period.

The total body weight gain during the study was in the main groups 148-143-142-125-147 g for males and 55-48-53-54-57 g for females, and in the recovery groups 208-220 g for males and 95-93 g for females (in increasing dose levels).

D. Food and water consumption and test item intake

Food consumption

No relevant effects were observed on the food intake per animal per day and per kg body weight per day during the treatment and recovery period.

Water consumption

There was no treatment-related effect observed in water consumption during treatment and recovery period.

E. Haematology

At the end of the treatment period the erythrocyte counts (ERY) were decreased at 50 and 1000 mg/kg bw/day, the counts of eosinophils (EOS) were decreased at 50 mg/kg w/day, and the mean corpuscular volume (MCV) of erythrocytes were increased at 1000 mg/kg bw/day in females. These effects are considered as not treatment related, since they occurred in one sex only, lay in the range or on the upper level of the range of historical control values (2s-range), and/or were not dose related (see Table 5.8.1/36- 1 and Table 5.8.1/36-2).

At the end of the recovery period, the counts of reticulocytes (RETI) were decreased in males. The increased counts in females are due to one extreme value.

No treatment-related effects were observed on the white blood cells and blood coagulation parameters.

Table 5.8.1/36- 1: Summary of haematological effects

Dose (mg/kg bw/day)	Males				Females			
	ERY (10^{12}/L)	MCV (10^{-15} L)	RETI (10^9/L)	EOS (10^9/L)	ERY (10^{12}/L)	MCV (10^{-15} L)	RETI (10^9/L)	EOS (10^9/L)
Main groups								
0	7.58	62.3	32	0.08	8.29	57.6	21	0.10
10	7.58	62.1	34	0.08	8.14	58.4	21	0.06
50	7.74	62.3	28	0.06	7.78^{+}	59.3	19	0.05^{+}
200	8.11	61.0	26	0.06	8.11	59.1	17	0.04
1000	7.50	63.2	33	0.06	7.73^{++}	59.9^{+}	21	0.05
Recovery groups								
0	8.68	56.7	19	0.08	8.11	58.0	21	0.08
1000	8.61	56.1	15^{+}	0.07	7.67	59.1	40	0.08

ERY = erythrocytes, MCV = mean corpuscular volume, RETI = reticulocytes, EOS = eosinophils
$^{+}$ = statistically significantly different at $p \leq 0.05$
$^{++}$ = statistically significantly different at $p \leq 0.01$

Table 5.8.1/36-2: Haematology – historical control values of Hsd Cpb:WU rats

Parameter	Unit	N	Mean	SD	Range		Range	
					-2s	+2s	-3s	+3s
Males 8-11 weeks								
RETI	$/10^{-3}$	74	30	6.9	20^{5P}	44	9	51
Females 8-11 weeks								
EOS	10^9/L	65	0.08	0.036	0.03^{5P}	0.15	Up to	0.19
ERY	10^{12}/L		7.92	0.362	7.20	8.65	6.84	9.01
MCV	(10^{-15} L)		58.5	2.06	54.3	62.6	52.3	64.6

^{5P} = 5^{th} percentile.

F. Clinical chemistry

Enzymes, electrolytes, substrates and plasma proteins

There were no treatment-related effects on activities of the liver enzymes ASAT, ALAT, alkaline phosphatase and glutamate dehydrogenase and on the concentrations of electrolytes during treatment and recovery period. Gamma-glutamyl transferase activities (GGT) were not affected during treatment, but were decreased in high dose males after recovery. Since the measurements were done at the detection limit of the method, variations of 1-2 U/L are considered normal. Thus, the finding was not considered to be toxicologically relevant (see Table 5.8.1/36-3).

At the end of the treatment period creatinine (Crea) values were slightly but significantly decreased in males at 200 mg/kg bw/day and above. The difference was only slight (44 µmol/L versus 48 µmol/L in controls; i.e. –9%), not dose-related and all the individual values were within the 2-s-range (i.e. 34-56 µmol/L) of historical controls. Thus, this finding was considered to be incidental. No changes in creatinine were observed after the recovery period (Table 5.8.1/36-3 and Table 5.8.1/36-4).

Thyroid parameters

No effects on T3, T4 and TSH were observed after the treatment period. At the end of the recovery period T3 and T4 values were increased in high-dose males. However, the values were within the 2-s range of historical controls. Therefore, the changes in thyroid hormone levels are considered to be of no relevance (Table 5.8.1/36-3 and Table 5.8.1/36-4).
There were no differences in TSH levels after recovery.

Table 5.8.1/36-3: Summary of effects on clinical chemistry parameters

Dose (mg/kg bw/day)	Males GGT (U/L)	Crea (µmol/L)	T3 (nmol/L)	T4	Females GGT (U/L)	Crea (µmol/L)	T3 (nmol/L)	T4
Main groups								
0	2	48	1.69	71	0	46	1.61	50
10	2	45	1.77	73	1	46	1.79	68
50	2	45	1.53	74	2	47	1.41	55
200	1	44^{+}	1.88	73	2	50	1.55	57
1000	3	44^{+}	1.91	73	1	46	1.84	59
Recovery groups								
0	3	48	1.49	62	2	51	1.77	63
1000	1^{+}	49	1.79^{++}	74^{+}	2	54	1.83	68

GGT = gamma glutamyl transferase, Crea = creatinine, T3 = triiodothyronine, T4 = thyroxine
$^{+}$ = statistically significantly different at $p \leq 0.05$
$^{++}$ = statistically significantly different at $p \leq 0.01$

Table 5.8.1/36-4: Clinical chemistry – historical control values of Hsd Cpb:WU rats

Parameter	Unit	N	Mean	SD	Range -2s	+2s	Range -3s	+3s
Males 8-11 weeks								
GGT	U/L	10	0	0.0	Up to	0	Up to	0
Crea	µmol/L	198	45	53	34	56	29	61
T3	nmol/L	123	1.40	0.238	0.92	1.87	0.69	2.11
T4	nmol/L	123	74	17.8	38	109	20	127

G. Determination of enzymes in liver tissue
There were no effects observed on the content of cytochrome P450 and on the activity of N- and O-demethylase.

H. Urinanalysis
In high-dose males creatinine was increased after the treatment period. This effect was reversible at the end of the recovery period. The pH was decreased in both sexes at 1000 mg/kg bw/day.
At the end of the recovery period urine volume was increased in high-dose males.
All the changes were within the 2-s- range of historical controls.
None of the other urine analysis parameters was affected in any dose group.

I. Gross necropsy
Organ weights
There were no treatment-related effects on organ weights observed.

Gross pathology
There were no treatment-related gross pathological findings in any dose group after treatment and recovery.

J. Histopathology
There were no treatment-related microscopic findings observed at any dose level tested.

K. Neurotoxicity evaluation

Functional observational battery (FOB)

The functional observation battery, reflex testing, and grip strength measurement, showed no relevant signs or symptoms indicating evidence for a neurotoxic potential.

Motor activity (MA)

There were no treatment-related effects observed for motor and locomotor activity in any dose group. There was no indication for a neurotoxic potential.

III. Conclusion

Based on the haematological, and urine analysis findings the NOEL is 200 mg/kg bw/day. The NOAEL is 1000 mg/kg bw/day.

Phthalic acid

Report:	KCA 5.8.1 /37; Lee, K.-H.; Lee, B. M.; 2007; M-462063-01
Title:	Study of mutagenicities of phthalic acid and terephthalic acid using in vitro and in vivo genotoxicity tests
Report No:	M-462063-01-1
Document No:	M-462063-01-1
Guidelines:	**not applicable; not applicable**
GLP/GEP:	**n.a.**

I. Materials and methods

A. Materials

1. Test material:

Name:	Phthalic acid (PA), CAS No. 88-99-3 terephthalic acid (TPA), CAS No. 100-21-0
Source:	Sigma (St. Louis, MO, USA)
Description:	Not reported
Lot/Batch no:	Not reported
Purity:	Not reported
Stability of test compound:	Not reported

2. Vehicle and or positive control:

Ames test:	Vehicle: not reported Positive controls: Without metabolic activation TA 98: 2-Nitrofluorene (2-NF); 1µg/plate TA 1535, TA 100: Sodium azide (NaN_3), 1.5 µg/plate TA 1537: acridine (ICR-191): 1µg/plate TA 102: mitomycin C (MMC): 1µg/plate With metabolic activation TA 1535, TA 1537, TA 98, TA 100, TA 102: 2-aminoanthracene (2-AA): 1 µg/plate
Chromosome aberration test:	Vehicle: not reported negative control: Without metabolic activation: Distilled water With metabolic activation: dimethylsulfoxid (DMSO) Positive controls: Without metabolic activation

	mitomycin C (MMC):
	With metabolic activation
	Benzo[a]pyrene (BaP)
Micronucleus test in vivo:	Vehicle: DMSO
	Positive control: MMC at 2 mg/kg bw

3. Test system:

Ames test:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537
Chromosome aberration test:	Chinese Hamster Ovary (CHO) cells
Medium:	The culture medium consisted of Eagle´sminimum essential medium (GIBCO, NY) supplemented with 10% fetal bovine serum (FBS), L-glutamine, and antibiotic
Metabolic activation:	Mammalian Microsomal Fraction (S9 Mix) prepared from livers of rats treated with Aroclor 1254 (Source: Woojung Chem. Co., Korea)

3. Test animals

Species:	mouse
Strain:	ICR
Age:	7 - 8 weeks
Weight at dosing:	Males: 23 ± 0.5 g
Source:	Not reported
Acclimatisation period:	7 days
Diet:	Not reported
Water:	Not reported
Housing:	Not reported

B. Study design and methods

1. Ames test

Dose	0-20-100-500-2500-12500 µM +/- S9 mix
	Positive controls
	Without metabolic activation
	TA 98: 2-Nitrofluorene (2-NF); 1 µg/plate
	TA 1535, TA 100: Sodium azide (NaN_3), 1.5 µg/plate
	TA 1537: acridine (ICR-191): 1 µg/plate
	TA 102: mitomycin C (MMC): 1 µg/plate
	With metabolic activation
	TA 1535, TA 1537, TA 98, TA 100, TA 102:
	2-aminoanthracene (2-AA): 1 µg/plate
Application volume:	100 µL (test solution)/culture (pre-incubation method)
Incubation time / temperature:	Pre-incubation. 30 min, 37°C
	48 hours, 37°C
Replicates:	3 plates per dose level
Evaluation:	Revertant colonies per plate were counted

2. Chromosome aberration test

Dose	0-20-100-500-12500-12500 µM/mL +/- S9-mix
Positive control:	Dose levels for positive controls not reported
Replicates:	2 cultures per dose level
Harvest time:	18 h after treatment
Incubation temperature:	37°C
Evaluation:	At least 100 metaphases per dose level were analysed.

3. Micronucleus test in vivo

Dose	0-20-100-500-2500-12500 µM/kg bw;
	positive control: 2 mg/kg bw

Application route:	i.p
Application volume:	None reported
Group size:	5 males / dose group
Observations:	None reported
Sacrifice/bone marrow preparation:	24 h after treatment
Evaluation:	At least 1000 polychromatic erythrocytes (PCE) per animal were analysed for micronuclei. Micronucleated polychromatic erythrocytes (MNPCE) that contained micronuclei were counted from at least 1000 PCE. Normochromatic erythrocytes (NCE) were also determined.
Statistics:	All results are expressed as means ± standard deviation (SD). The significance of differences was assessed by Student´s t-test ($p<0.05$).

II. Results and discussion

A. Ames test

A significant increase in the number of revertants was observed in the presence of the positive control compounds. Negative and strain-specific positive control values were within historical lab range, demonstrating that the test conditions were effective and that the metabolic activation system functioned properly.

Phthalic acid (PA) did not produce an increase in the revertant frequencies in any of the five bacterial strains tested with and without metabolic activation.

Table 5.8.1/37-1: Result of the reverse mutation test (mean values of n = 3 plates per test solution/control)

Compound	S9 mix	Concen-tration (µg/plate)	Base-pair substitution type			Frameshift type	
			TA 1535	TA 100	TA 102	TA 1537	TA 98
Control	–		9.2	114.3	257.6	5.2	22.6
PA	–	20	16.8	162.3	286.3	13.5	29.3
	–	100	18.5	156.8	294.5	16.2	35.2
	–	500	17.4	165.4	300.5	15.8	31.8
	–	2500	17.6	159.6	298.8	15.2	29.8
	–	12500	15.9	160.8	297.6	13.4	33.5
NaN_3	–	1.5	103.5	1172			
2-NF	–	1.0					386.7
MMC	–	1.0			1256.9		
ICR-191	–	100				88.5	
Control	+		8	101.2	261.3	4	26.8
PA	+	20	13.6	158.6	296.7	11.3	36.2
	+	100	16.2	148.6	302.2	14.3	32.4
	+	500	14.4	152.9	295.3	14.9	36.4
	+	2500	14.9	139.7	297.6	13.7	29.7
	+	12500	15.3	154.2	296.3	14.1	32.9
2-AA	+	1.0	97.8	1278.5	1358.2	74.5	402.3

Positive controls: NaN_3 = Sodium azide; MMC = Mitomycin C, 2-NF = 2-nitrofluorene, ICR 191 = acridine, 2-AA = 2-aminoanthracene

The test item PA did not induce gene mutations by base pair changes or frameshifts in the genome of *S. typhimurium* TA98, TA100, TA102, TA1535, and TA1537.
Therefore, PA is considered to be non-mutagenic in this assay.

B. Chromosome aberration test

Cytotoxicity

None of the test substance concentrations produced any cytotoxic effects under the experimental conditions described.

Clastogenicity

PA at concentrations ranging from 20 µM/mL to 12,500 µM/mL induced chromosome aberrations including gaps, breakages and exchanges. However, there was not significant increase in the incidence of chromosome aberration as compared to controls. With the positive control substances mitomycin C and benzo[a]pyrene a clear increase in the aberration frequency was noted.

The results are summarized in the following Table 5.8.1/37-2:

Table 5.8.1/37-2: Summary of the results of the chromosome aberration test

Substance	Concentration [µM/kg]	Cells scored [number]	Cells with aberrations incl. gaps [total number]	Cells with aberrations excl. gaps [total number]
Experiment without S9-mix (6 h treatment; 18 h harvest)				
Solvent control[1]		100	2	2
PA	20	100	4	2
	100	100	2	2
	500	100	2	1
	2500	100	3	3
	12500	100	5	3
MMC[2]	Not reported	100	32	25
Experiment with S9-mix (6 h treatment; 18 h harvest)				
Solvent control[3]		100	3	1
PA	20	100	4	4
	100	100	4	2
	500	100	6	5
	2500	100	4	1
	12500	100	6	4
BaP[4]	Not reported	100	57	40

[1] Distilled water [2] MMC = mitomycin C [3] DMSO [4] BaP = Benzo[a]pyrene

In all experiments performed without and with metabolic activation no significant increase in the number of metaphases containing chromosomal aberrations was observed.
In conclusion, it can be stated that under the experimental conditions reported no evidence of clastogenic effects was observed in Chinese hamster ovary cells *in vitro* in the absence and presence of S9 mix. Thus, the test item PA is not considered to be clastogenic.

C. Micronucleus test in vivo

No mortalities were observed at dose levels up to and including 12500 µM/kg bw.

Ratio of MNPCE (micronucleated polychromatic erythrocytes/1000 polychromatic erythrocytes) in the vehicle control group was 0.24%, whereas MNPCE ratio in the positive control groups were 6.47%. In general, MNPCE% was higher for mouse bone marrow cells treated with the test substance, but no concentration response relationship was observed. PCE/(PCE + NCE) values were also elevated in cells treated with the test agents, but again no concentration-response relationship was found.

Table 5.8.1/37-3: Summary of the results of the micronucleus test *in vivo*

Substance	Concentration [µM/kg bw]*	Animals used [number]	MNPCE[1] [%]	PCE/(PCE + NCE)[2]
Solvent control[3]	0	5	0.24	0.11
PA	20	5	0.32	0.15
	100	5	0.38	0.13
	500	5	0.41	0.16
	2500	5	0.31	0.14
	12500	5	0.35	0.18
MMC[4]	2	5	6.47	0.62

* Positive control in [mg/kg bw]
1 Micronucleated polychromatic erythrocytes/1000 polychromatic erythrocytes
2 Polychromatic erythrocytes/1000 erythrocytes
3 Sample dilution buffer
4 MMC = mitomycin C

There was no indication of a genotoxic effect after intraperitoneal administration of PA in the micronucleus test on the mouse *in vivo*.

III. Conclusions

Based on the study results the test item phthalic acid is considered to be non-genotoxic.

CA 5.8.2 Supplementary studies on the active substance

Summary of supplementary studies

During the EU review process toxicological data were evaluated on CGA 340161 (= CA 2446 A) and CGA 289998 in order to support the limits of specified impurities.
In addition, toxicological studies conducted with CA 2249 A are also considered supportive to justify the limits of specified impurities.

CA 2446 A

CA 2446 A showed no genotoxicity potential in the bacterial reverse mutation assay or in the chromosome aberration test in CHO cells. The substance was not toxic after acute oral, dermal and inhalation exposure. CA 2446 A was not irritating to the skin and eyes of rabbits, but showed a skin sensitizing potential under the conditions of the Magnusson-Kligman test. After 28-day oral exposure a NOEL of 10 mg/kg bw/day in males/females based on effects on liver, thyroid, and kidney at 100 mg/kg bw/day and above.

Table 5.8.2-1: Summary of studies with CA 2446 A

Study	Concentration range Dose level tested	Result	Author / Reference
Bacterial reverse mutation assay	312.5-5000 mg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (1998) M-072538-01-1
Mammalian chromosome aberration, CHO cells	0.78-12.5 µg/mL (-S9 mix) 25-100 µg/mL (+ S9 mix)	Negative (+/- S9 mix)	[redacted] (1998) M-072553-01-1
Acute oral, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1998) M-072479-01-1
Acute dermal, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1998) M-072484-01-1
Acute inhalation, rat	5 mg/L (4h)	LC_{50} > 5.657 mg/L	[redacted] (1998) M-072489-01-1
Skin irritation, rabbit	0.5 mL/patch (undiluted)	Not irritating	[redacted] (1998) M-072502-01-1
Eye irritation, rabbit	0.1 mL/animal (undiluted)	Not irritating	[redacted] (1998) M-072511-01-1
Skin sensitization, Guinea pig (MKT**)	Intradermal: 5% Topical: 100% Challenge: 30%	Sensitizing	[redacted] (1998) M-072530-01-1
28-day oral rat (gavage) 4 week recovery	0-10-100-300-1000 mg/kg bw/day	NOEL10 mg/kg bw/d in males/females based on hepato-tropic, thyreotrophic, nephrotropic effects ≥ 100 mg/kg bw/d	[redacted] (1999) M-072591-01-1

**MKT = Magnusson Kligman maximisation test

These toxicity studies for CA 2446 A were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CGA 289998

CGA 289998 is not mutagenic in bacteria and showed a low acute toxicity (LD50 >2000 mg/kg bw) after acute oral exposure.

Table 5.8.2-2: Summary of studies with CGA 289998

Study	Concentration range Dose level tested	Result	Author / Reference
Bacterial reverse mutation assay	312.5-5000 mg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted] (2000) M-073574-01-1
Acute oral, rat	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted] (1998) M-137220-01-1

These toxicity studies for CGA 289998 were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of trifloxystrobin.

CA 2249 A

CA 2249 A is not mutagenic in bacteria and mammalian cells in vitro. There is no indication of a clastogenic potential in mammalian cells *in vitro* without metabolic activation, but with metabolic activation an increase of chromosome aberrations occurred. However, in the *in vivo* micronucleus test in mice resulted negative. Overall, CA 2249 A is considered to be non-genotoxic.

The substance was not toxic after acute oral and dermal application. CA 2249 A was not irritating to the skin, but was severely irritating to eyes, and showed no sensitizing potential under the conditions of the Magnusson-Kligman test.

After sub-acute oral exposure hepatotrophic and nephrotoxic effects were observed in both sexes. In the high-dose group at 1000 mg/kg bw/day slight axonal degeneration in the sciatic nerves was observed that were not resolved in female animals after 4 weeks recovery. The NOEL is 10/100 mg/kg bw/day in males/females based on hepatotropic and nephrotropic effects at doses ≥100/300 mg/kg bw/day in males/females.

Table 5.8.2-3: Summary of studies with CA 2249 A (intermediate of CGA 279202)*

Study	Concentration range Dose level tested	Result	Author / Reference
Bacterial reverse mutation assay	**62.5 - 2000 mg/plate (+/- S9 mix)**	**Negative (+/- S9 mix)**	**[redacted] (1998) M-073323-01-1**
Mammalian chromosome aberration, CHO cells	**15.63 - 125 mg/mL (+/- S9 mix)**	**Negative (- S9 mix) Positive (+ S9 mix)**	**[redacted] (1998) M-137214-01-1**
In vivo Micronucleus test, mice	**0-500-1000-2000 mg/kg bw**	**Negative**	**[redacted] (1998) M-073331-01-1**
Acute oral, rat	**2000 mg/kg bw**	**LD_{50} > 2000 mg/kg bw**	**[redacted] (1998) M-073084-01-1**
Acute dermal, rat	**2000 mg/kg bw**	**LD_{50} > 2000 mg/kg bw**	**[redacted] (1998) M-073088-01-1**
Acute inhalation, rat	**--**	**No suitable dust aerosol could be generated.**	**[redacted] (1998) M-073091-01-1**
Skin irritation, rabbit	**0.5 mL/animal**	**Not irritating**	**[redacted] (1998) M-073294-01-1**
Eye irritation, rabbit	**0.1 mL/animal**	**Severely irritating**	**[redacted] (1998) M-073304-01-1**
Skin sensitization, Guinea pig, (MKT)**	**Intradermal: 5% Topical: 70% Challenge: 30%**	**Not sensitising**	**[redacted] (1998) M-073314-01-1**
28-day oral (gavage) rat 4 week recovery	**0-10-100-300-1000 mg/kg bw/d**	**NOEL10/100 mg/kg bw/d in males/females based on hepatotropic, nephrotoxic effects ≥100/300 mg/kg bw/d (m/f)**	**[redacted] (1999) M-137216-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold
**MKT = Magnusson Kligman maximisation test

Trifloxystrobin

For the EU review process two studies were submitted investigating the potential induction of hepatocellular proliferation in rats and mice dosed with trifloxystrobin for 90 days by PCNA staining. There was no evidence for an induction of hepatocellular proliferation in either species (M-039187-01-1, M-039206-01-1).

No immuno-suppressive potential of trifloxystrobin was observed in rats after 28-day dietary exposure to doses up to and including 4000 ppm (263 mg/kg bw/day). The only observed treatment-related effect was a reduction of body weight and body weight gain at 4000 ppm.

Table 5.8.2-4: Summary of studies with trifloxystrobin (CGA 279202)*

Study	Concentration range Dose level tested	Result	Author / Reference
28-day immuno-toxicity study, rat	**0-200-1000-4000 ppm 0-14.2-71- 263 mg/kg bw/day**	**No impairment of immunological IgM response up to and including 4000 ppm NOAEL 1000 ppm (70 mg/kg bw/day) based on body weight effects**	**[redacted] (2012) M-429141-01-1**

* New studies, i.e. studies that were not previously submitted, are written in bold

CA 2249 A

Report: KCA 5.8.2 /12; [redacted] 1998; M-073323-01
Title: CA 2249 A (intermediate of CGA 279202) - Salmonella and Escherichia/mammalian-microsome mutageneity test
Report No: 983025
Document No: M-073323-01-1
Guidelines: **OECD 471 (1983), EEC Directive 92/69 B.14 (1992), MITI Japan (1986), US-EPA CRF 798.5265 (1987)**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:
Name: CA 2249 A (Intermediate of CGA 279202)
Description: solid
Lot/Batch no: P.705010
Purity: 89.1%
Stability of test compound: guaranteed for study duration; expiry date: 2000-01-31

2. Vehicle and or positive control: Vehicle: dimethylsulfoxide (DMSO)
Positive controls without metabolic activation
Sodium azide (NaN_3) in bidistilled water
4-Nitroquinoline (4-NQO) in DMSO
Mitomycin C (MMC) in bidistilled water
2-nitrofluorene (2-NF) in DMSO
9-Aminoacridine (9-AA) in DMSO
With metabolic activation
2-aminoanthracene (2-AA) in DMSO
Cyclophosphamide (CPA) in bidistilled water

3. Test system: *Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537;
E. coli WP2 uvrA

Metabolic activation: Mammalian Microsomal Fraction (S9 Mix) prepared from livers male rats treated with Aroclor 1254 (500 mg/kg, i.p) 5 days prior to sacrifice.
The protein concentration was 34.72 and 34.79 mg/mL.
The amount of S9 supernatant was 10% v/v in the S9 mix.
Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix: 100 µL/mL S9-fraction, 8

µmol/mL $MgCl_2$, 33 µmol/mL KCl, 5 µmol/mLGlucose-6-phosphate, 4 µmol/mL NADP in 100 µmol/mL sodium-ortho-phosphate-buffer, pH 7.4.

B. Study design and methods

1. Treatment

Dose	Experiment I: 125.0 - 2000 µg/plate +/- S9 mix Experiment II: 62.5 - 1000 µg/plate +/- S9 mix Positive controls with metabolic activation: 2-AA: 1.5 µg/plate (TA 98, TA 100 ; TA 1537) 2-AA: 4.0 µg/plate (TA 102) 2-AA: 20.0 µg/plate (WP2 uvrA) CPA: 200.0 µg/plate (TA 1535) without metabolic activation: NaN_3: 2.0 µg/plate (TA 100, TA 1535) 4-NQO: 2.0 µg/plate (WP2 uvrA) MMC: 0.5 µg/plate (TA 102) 4-NQO: 5.0 µg/plate (TA 98) 2-AA: 80 µg/plate
Application volume:	100 µL(test solution)/plate 100 µL (test solution)/culture (pre-incubation method)
Incubation time / temperature:	Pre-incubation. 30 min, 37°C 48 hours, 37°C
Replicates:	3 plates per dose level

II. Results and discussion

The original experiment with and without metabolic activation and the confirmatory experiment without activation was performed as standard plate incorporation assay. The confirmatory experiments with metabolic activation were carried out as preincubation assay.

In the original experiment, performed with and without metabolic activation, treatment of strains TA 98, TA 100, TA 102, TA 1535, TA 1537 and WP2 uvrA with CA 2249 A did not lead to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants in comparison with the negative control (see Table 5.8.2/12-1).

In the confirmatory experiment performed with and without metabolic activation, again after treatment of strains TA98, TA 100, TA 102, TA 1535, TA 1537 and WP2uvrA with CA 2249 A no increase in the incidence of either histidine- or tryptophanprototrophic mutants was observed in comparison with the negative control (see Table 5.8.2/12-2).

Due to a growth inhibiting effect of the test substance the number of revertant colonies was reduced in the experiment with metabolic activation on strains TA 100, *E. coli* WP2 uvrA (1000.0 and 2000.0 µg/plate), TA 1535, TA 98, TA 1537 (2000.0 µg/plate) and TA 102 (500.0 to 2000.0 µg/plate. In the pre-incubation assay, which was performed with metabolic activation, the number of revertant colonies was reduced on strains TA 100, TA 102 (250.0 to 1000.0 µg/plate), TA 1535, TA 98, TA 1537 and E. coli (500.0 and 1000.0 µg/plate). In the experiments without metabolic activation a reduction in the number of revertant colonies occurred with strains TA 100, TA 1535, TA 98, TA 1537, *E. coli* WP2 uvrA (1000.0 and 2000.0 µg/plate) and TA 102 (500.0 to 2000.0 µg/plate). The growth of the background lawn was totally inhibited at the highest concentration and was occasionally reduced at lower concentrations. The test substance did not precipitate on the surface of the agar plates.

Table 5.8.2/12-1: Result of the original experiment (mean values of n = 3 plates per test solution/control)

Compound	S9 mix	Conc. (µg/plate)	Revertants / plate Base-pair substitution type TA 1535	TA 100	TA 102	WP2 uvrA	Frameshift type TA 1537	TA 98
DMSO	–		19.67	108.33	337.67	27.00	13.67	20.00
CA 2249 A	–	125.0	21.00	97.67	294.33	36.00	16.33	23.67
	–	250.0	18.00	98.00	237.67	25.00	14.00	25.00
	–	500.0	17.33	90.67	152.67	28.67	8.67	24.00
	–	1000.0	14.33	18.33	9.33	10.33	3.67	24.33
	–	2000.0	0.00	0.00	0.00	0.00	0.00	0.00
NaN_3	–	2.0	678.00	1090.00				
4-NQO	–	2.0				645.00		
MMC	–	0.5			1960.67			
2-NF	–	5.0						919.33
9-AA	–	80.0					1701.00	
DMSO	+		17.33	88.33	320.33	40.00	12.00	27.00
CA 2249 A	+	125.0	19.67	92.67	280.67	34.33	17.33	35.00
	+	250.0	19.67	94.67	260.33	27.00	14.33	39.33
	+	500.0	21.33	91.00	144.33	23.00	15.00	41.00
	+	1000.0	16.67	23.00	24.00	18.00	7.00	40.33
	+	2000.0	0.00	0.00	0.00	0.00	0.00	0.00
2-AA	+	1.5		1457.67			275.67	1286.33
2-AA	+	4.0			1720.00			
2-AA	+	20.0				1140.67		
CPA	+	200.0	234.00					

NaN_3 = Sodium azide, 4-NQO = 4-Nitroquinoline, MMC = Mitomycin C, 2-NF = 2-nitrofluorene, 9-AA = 9-Aminoacridine, 2-AA = 2-aminoanthracene, CPA = Cyclophosphamide

Table 5.8.2/12-2: Result of the confirmatory experiment (mean value of n = 3 plates per test solution/control)

Compound	S9 mix	Conc. (µg/plate)	Revertants / plate Base-pair substitution type TA 1535	TA 100	TA 102	WP2 uvrA	Frameshift type TA 1537	TA 98
DMSO	–		16.67	93.00	301.67	23.67	12.67	25.33
CA 2249 A	–	62.5	19.33	96.00	295.00	18.00	12.00	27.67
	–	125.0	16.67	89.00	291.33	18.33	15.67	20.67
	–	250.0	17.00	91.67	213.67	19.33	16.00	22.67
	–	500.0	19.00	81.33	138.67	13.67	17.33	25.67
	–	1000.0	0.00	9.67	0.00	10.00	5.67	9.67
NaN_3	–	2.0	673.67	1154.00				
4-NQO	–	2.0				581.33		
MMC	–	0.5			1540.33			
2-NF	–	5.0						266.33
9-AA	–	80.0					1171.00	
DMSO	+		18.67	84.33	307.00	21.33	13.00	28.67
CA 2249 A	+	62.5	17.33	79.33	258.00	20.67	16.33	33.67
	+	125.0	18.33	64.33	226.00	20.00	12.67	28.67
	+	250.0	11.00	37.00	12.67	14.67	11.33	25.67
	+	500.0	0.00	0.00	0.00	0.00	0.00	0.00
	+	1000.0	0.00	0.00	0.00	0.00	0.00	0.00
2-AA	+	1.5		759.67			163.67	862.00
2-AA	+	4.0			1336.67			
2-AA	+	20.0				578.00		
CPA	+	200.0	467.00					

NaN_3 = Sodium azide, 4-NQO = 4-Nitroquinoline, MMC = Mitomycin C, 2-NF = 2-nitrofluorene, 9-AA = 9-Aminoacridine, 2-AA = 2-aminoanthracene, CPA = Cyclophosphamide

III. Conclusions

Based on the results of these experiments and on standard evaluation criteria, it is concluded that CA 2249 A and its metabolites did not induce gene mutations in the strains of S. typhimurium and E. coli used. Therefore, CA 2249 A is considered to be non-mutagenic in this assay.

Report: KCA 5.8.2/13; [redacted]; 1998; M-137214-01
Title: CA 2249 A (intermediate of CGA 279202) - Cytogenic test on the chinese hamster cells in vitro
Report No: 983026
Document No: M-137214-01-1
Guidelines: OECD 473 (1983), EEC Directive 92/69 B.10 (1992), MAFF Japan (1985), US-EPA CRF 798.5375 (1987)
Deviation(s): none
GLP/GEP: yes

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249 A (Intermediate of CGA 279202)
Article no:	Not stated
Description:	solid

Lot/Batch no: P.705010
Purity: 89.1%
Stability of test compound: guaranteed for study duration; expiry date: 2000-01-31

2. Vehicle and or positive control: Vehicle: diemthylsulfoxide (DMSO).
Positive controls without metabolic activation
0.2 µg/mL Mitomycin C (MMC)
With metabolic activation
20 µg/mL cyclophosphamide (CPA)

3. Test system: Chinese hamster ovary (CHO) cells

Cell line: CCL 61 (CHA K1)
Medium: The culture medium consisted of Nutrient Mixture F-12 supplemented with 10% fetal calf serum and Penicillin/Streptomycin 100 units/ml/100 µg/ml.
Metabolic activation: Rat-liver post mitochondrial supernatant (S9 fraction) was prepared from Aroclor 1254-treated male rats. The protein content was 34.72 mg/mL.

B. Study design and methods

1. Treatment

Dose

Exposure period	S9 mix	Concentrations in µg/mL
21 h	–	3.91, 7.81, **15.63**, **31.25**, **62.50**, 125.0, 250.0, 500.0
21 h*	–	0.98, 1.95, 3.91, 7.81, 15.63, **31.25**, **62.50**, **125.0**
45 h*	–	0.98, 1.95, 3.91, 7.81, **15.63**, **31.25**, **62.50**, 1250.
3 h	+	3.91, 7.81, **15.63**, **31.25**, **62.50**, 125.0, 250.0, 500.0
3 h*	+	0.98, 1.95, 3.91, 7.81, 15.63, **31.25**, **62.50**, **125.0**
3 h**	+	0.98, 1.95, 3.91, 7.81, 15.63, **31.25**, **62.50**, **125.0**

* confirmatory experiment, ** confirmatory experiment with harvest 42 h after treatment
Concentrations in bold letters chosen for mutation rate analysis; all concentrations used for cytotoxicity assessment.

Replicates: 2 cultures per concentration
Harvest time: 18 h after treatment; 42 h after treatment (confirmatory experiment with S9 only)

Incubation temperature: 37°C

2. Evaluation

100 metaphases per replicate culture were evaluated for structural chromosome aberrations in vehicle control and test substance groups.
At least 50 metaphases were scored in positive controls (25/replicate culture).

II. Results and discussion

A. Cytotoxicity

Experiment 1 without S9-mix (21 h treatment)

The highest concentration of 62.50 µg/ml selected for chromosome analysis caused 72.3% suppression of mitotic activity. The next higher concentration of 125.00 µg/ml completely suppressed mitotic activity due to toxicity.

Experiment 2 with S9-mix (3 h treatment; 18 h recovery)
The highest concentration of 62.50 µg/ml selected for chromosome analysis caused 17.2% suppression of mitotic activity. The next higher concentration of 125.00 µg/ml completely suppressed mitotic activity.

Experiment 1 confirmatory without S9-mix (21 h treatment)
The highest concentration of 125.00 µg/ml selected for chromosome analysis caused 76.5% suppression of mitotic activity.

Experiment 2 confirmatory with S9-mix (3 h treatment; 18 h recovery)
In experiment 4 the highest concentration of 125.00 µg/ml selected for chromosome analysis caused 59.3% suppression of mitotic activity.

Experiment 3 confirmatory without S9-mix (45 h treatment)
The highest concentration of 62.50 µg/ml selected for chromosome analysis caused 29.4% suppression of mitotic activity. The next higher concentration of 125.00 µg/mL suppressed mitotic activity by 92.2% and was not scored according to the selection criteria.

Experiment 4 confirmatory with S9-mix (3 h treatment; 42 h recovery)
In experiment 6 the highest concentration of 125.00 µg/ml selected for chromosome analysis caused 37.2% suppression of mitotic activity.

The results are summarized in Table 5.8.2/13-1

Table 5.8.2/13-1: Results of the cytotoxicity determinations

Substance	Concentration [µg/mL]	Cells scored [number]	Mitosis [number]	Frequency [% of control]	Mitotic index [%]	Cell density
Experiment 1 without S9-mix (21 h treatment)						
Solvent control[1]		2000	166	100.00	8.30	+++
CA 2249 A	7.81	2000	176	106.02	8.80	+++
	15.63	2000	162	97.59	8.10	+++
	31.25	2000	131	78.92	6.55	+++
	62.50	2000	46	27.71	2.30	++
	125.00	2000	0	0.00	0.00	+
	250.00	nc				--
	500.00	nc				--
Experiment 2 with S9-mix (3 h treatment; 18 h recovery)						
Solvent control[1]		2000	221	100.00	11.05	+++
CA 2249 A	15.63	2000	273	123.53	13.65	+++
	31.25	2000	214	96.83	10.70	+++
	62.50	2000	183	82.81	9.15	+++
	125.00	2000	0	0.00	0.00	+
	250.00	nc				
	500.00	nc				
Experiment 1 (confirmatory) without S9-mix (21 h treatment)						
Solvent control[1]		2000	132	100.00	6.60	+++
CA 2249 A	7.81	2000	128	96.97	6.40	+++
	15.63	2000	146	110.61	7.30	+++
	31.25	2000	120	90.91	6.00	+++
	62.50	2000	68	51.52	3.40	+++
	125.00	2000	31	23.48	1.55	++

Substance	Concentration [µg/mL]	Cells scored [number]	Mitosis [number]	Frequency [% of control]	Mitotic index [%]	Cell density
Experiment 2 (confirmatory) with S9-mix (3 h treatment; 18 h recovery)						
Solvent control[1]		2000	231	100.00	11.55	+++
CA 2249 A	15.63	2000	229	99.13	11.45	+++
	31.25	2000	226	97.84	11.30	+++
	62.50	2000	199	86.15	9.95	+++
	125.00	2000	94	40.69	4.70	++
Experiment 3 (confirmatory) without S9-mix (45 h treatment)						
Solvent control[1]		2000	153	100.00	7.65	+++
CA 2249 A	15.63	2000	174	113.73	8.70	+++
	31.25	2000	115	75.16	5.75	+++
	62.50	2000	108	70.59	5.40	+++
	125.00	2000	12	7.84	0.60	++
Experiment 4 (confirmatory) with S9-mix (3 h treatment; 42 h recovery)						
Solvent control[1]		2000	191	100.00	9.55	+++
CA 2249 A	15.63	2000	226	118.32	11.30	+++
	31.25	2000	208	108.90	10.40	+++
	62.50	2000	202	105.76	10.10	+++
	125.00	2000	120	62.83	6.00	++

[1] DMSO
nc no cells due to toxicity
\+ Less than 50% of solvent control; ++ about 50% of solvent control; +++ similar to solvent control

B. Clastogenicity

Main study (experiments 1 and 2)

Without metabolic activation 0.5% of metaphases with specific chromosomal aberrations were detected in the negative control. At 15.63 µg/ml, 31.25 µg/ml and 62.50 µg/ml 4.0%, 2.5% and 1.0% of cells with specific chromosomal aberrations were found.

With metabolic activation 0.5% of metaphases with specific chromosomal aberrations were seen in the negative control. At 15.63 µg/ml, 31.25 µg/ml and 62.50 µg/ml the respective values were 2.0%, 2.5% and 3.0%.

Confirmatory study (experiments 1, 2, 3 and 4)

Without metabolic activation after 21 hours treatment (experiment 1), 0.5% of metaphases with specific chromosomal aberrations were detected in the negative control. At 31.25 µg/ml, 62.50 µg/ml and 125.00 µg/ml 2.5%, 1.5% and 3.0% of cells with specific chromosomal aberrations were registered.

With metabolic activation after 3 hours treatment and 18 hours recovery (experiment 2), 1.5% of metaphases with specific chromosomal aberrations were seen in the negative control. At 31.25 µg/ml, 62.50 µg/ml and 125.00 µg/ml 4.5%, 4.5% and 15.0% of cells showed specific chromosomal aberrations.

In experiment 3 without metabolic activation after 45 hours treatment 1.0% of metaphases with specific chromosomal aberrations were detected in the negative control cultures. At 15.63 µg/ml, 31.25 µg/ml and 62.50 µg/ml the corresponding values were 0%, 13.5% and 1.0%.

In experiment 4 performed with metabolic activation after 3 hours treatment and 42 hours recovery, 2.0% of metaphases with specific chromosomal aberrations were registered in the negative control cultures. At 31.25 µg/ml, 62.50 µg/ml and 125.00 µg/ml 2.0%, 1.5% and 10.5% of cells with specific chromosomal aberrations were found.

The results are summarized in the following Table 5.8.2/13-2.

Table 5.8.2/13-2: Summary of the results

Substance	Concen-tration [µg/mL]	Cells scored [number]	Polyploid cells [%]	Cells with aberrations incl. gaps [total number]	Cells with aberrations excl. gaps# [%]	Frequency [% of control]	Mitotic index [%]
Experiment 1 without S9-mix (21 h treatment)							
Solvent control[1]		200	0.0	3	0.5	100.00	8.30
CA 2249 A	15.63	200	1.5	3	4.0*	97.59	8.10
	31.25	200	1.5	1	2.5	78.92	6.55
	62.50	200	1.0	2	1.0	27.71	2.30
MMC[2]	0.2	50	0.5	14	80.0***		
Experiment 2 with S9-mix (3 h treatment; 18 h recovery)							
Solvent control[1]		200	1.5	4	0.5	100.00	11.05
CA 2249 A	15.63	200	0.0	5	2.0	123.53	13.65
	31.25	200	1.0	4	2.5	96.83	10.70
	62.50	200	2.5	3	3.0*	82.81	9.15
CPA[3]	20.0	50	0.5	6	74.0***		
Experiment 1 (confirmatory) without S9-mix (21 h treatment)							
Solvent control[1]		200	3.0	0	0.5	100	6.60
CA 2249 A	31.25	200	0.5	7	2.5	90.91	6.00
	62.50	200	1.5	5	1.5	51.52	3.40
	125.00	200	1.0	8	3.0*	23.48	1.55
MMC[2]	0.2	50	0.5	9	74.00***		
Experiment 2 (confirmatory) with S9-mix (3 h treatment; 18 h recovery)							
Solvent control[1]		200	3.0	5	1.5	100.00	11.55
CA 2249 A	31.25	200	1.5	10	4.5	97.84	11.30
	62.50	200	2.5	5	4.5	86.15	9.95
	125.00	200	2.0	15	15.0***	40.69	4.70
CPA[3]	20.0	50	0.0	5	64.0***		
Experiment 3 (confirmatory) without S9-mix (45 h treatment)							
Solvent control[1]		200	3.0	4	1.0	100.00	7.65
CA 2249 A	15.63	200	0.5	1	0.0	113.73	8.70
	31.25	200	1.5	1	1.5	75.16	5.75
	62.50	100	2.0	3	1.0	70.59	5.40
Experiment 4 (confirmatory) with S9-mix (3 h treatment; 42 h recovery)							
Solvent control[1]		200	1.0	6	2.0	100.00	9.55
CA 2249 A	31.25	200	2.0	5	2.0	108.90	108.90
	62.50	200	1.0	6	1.5	105.76	105.76
	125.00	200	5.0	11	10.5***	62.83	62.83

\# excluding gaps and numerical aberrations

* Statistically significance compared to control ($0.05 \geq p > 0.01$)

** Statistically significance compared to control ($0.01 \geq p > 0.001$)

*** Statistically significance compared to control ($p \leq 0.001$)

[1] DMSO, [2] MMC = mitomycin C, [3] CPA = cyclophosphamid

In all three experiments performed without metabolic activation no biologically relevant increase in the number of metaphases containing specific chromosomal aberrations was observed.

In the experiments carried out with metabolic activation, also no biologically relevant increase in the number of aberrant metaphases was seen. In the two confirmatory experiments (experiments 2 and 4) performed with activation, clearly increased numbers of metaphases with specific chromosomal aberrations were registered at the highest concentration.

These effects occurred at the limit of toxicity. In the first experiment with activation, this concentration was completely toxic. Lower concentrations induced no chromosomal aberrations (see Table 5.8.2/13-2).

In the original experiment without metabolic activation (experiment 1) at the concentration of 15.63 µg/ml, in the original experiment with metabolic activation (experiment 2) at the concentration of 62.50 µg/ml, and in the confirmatory experiment without metabolic activation (experiment 4) at the concentration of 125.00 µg/mL slight, but statistically significant increased numbers of metaphases with specific chromosomal aberrations were observed. Since these incidences of metaphases with aberrations (3.0 to 4.0%) are within the historical negative control range (without S9, 21 h treatment: 0-7; with S9, 3 h treatment: 0-6) and do not meet the criteria for a positive response (frequency of aberrant metaphases >6%), they are considered to be of spontaneous origin and not related to treatment with the test material.

III. Conclusions

In conclusion, under the experimental conditions reported CA 2249 A revealed a clastogenic activity in Chinese hamster ovary cells *in vitro* in the presence of S9 mix. No clastogenicity was observed without metabolic activation.

Report:	KCA 5.8.2 /14; [redacted];1998;M-073931-01
Title:	CA 2249 A (intermediate of CGA 279202) - Micronucleus test, mouse (OECD conform)
Report No:	983067
Document No:	M-073931-01-1
Guidelines:	**OECD 474 (1983), EEC Directive 92/69 B.12 (1992), US-EPA CRF 798.5395 (1987), EEC Directive 92/69 B.10 (1992), MITI Japan (1987)**
	Deviation(s): none
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249 A (Intermediate of CGA 279202)
Description:	Solid
Lot/Batch no:	P.705010
Purity:	89.1%
Stability of test compound:	guaranteed for study duration; expiry date: 2000-01-31

2. Vehicle / positive control: Vehicle: Carboxymethylcellulose (CMC), 0.5% in water supplemented with 0.1% Tween 80
positive control: cyclophosphamide (CPA) 64 mg/kg bw

3. Test animals

Species:	mouse
Strain:	(ICO:CD1[CRL]
Age:	approx. 7 - 8 weeks
Weight at dosing:	Males: 28 - 59 g; females: 22 - 27 g
Source:	[redacted], France
Acclimatisation period:	at least five days
Diet:	Pelleted, certified standard diet ([redacted] No. 890 Tox), *ad libitum*, except for 12 h prior to dosing

Water:	tap water , *ad libitum*
Housing:	2 per cage or individually

B. Study design and methods

1. Animal assignment and treatment

Dose:	0-500-1000-2000 mg/kg bw; positive control: 64 mg/kg bw
Application route:	Oral, gavage
Application volume:	10 mL/kg bw
Group size:	5/sex/dose group
Observations:	mortality, clinical signs, body weights
Sacrifice/bone marrow preparation:	Negative control and high-dose: 16, 24 and 48 h after treatment positive control, low- and intermediate dose: 24 h after treatment
No. of cells scored:	2000 per animal

2. Evaluation

Scoring:	The incidence of micronucleated polychromatic erythrocytes (MNPCE) among at least 2000 polychromatic erythrocytes (PCE), and the ratio of PCE to normochromatic erythrocytes (NCE) among a total of at least 1000 erythrocytes was determined for each slide.
Statistics:	The significance of differences was assessed by the Chi-Squared-Contingency-Test ($F=4$, $p<0.05$).

II. Results and discussion

A. Clinical observations

In the high dose group of the 16 and 24 hours sampling time irritability, anxious behavior and circling were occasionally observed immediately before sacrifice.

B. Microscopic Evaluation

At all sampling times (16, 24 and 48 hours) there was no statistically significant increase in the number of micronucleated polychromatic erythrocytes in the animals treated with the respective doses of CA 2249 A as compared with the negative control animals.

In the positive control the percentage of micronucleated cells within polychromatic erythrocytes was significantly increased (1.77% micronucleated PCEs) when compared to the negative control (0.02% micronucleated PCEs)).

There was no change in the PCE/NCE-ratio at any dosage compared to the respective negative control. However, the PCE/NCE-ratio observed in the positive control was markedly reduced due to an influence on the erythropoiesis.

The results are summarized in the following Table 5.8.2/14-1.

Table 5.8.2/14-1: Summary of results

Experimental groups		Sacrifice after treatment [hours]	PCEs counted # [number]	Ratio PCE/NCE	MNPCE found# [number]	% of MNPCE [%]
Negative control		16	20000	0.90	6	0.03
0.5% CMC		24	20000	0.93	3	0.02
		48	20000	0.83	4	0.02
CA 2249 A	500 mg/kg bw	24	20000	0.73	5	0.03
CA 2249 A	1000 mg/kg bw	24	20000	0.87	9	0.05
CA 2249 A	2000 mg/kg bw	16	20000	0.86	6	0.03
		24	20000	0.78	9	0.05
		48	20000	0.77	7	0.04
CPA:	64 mg/kg bw	24	20000	0.56	353	1.77*

* Statistically significant different from control p=0.05

III. Conclusions

There was no indication of a clastogenic/aneugenic activity after oral administration of CA 2249 A in the micronucleus test on the mouse.

Report: KCA 5.8.2 /15; [redacted]; 1998; M-073084-01-1
Title: CA 2249 A (intermediate of CGA 279202) - Acute oral toxicity in the rat (limit test)
Report No: 983019
Document No: M-073084-01-1
Guidelines: **Commission Directive 92/69/EEC Method B.1 (1992); OECD 401 (1987); Deviations: none**
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249 A (Intermediate of CGA 279202)
Description:	Yellowish solid
Batch / Lot No.:	P.705010
Purity:	89.1%
Stability of test compound:	guaranteed for study duration; expiry date: 2000-01-31

2. Vehicle: 0.5% (w/v) carboxymethylcellulose in 0.1 (w/v) aqueous polysorbate 80

3. Test animals

Species:	Rat,
Strain:	Wistar, HanIbm_WIST
Age:	Young adults, approx. 7-11 weeks
Weight at dosing:	males: 178.2 g - 195.2 g; females: 154.3 g - 174.2 g
Source:	[redacted] Ltd.; [redacted], Switzerland
Acclimatisation period:	at least 5 days
Diet:	[redacted] No. 890 ([redacted], Switzerland), *ad libitum*

Water:	Municipal water in bottles, *ad libitum*
Housing:	5 per sex in Makrolon Type 4 cages with soft wood bedding

B. Study design and methods

1. Animal assignment and treatment

Dose:	2000 mg/kg bw
Application route:	Oral, gavage
Application volume:	10 mL/kg bw
Fasting time:	before administration: overnight
Group size:	5 rats/sex
Post-treatment observation period:	14 days
Observations:	clinical signs, mortality, body weight, gross necropsy

II. Results and discussion

A. Mortality
There were no mortalities.

Table 5.8.2/15-1: Result summary

Dose (mg/kg bw)	Toxicological result*	Onset and duration of signs	Onset of death after	Mortality (%)
Male rats				
2000	0 5 5	1 - 5 h	--	0
Female rats				
2000	0 5 5	1 - 5 h	--	0
LD_{50}: > 2000 mg/kg bw (males and females)				

* 1st number = number of dead animals, 2nd number = number of animals with toxic signs
3rd number = number of animals used

B. Clinical observations
Hypoactivity, piloerection and hunched posture were observed in all animals on the treatment day. All animals appeared normal by day 1 after treatment.

C. Body weight
There were no effects on body weight noted.

D. Necropsy
There were no abnormalities observed at necropsy.

III. Conclusion

CGA 2249 A is considered to be non-toxic after oral administration. The acute oral LD_{50} of male and female rats was greater than 2000 mg/kg bw.

Report: KCA 5.8.2 /16; [redacted];1998;M-073088-01
Title: CA 2249 A (intermediate of CGA 279202) - Acute dermal toxicity in the rat (limit test)
Report No: 983020
Document No: M-073088-01-1
Guidelines: **Commission Directive 92/69/EEC Method B.3 (1992); OECD 402 (1992); Deviations: none**
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249 A (intermediate of CGA 279202)
Description:	Yellow solid
Lot/Batch no:	P.705010
Purity:	89.1 %
Stability of test compound:	guaranteed for study duration; approved until not reported

2. Vehicle: 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80

3. Test animals

Species:	Rat albino
Strain:	Wistar, HanIbm_WIST
Age:	Young adults, approx. 8-12 weeks
Weight at dosing:	males: 221.2 g – 247.4 g; females: 187.6 g – 214.4 g
Source:	[redacted] Ltd.; [redacted], Switzerland
Acclimatisation period:	at least 5 days
Diet:	[redacted] No. 890 ([redacted], Switzerland), *ad libitum*
Water:	Municipal water in bottles, *ad libitum*
Housing:	Individually in Makrolon Type 3 cages with soft wood bedding

B. Study design and methods

1. Animal assignment and treatment

Dose

Dose (mg/kg bw)	Surface area (cm^2)	Range (mg/cm^2)
2000	36	Not reported

Quantity applied:	400 mg/100 g bodyweight the test substance was moistened with vehicle (1:1)
Application route:	Dermal, semi-occlusive dressing
Duration:	24 hours
Group size:	5 per sex
Post-treatment observation period:	14 days
Observations:	Mortality, clinical signs, skin effects, body weight, gross necropsy

II. Results and discussion

A. Mortality

No deaths occurred during the study.

Table 5.8.2/16-1: Result summary

Dose (mg/kg bw/day)	Toxicological result*			Onset and duration of signs	Onset of death after	Mortality (%)
Male rats						
2000	0	0	5	--	--	0
Female rats						
2000	0	0	5	--	--	0
LD_{50} > 2000 mg/kg bw						

* 1st number = number of dead animals, 2nd number = number of animals with toxic signs, 3rd number = number of animals used

B. Clinical observations
There were no remarkable clinical signs observed. There were no local skin findings at the application site observed.

C. Body weight
A slight loss of body weight was observed in two females during the first week after treatment. No other effects on body weights were noted.

D. Necropsy
Macroscopic examination of the animals revealed no apparent abnormalities.

III. Conclusion

CGA 2249 A is considered to be non-toxic after dermal administration. The acute dermal LD_{50} of male and female rats was greater than 2000 mg/kg bw.

Report: KCA 5.8.2 /17; [redacted];1998;M-073091-01
Title: Technical trials preceding a proposed 4 hour acute inhalation study with CA 2249 A (intermediate of CGA 279202) in rats
Report No: 685113
Document No: M-073091-01-1
Guidelines: **EC Directive 92/69/EEC, Part B.2, (1992), OECD 403 (1981), U.S. EPA, 40 Part 798, B-, Section 798.1150, revised (1994), U.S. EPA, OPPTS 870.1300, JUNE 1996 ("Public Draft")**
Deviation(s): none
GLP/GEP: **no**

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249 A (Intermediate of CGA 279202
Description:	solid
Lot/Batch no:	P.705010
Purity:	93.6%
Stability of test compound:	guaranteed for study duration, approved until 2000-01-31

2. Vehicle: none

II. Results and discussion

The purpose of this acute 4-hour inhalation toxicity study was to assess the acute inhalation toxicity of CA 2249 A when administered to rats for a single continuous 4-hour period. The target concentration of the exposure aerosol was 5 mg/L air.

A dust aerosol suitable for administration by inhalation could not be generated from CA 2249 A, because of adhesive properties of the test article. An aerosol was generated from liquefied test article, but was not considered to be suitable for acute inhalation toxicity testing, because accumulation of the test article in the exposure system was evident and would have led to blockage of the exposure system during the 4-hour exposure period, and because the particle size distribution obtained was considered to be unrepresentative.

Therefore, the objective of this study, i.e. the exposure and observation of rats, was not achieved. An LC_{50} of CA 2249 A was not obtained.

III. Conclusion

An LC_{50} of CA 2249 A was not obtained.

Report: KCA 5.8.2 /18; [redacted];1998;M-073294-01
Title: CA 2249 A (intermediate of CGA 279202) - Acute dermal irritation/corrosion in the rabbit
Report No: 983022
Document No: M-073294-01-1
Guidelines: **Directive 92/69/EEC B.2 (1992); OECD 404 (1992)**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249 A (Intermediate of CGA 279202)
Description:	Yellow solid
Lot/Batch no:	P.705010
Purity:	89.1%
Stability of test compound:	guaranteed for study duration; approved until 2000-01-31

2. Vehicle: None, the test item was used in its original form.

3. Test animals

Species:	Rabbit
Strain:	New Zealand White
Sex:	males
Age:	approx. 2/3 months
Weight at dosing:	2.4–2.56 kg
Source:	[redacted]; France
Acclimatisation period:	At least 5 days
Diet:	[redacted] No. 814 ([redacted], Switzerland), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in Techniplast batteries (Techniplast F&L, Italy)

B. Study design and methods

1. Animal assignment and treatment

Dose	0.5 g
Application route:	Dermal (area: approx. 6 cm^2)
Duration:	4 hours
Group size:	3 males
Observations:	Mortality, clinical signs, skin effects, body weight (at beginning and end of study)

II. Results and discussion

A. Findings

There were no mortalities or systemic intolerance reactions. Body weights were not affected by the treatment.

A very slight erythema (score 1) was observed in all rabbits at the 1-hour examination and in one rabbit at the 24-hours examination. All skin reactions were fully reversed within 48 hours after patch removal.

The mean irritation scores for the individual animals were 0.33, 0.0 and 0.0 for erythema, and 0.0, 0.0 and 0.0 for oedema. The overall mean irritation score for erythema was 0.11, and for oedema 0.0.

The skin irritation observations are summarized in the Table 5.8.2/18-1.

Table 5.8.2/18-1: Summary of irritant effects (Score)

Time after patch removal	Animal #1		Animal #2		Animal #3	
	Erythema and eschar formation	Oedema	Erythema and eschar formation	Oedema	Erythema and eschar formation	Oedema
60 min	1	0	1	0	1	0
24 h	1	0	0	0	0	0
48 h	0	0	0	0	0	0
72 h	0	0	0	0	0	0
Mean 24-72 h	**0.33**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

III. Conclusion

The test item CA 2249 A was not irritating to the skin.

Report: KCA 5.8.2 /19[redacted];1998;M-073304-01
Title: CA 2249 A (intermediate of CGA 279202) - Acute eye irritation/corrosion in the rabbit
Report No: 983023
Document No: M-073304-01-1
Guidelines: **Directive 92/69/EEC B.5 (1992); OECD 405 (1987)**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name: CA 2249 A (Intermediate of CGA 279202)
Description: White solid
Lot/Batch no.: P.705010
Purity: 89.1%
Stability of test compound: guaranteed for study duration; approved until 2000-01

2. Vehicle: None, the test item was used in its original form

3. Test animals

Species: Rabbit
Strain: New Zealand White
Sex: males
Age: approx. 8 - 12 weeks
Weight at dosing: 2.46 - 2.97 kg
Source: [redacted]; France
Acclimatisation period: At least 5 days
Diet: [redacted] No. 814 ([redacted], Switzerland), *ad libitum*
Water: Tap water, *ad libitum*
Housing: Individually in Techniplast batteries (Techniplast FRL, Italy)

B. Study design and methods

1. Animal assignment and treatment

Dose: 0.1 mL/animal
Application route: Single instillation to the conjunctival sac of the left eye (eyes

	were rinsed 24 h after application)
Group size:	3 males
Observations:	Mortality, clinical signs, eye effects, body weight (at beginning and end of study)

II. Results and discussion

A. Findings

There were no mortalities or systemic intolerance reactions. One animal vocalised upon the instillation of the test article. Body weights were not affected by treatment.
Scattered areas of corneal opacity (score of 1) were seen in animal #3 from 1 hour through 48 hours and in animal #1 through 7 days after treatment.
Scattered areas of corneal opacity up to slightly opaque cornea (scores of 1-2) were seen in animal #2 from 1 hour through 24 days after treatment. Moderate circumcorneal hyperaemia of the iris (score of 1) was seen at the 1-hour, 24-hours and 48 hours examinations in all animals, and at the 72-hours examination in two animals.
Hyperaemic conjunctival blood vessels up to diffuse, crimson coloured conjunctival redness (scores of 1-2) were observed in one rabbit (no 3) from 1 hour through 7 days and in two rabbits (no. 1 and 2) through 10 days after treatment. Above-normal swelling of the eyelids and nictitating membranes (score of 1) was seen in two animals from 1 hour through 72 hours after treatment. In animal #1, above-normal swelling of the eyelids and nictitating membranes (score of 1) was seen at the 1-hour reading and obvious swelling with partial eversion of lids (score of 2) at the 24-hour reading.
Eye reactions are not fully reversible within 21 days. All eye reactions were resolved by day 28.
The eye observations are summarized in the Table 5.8.2/19-1.

Table 5.8.2/19-1: Summary of irritant effects (Score)

Animal No.	Observation	1 h	24 h	48 h	72 h	Mean scores	Response	Reversibility (days)
1	Corneal opacity	1	1	1	1	1.0	+	10
	Iris	1	1	1	1	1.0	+	7
	Redness conjunctivae	2	2	2	2	2.0	+	14
	Chemosis conjunctivae	1	2	0	0	0.67	−	2
2	Corneal opacity	1	2	2	2	2.0	++	28
	Iris	1	1	1	1	1.0	+	7
	Redness conjunctivae	1	2	2	2	2.0	+	14
	Chemosis conjunctivae	1	1	1	1	1.0	−	7
3	Corneal opacity	1	1	1	0	0.67	−	3
	Iris	1	1	1	0	0.67	−	3
	Redness conjunctivae	1	2	2	2	2.0	+	10
	Chemosis conjunctivae	1	1	1	1	1.0	−	7

Response for mean scores		Corneal opacity	Iritis	Conjunctival redness	Conjunctival oedema	
−	= negative	<1	<1	<2	<2	(Regulation (EC) No 1272/2008 and GHS)
		<2	<1	<2.5	<2	(Directive 1999/45/EC as amended)
(+)	= mild irritant	≥1 - <3	≥1 - <2	≥2	≥2	(GHS category 2B (effects reversible within 7 days))
+	= irritant	≥1 - <3	≥1 - <2	≥2	≥2	(Regulation (EC) No 1272/2008 (GHS) category 2)
		≥2 - <3	≥1 - <2	≥2.5	≥2	(Directive 1999/45/EC as amended)
++	= irreversible effects	≥3	≥1.5			(Regulation (EC) No 1272/2008 and GHS category 1)
	serious damage	≥3	≥2			(Directive 1999/45/EC as amended)
na	not applicable					

III. Conclusion

Based on the study results the test substance CA 2249 A is severely irritating to eyes of rabbits, since the eye findings were not resolved after 21 days.

Report:	KCA 5.8.2 /20; [redacted];1998;M-073314-01
Title:	CA 2249 A (intermediate of CGA 279202) - Skin sensitization in the guinea pig (maximization test)
Report No:	983024
Document No:	M-073314-01-1
Guidelines:	**OECD 406 (1992); Commission Directive 96/54/EC, section IV C (1996)** **Deviations: none**
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test materials:

Name:	CA 2249 A (Intermediate of CGA 279202)
Description:	Yellow solid
Lot/Batch no:	P.705010
Purity:	89.1%
Stability of test compound:	guaranteed for study duration; expiry date: 2000-01

2. Vehicle: Peanut oil (intradermal induction), Vaseline (topical induction, irritant treatment, and challenge)

3. Test animals:

Species:	Guinea pig
Strain:	Himmalayan Spotted (GOHI)
Age:	Approx. 1 - 3 month
Sex:	Males and females
Weight at dosing:	326 - 427 g
Source:	[redacted] Ltd. 4414 [redacted], Switzerland
Acclimatisation period:	At least 5 days
Diet:	[redacted] No. 845 ([redacted], [redacted]/SG, Switzerland), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in Makrolon® type 3 cages with soft wood bedding

B. Study design and methods

1. Animal assignment and treatment

Dose	
Intradermal induction:	5% (= 20 mg test substance/animal)
Irritant treatment:	10% (= 0.5 g) sodium laurylsulfate
Topical induction:	70% (approx. 0.4g test substance/animal)
Challenge:	30% (highest non-irritant dose)
Application route:	Intradermal, dermal
Application volume:	intradermal induction: 0.1 mL/injection

	callenge: approx. 0.35 mL
Duration:	topical induction: 48 hours, callenge: 24 hours
Group size:	20 in test item groups; 10 in control group
Observations::	mortality, clinical signs, skin effects, body weight (at beginning and termination of study)

II. Results and diseussion

A. Findings

One hour after epidermal induction positive skin reactions were noted in all animals of the test substance and in the vehicle control group.

After callenge exposure positive reactions were observed in 2 males and 1 female of the test substance group at the 24-hour examination, no positive skin responses were observed at the 48-hour examination; the sensitization rate for CA 2249 A was therefore 15%. There were no positive skin responses among the vehicle control group.

There were no mortalities or systemic intolerance reactions. A loss of body weight was recorded in one female guinea pig of the control group. No other effects on body weight were noted.

A summary of the skin reactions observed after callenge exposure are given in the Table 5.8.2/20-1.

Table 5.8.2/20-1: Number of animals exhibiting skin effects

	Test item group (20 animals)					Control group (10 animals)				
	Test item patch			Control patch		Test item patch			Control patch	
Hours	24	48	Total	24	48	24	48	Total	24	48
Callenge 30%	3/20	0/20	3/20	0/20	0/20	0/10	0/10	0/10	0/10	0/10

III. Conclusions

Under the experimental conditions employed 15% of the test group showed skin reactions 24 hours after exposure. Thus, CGA 2249 A is not considered to be a sensitizer in guinea pigs.

Report: KCA 5.8.2 /21; [redacted];1999;M-137216-01
Title: CA 2249 A (intermediate of CGA 279202) - 28 days subacute, oral toxicity study in rats (gavage)
Report No: 983033
Document No: M-137216-01-1
Guidelines: **OECD 407 (1995); Commission Directive 96/54/EC B.7 (1996)**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	CA 2249A (Intermediate of CGA 279202)
Article no:	Not reported
Description:	solid
Lot/Batch no:	P.705010
Purity:	89.1 %

Stability of test compound:	guaranteed for study duration; expiry date: 2000-01
2. Vehicle and positive control:	Vehicle: 0.5% carboxymethylcellulose (CMC) / 0.1% Tween 80
3. Test animals	
Species:	Rat, albino
Strain:	HanIbm:WIST (SPF)
Sex:	Males and females
Age:	About 6 weeks
Weight at dosing:	Males: 142.6 - 168.0 g; females: 102.5 - 140.6 g
Source:	[redacted], Switzerland
Acclimatisation period:	At least 15 days
Diet:	Pelleted, certified standard diet ([redacted] no. 8900 For GLP), *ad libitum*, except for overnight fasting prior to blood collection
Water:	Tap water, *ad libitum*
Housing:	Individually in Makrolon type 3 cages with wire-mesh tops and sterilized granulated soft wood bedding

B. Study design and methods

1. Animal assignment and treatment

Dose	0, 10, 100, 300, 1000 mg/kg bw/day
Duration:	28 days
Application route:	Oral, gavage
Application volume:	10 mL/kg bw
Recovery period:	4 weeks for control and high-dose recovery animals
Group size:	5/sex/dose
Observations:	Mortality, clinical signs, body weight, food consumption and water intake, food consumption ratios, detailed clinical examinations, functional observational battery, motor acrtivity, haematology, clinical chemistry, urine analyses, gross necropsy, organ weight (brain, heart, liver, kidneys, adrenals, thymus, ovaries/testes, epididymides, spleen, thyroid), histopathology

II. Results and discussion

A. Mortality

There were no mortalities in any dose group.

B. Clinical signs

Treatment-related clinical signs observed during the treatment period were salivation in two males and two females of the high-dose group, as well as hunched posture of one female of this dose group.

C. Body weight

There were no treatment-related effects on body weight or body weight gain observed at doses up to and including 1000 mg/kg bw/day.

D. Food consumption and water intake

There was a slightly depressed mean food intake during week 1 in both sexes at 1000 mg/kg bw/day. However, the overall mean food intake in treated males and females was similar to their respective control values during treatment and recovery.

The mean food consumption ratios were slightly depressed during week 1 for males and females at 300 and 1000 mg/kg bw/day.

During recovery, ratios for controls and treated animals showed no relevant differences.

The mean water intake in males and females of groups at 300 and 1000 mg/kg bw/day was considered slightly increased by treatment.
During recovery, the water intake in high dose males persisted on an elevated level, whereas females consumed a similar amount as female controls.

E. Neurotoxicology
Functional observational battery and motor activity measurements revealed no effects which could be related to the treatment with the test item

F. Laboratory investigations
<u>Haematology</u>
At the end of the treatment period, a slight normochromic anemia was recorded for males and females of the high-dose group with a tendency to anisocytosis and macrocytosis (females) of red blood cells. In addition, slightly lower mean values were recorded for erythrocyte count, hemoglobin concentration and hematocrit for females at 300 mg/kg bw/day of which only erythrocyte count attained a level of statistical significance from control values. The higher reticulocyte count in high dose animals is evidence of the regenerative nature of the anemia. Furthermore, higher platelet counts were recorded for males and females at 1000 mg/kg bw/day.
Following the 4-week recovery period, values for all the above parameters were similar to those of the respective controls.

Table 5.8.2/21-1: Haematology findings in males after treatment and recovery

Dose group (mg/kg bw/day)	RBC (T/L)	Hb (mmol/L)	Hct (L)	RDW (L)	Reti (L)	PLT (g/L)
4 week treatment						
0	8.064	9.450	0.470	0.114	0.054	1109
10	8.256	9.520	0.478	0.106	0.048	822.2*-
100	8.182	9.360	0.473	0.111*	0.056	952.4
300	8.216	9.280	0.463	0.111	0.054	956.6
1000	7.599*-	8.740*-	0.443*-	0.121+	0.080*+	1142*+
4 week recovery						
0	8.708	9.600	0.476	0.122	0.048	874.0
1000	8.348	9.560	0.471	0.119	0.045	850.2

RBC: erythrocyte count, Hb: haemoglobin, Hct: hematocrit, RDW: red cell volume distribution width, Reti: reticulocytes; PLT: platelets
* Statistically significantly different at $p < 0.05$ (Lepage test)
\+ - Statistically significantly different at $p < 0.01$ (Jonckheere test)

Table 5.8.2/21-2: Haematology findings in females after treatment and recovery

Dose group (mg/kg bw/day)	RBC (T/L)	Hb (mmol/L)	Hct (L)	RDW (L)	Reti (L)	PLT (g/L)
4 week treatment						
0	7.849	9.025	0.439	0.105	0.065	898.9
10	7.970	9.140	0.450	0.101	0.061	970.8
100	7.929	9.120	0.446	0.100	0.065	898.6
300	7.500*	8.680	0.424	0.107	0.075	976.6
1000	6.858*-	8.000*-	0.410*-	0.126*+	0.122*+	1059*+
4 week recovery						
0	7.938	9.360	0.452	0.112	0.047	816.8
1000	7.834	9.280	0.446	0.131	0.042	972.2

RBC: erythrocyte count, Hb: haemoglobin, Hct: hematocrit, RDW: red cell volume distribution width, Reti: reticulocytes; PLT: platelets
* Statistically significantly different at $p < 0.05$ (Lapage test)
\+ - Statistically significantly different at $p < 0.01$ (Jonckheere test)

Clinical chemistry

At the end of the treatment period, males and females at 1000 mg/kg bw/day had significantly higher mean values for bilirubin, protein, albumin, globulin and cholesterol. In addition, high dose level males had a higher mean value for chloride and high dose level females had a raised mean calcium level. These findings were considered to be related to treatment.
Although some inter-group differences attained a level of statistical significance they were not related to treatment as they did not form a dose-response relationship and/or the magnitude of the change was too small to be of toxicological relevance.

After the recovery period, the above values had returned to values similar to those of the respective controls.

Table 5.8.2/21-3: Clinical chemistry findings in males after treatment and recovery

Dose group (mg/kg bw/day)	Bilirubin (µmol/L)	Protein (g/L)	Albumin (g/L)	Globulin (g/L)	Cholesterol (mmol(L)	Chloride (mmol/L)
After 4 week treatment						
0	1.314	66.44	32.85	33.59	1.572	98.58
10	1.552*	64.92	32.72	32.20	1.356	99.62
100	1.436	67.06	32.88	34.18	1.584*	100.1
300	1.318	66.30	32.69	33.60	2.064*	97.06
1000	1.776*+	73.06*+	36.05*+	37.01*+	3.185*+	102.1*
After 4 week recovery						
0	1.356	66.96	33.34	33.62	1.632	99.00
1000	1.556	66.49	33.10	33.38	1.716	101.8

* Statistically significantly different at $p < 0.05$ (Lapage test)
\+ - Statistically significantly different at $p < 0.01$ (Jonckheere test)

Table 5.8.2/21-4: Clinical chemistry findings in females after treatment and recovery

Dose group (mg/kg bw/day)	Bilirubin (µmol/L)	Protein (g/L)	Albumin (g/L)	Globulin (g/L)	Cholesterol (mmol(L)	Calcium (mmol/L)
After 4 week treatment						
0	1.937	65.72	33.88	31.84	1.488	2.623
10	2.260	67.77	35.27	32.51	1.268	2.624
100	2.172	66.53	34.15	32.38	1.286	2.630
300	1.814	68.60	35.22	33.38	1.926	2.588
1000	2.538*	74.61*+	38.13*+	36.49*+	2.798*+	2.727
After 4 week recovery						
0	1.582	65.82	34.01	31.82	1.472	2.608
1000	1.440	69.56*+	34.66	34.90*+	1.564	2.668

* Statistically significantly different at $p < 0.05$ (Lapage test)
\+ - Statistically significantly different at $p < 0.01$ (Jonckheere test)

Urine analyses

At the end of the treatment period, proteinuria, ketonuria and leukocyturia was observed in males and females at 1000 mg/kg bw/day. In addition these animals excreted slightly higher volumes of urine. Higher leukocyte content was also recorded in the urine of males and females of the 300 mg/kg bw/day dose group.

Following the recovery period, urinary parameters for previously treated animals were similar to those of the control group.

Table 5.8.2/21-5: Urine analysis findings in males after treatment and recovery

Dose group (mg/kg bw/day)	Volume (mL)	Protein (g/L)	Ketones (mmol/L)	Leukocytes (per µL)
After 4 week treatment				
0	5.680	0.300	0.650	40.00
10	5.560	0.250	0.400	25.00
100	6.1000	0.250	0.700	70.00
300	6.500	0.500	1.100	180.0*+
1000	7.240*+	1.050*+	2.900*+	172.5*+
After 4 week recovery				
0	4.740	0.350	0.500	25.00
1000	4.180	0.450	0.600	40.00

* Statistically significantly different at $p < 0.05$ (Lapage test)
\+ - Statistically significantly different at $p < 0.01$ (Jonckheere test)

Table 5.8.2/21-6: Urine analysis findings in females after treatment and recovery

Dose group (mg/kg bw/day)	Volume (mL)	Protein (g/L)	Ketones (mmol/L)	Leukocytes (per µL)
After 4 week treatment				
0	3.990	0.300	0.550	15.00
10	3.300	0.250	0.500	10.00
100	3.980	0.250	0.700	25.00
300	4.320	0.250	0.900	40.00
1000	5.270+	0.625*	1.100*	32.50*
After 4 week recovery				
0	4.360	0.250	0.200	15.00
1000	3.780	0.250	0.200	0.000*

* Statistically significantly different at $p < 0.05$ (Lapage test)
\+ - Statistically significantly different at $p < 0.01$ (Jonckheere test)

I. Gross necropsy

Terminal body and organ weights

At the end of the treatment period the absolute/relative mean liver weights were increased in males at 300 mg/kg bw (+11%/+12%) and 1000 mg/kg bw (+45%/+52%) and in females at 300 mg/kg bw (+26%/+19%) and 1000 mg/kg bw (+53%/+49%).
The absolute / relative mean kidney weights were increased in male at 100 mg/kg bw (+7%/+9%), 300 mg/kg bw (+8%/+8%), and 1000 mg/kg (+15%/+20%) and in females of group 5 (+20%/+18%).
After the recovery period higher liver to body weight and higher kidney to body weight ratios were recorded for both sexes. In addition, a higher spleen to body weight ratio was noted in females.

Table 5.8.2/21-7: Terminal body and organ weights (mean values) in male animals

Dose (mg/kg bw/day)	Terminal body weight (g)	Liver weight Absolute (g)	Liver weight Relative (%)	Kidney weight Absolute (g)	Kidney weight Relative (%)
After 4 week treatment					
0	285.6	13.22	46.12	2.174	7.610
10	262.6	12.29	46.79	2.057	7.840
100	279.7	14.16	50.53	2.329	8.316*
300	284.2	14.66	51.65	2.342	8.248*+

Dose (mg/kg bw/day)	Terminal body weight (g)	Liver weight Absolute (g)	Liver weight Relative (%)	Kidney weight Absolute (g)	Kidney weight Relative (%)
1000	274.3	19.19*+	69.98*+	2.508	9.149*+
After 4 week recovery					
0	329.8	12.82	38.79	2.303	7.011
1000	301.6	12.86	42.59*	2.206	7.309

* Statistically significantly different at $p \leq 0.05$ (Wilcoxon test)
\+ Statistically significantly different at $p \leq 0.01$ (Jonckheere test)

Table 5.8.2/21-8: Terminal body and organ weights (mean values) in female animals

Dose (mg/kg bw/day)	Terminal body weight (g)	Liver weight Absolute (g)	Liver weight Relative (%)	Kidney weight Absolute (g)	Kidney weight Relative (%)
After 4 week treatment					
0	164.8	7.110	43.12	1.397	8.461
10	174.8	7.877	45.07	1.504	8.638
100	177.2	7.926	44.71	1.609	9.068
300	174.9	8.956*+	51.25*+	1.576*	9.046
1000	169.0	10.83*+	64.09*+	1.682*+	9.951*+
After 4 week recovery					
0	204.0	7.837	38.37	1.499	7.318
1000	193.9	8.437	43.44*	1.601	8.264*

* Statistically significantly different at $p \leq 0.05$ (Wilcoxon test)
\+ Statistically significantly different at $p \leq 0.01$ (Jonckheere test)

Gross pathology
There were no treatment-related gross pathological findings in any dose group.

Histopathology
There were no histopathological lesions in control and low dose animals.

After the treatment period microscopic examination revealed the following findings.

In the 100 mg/kg bw/day dose group minimal to slight hyaline change of renal tubular epithelium and centrilobular hepatocellular hypertrophy was observed in males. In addition, an increased incidence and/or severity of extramedullary hematopoiesis was observed in the spleen of males. Furthermore, in males of this dose group an increased incidence of hypertrophy of thyroid follicular epithelium was found.

At 300 mg/kg bw/day tubular basophilia in kidney and hyaline change of renal tubular epithelium in males was observed. In addition, centrilobular hepatocellular hypertrophy and an increased incidence of thyroid follicular epithelium was observed in both sexes. In addition, an increased incidence and/or severity of extramedullary hematopoiesis was observed in the spleen of male animals of this group.

At 1000 mg/kg bw/day tubular basophilia in kidneys and hyaline change of renal tubular epithelium was observed in male animals. In both sexes of the high-dose group centrilobular hepatocellular hypertrophy and minimal to slight degenerating nerve fibers in the sciatic nerve were also noted. In addition, an increased incidence and/or severity of extramedullary hematopoiesis was observed in the spleen of male and female animals of this group.
Furthermore, an increased incidence of hypertrophy of thyroid follicular epithelium was found in animals of both sexes.

After the 4-week recovery period microscopical examination of high-dose animals revealed tubular basophilia in the kidney and hypertrophy of thyroid follicular epithelium in males, as well as degenerating nerve fibers in the sciatic nerve of females.

III. Conclusion

Based on the study results, oral exposure with CA 2249 A for 28 consecutive days resulted in hepatotropic and nephrotoxic effects at doses ≥100/300 mg/kg bw/day in males/females. In addition, slight axonal degeneration in the sciatic nerves was detected in few animals treated at 1000 mg/kg bw/day. Incidence and severity of this finding point to an equivocal nature of this effect.
At the end of a 4 week recovery period hepatotropic effects (except for liver to body weight ratio) were resolved. However, incomplete recovery of nephrotoxic effects was observed and degeneration of peripheral nerve fibers was still detected in one female.

Thus, the NO(A)EL for CA 2249 A is 10 mg/kg bw/day for males and 100 mg/kg bw/day for females.

Trifloxystrobin

Immunotoxicity

Report: KCA 5.8.2 (2; [redacted];2012;M-429141-01
Title: Trifloxystrobin - 28-day immunotoxicity study in the male Sprague-Dawley rat by dietary administration
Report No: SA 10359
Document No: M-429141-01-1
Guidelines: **US-EPA OPPTS 870.7800 (1998)**
Deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	Trifloxystrobin (CGA 279202)
Spec. no:	102000007792
Description:	Light beige powder
Lot/Batch no:	EDFL006101
Purity:	99.1 %
Stability of test compound:	guaranteed for study duration; expiry date: 2013-01-14

2. Vehicle and positive control: Vehicle: plain diet
Positive control: cyclophosphamid

3. Test animals

Species:	Rat
Strain:	Sprague-Dawley Crl:CD (SD)
Sex:	Male
Age:	About 7 weeks
Weight at dosing:	Males: 248 - 319 g (mean)
Source:	[redacted], [redacted], France
Acclimatisation period:	At least 10 days
Diet:	A04CP1-10 (SAFE Scientific Animal Food and Engineering,

	Augy, France), *ad libitum*
Water:	Tap water (filtered and softened), *ad libitum*
Housing:	Individually in suspended, stainless steel, wire-mesh cages.

B. Study design and methods

1. Animal assignment and treatment

Dose	0, 200, 1000, 4000 ppm equivalent to 14.2, 70.5 and 263 mg/kg bw/day positive control: 3.5 mg/kg bw/day cyclophosphamide
Duration:	28 days
Application route:	Oral, diet Positive control: oral, gavage
Group size:	10 males
Antigen stimulation:	Sheep red blood cell (SRBC) sensitization
Identification:	Sheep red blood cell (SRBC)
Source of SRBC:	BioMérieux
Preparation of SRBC:	On the day of injection, Sheep Red Blood Cells were washed in PBS (Phosphate Buffered Saline), counted using a cell counting instrument (Siemens Advia 120) and diluted in PBS in order to obtain a 5 x 10^9 cells/mL preparation. SRBC preparation was kept on ice until use.
Administration of SRBC:	On Study Day 26 after the start of treatment, all animals in all groups were immunized by intravenous injection in the tail vein (0.5 mL/animal) with SRBC-preparation under Isoflurane anesthetisation.
Observations:	Mortality, clinical signs, body weight, food intake, determination of SRBC-specific IgM (using ELISA), gross necropsy, organ weight (spleen and thymus only)

II. Results and discussion

A. Mortality

There were no mortalities in any dose group.

B. Clinical signs

There were no clinical signs observed in any dose group.

C. Body weight

There were no treatment-related effects on body weight or body weight gain observed at doses up to and including 1000 ppm.

At 4000 ppm, mean absolute body weight gain was markedly lower during the first week of treatment (-65%, $p<0.01$) when compared to controls. Body weight in this group remained significantly lower throughout the study. Similarly, mean body weight gain per day was lower than controls by 67%, 22% and 24% during weeks 1, 3 and 4, respectively.

As a consequence, mean body weight was significantly lower compared to controls (-8% to -10%, $p<0.01$ or $p<0.05$) throughout the study.

The results are summarized in Table 5.8.2/22-1.

Table 5.8.2/22-1: Summary of body weight development

	Mean body weight (g)				
	Day 1	**Day 8**	**Day 15**	**Day 22**	**Day 29**
Dose (ppm)	Males				
0	283	332	372	406	433
200	283	331	374	410	444
1000	284	331	373	411	435
4000	289	300^{++}	342^{+}	370^{+}	390^{+}
Cyclophosphamid*	285	326	361	392	414
	Mean absolute body weight gain (g)				
	Days 1-8	**Days 8-15**	**Days 15-22**	**Days 22-29**	
Dose (ppm)	Males				
0	49	88	123	150	
200	48	90	127	160	
1000	47	89	127	151	
4000	17^{++}	59^{++}	86^{++}	107^{++}	
Cyclophosphamid*	41	76	107	130	

* Positive control group (3.5 mg/kg bw/day by gavage). This group received plain diet.
$^{+}$ Statistically significantly different at $p \leq 0.05$
$^{++}$ Statistically significantly different at $p \leq 0.01$

D. Food consumption and test substance intake

At 4000 ppm, mean food consumption was significantly lower than controls during the first week of treatment (-31%, p<0.01) and to a lesser extent thereafter (-8% to -15%).
At 1000 ppm and 200 ppm, mean food consumption was not affected during the study.

Based on food consumption the mean achieved dietary trifloxystrobin intake at 200, 1000, 4000 ppm was 14.2, 70.5, 263 mg/kg bw/day, respectively.

E. SRBC-specific IgM response

A high inter-individual variability was noted in all the groups as usually observed with SRBC sensitization. The high mean anti-SRBC IgM concentration observed in the control group confirmed the sensitization of the animals.
No treatment-related change was noted up to and including 4000 ppm trifloxystrobin.

Relative to the control group, mean anti-SRBC IgM concentration was higher at 4000 ppm and lower at 200 ppm. However, these differences were not statistically significant and only few values were out of the control range. In addition, there was no consistency within the doses.
The mean anti-SRBC IgM concentration in the positive control group was markedly lower when compared to controls (-90%, p ≤0.01), and thus confirming the ability of the test system to detect immuno-suppressive effects.

Table 5.8.2/22-2: SRBC-specific IgM (mean ± SD) on study day 30

Dose level trifloxystrobin (ppm)	**0**	**200**	**1000**	**4000**	**Cyclophosphamide 3.5 mg/kg bw/day**
SRBC-specific IgM (µ/mL)	4742 ± 3126	2688 ± 2899	4985 ± 10855	6005 ± 5296	475 ± 1013^{++}
% change (compared to control)	--	-43	+5	+27	-90

SD = standard deviation
$^{++}$ Statistically significantly different at $p \leq 0.01$

I. Gross necropsy

Terminal body and organ weights

Terminal body weights were not affected at dietary doses up to and including 1000 ppm trifloxystrobin. At 4000 ppm mean body weights were statistically significantly reduced when compared to controls (-10 %, $p \leq 0.05$).

In the positive control group a lower mean terminal body weight was observed (-5%, not statistically significant). Absolute and relative mean spleen weight was statistically significantly reduced in this group (-28% and -25%, respectively, $p \leq 0.01$).

Table 5.8.2/22-2: Terminal body and organ weights

Dose (ppm)	Terminal body weight (g)	Spleen weight Absolute (g)	Spleen weight Relative (%)	Thymus weight Absolute (g)	Thymus weight Relative (%)
0	432.2	0.829	0.1926	0.435	0.1012
200	444.1	0.888	0.2003	0.510	0.1158
1000	433.4	0.762	0.1755	0.480	0.1103
4000	389.3+	0.748	0.1931	0.413	0.1064
Cyclophosphamide*	412.5	0.595++	0.1454++	0.399	0.0973

* Positive control (3.5 mg/kg bw/day, by gavage)
\+ Statistically significantly different at $p \leq 0.05$
++ Statistically significantly different at $p \leq 0.01$

Gross pathology

There were no treatment-related gross pathological findings in the negative (diet) control or trifloxystrobin groups at doses up to and including 4000 ppm.

In the positive control group an atrophic/small spleen was noted in 5 out of 10 animals. This finding correlates with the lower spleen weights observed in this group.

III. Conclusion

Based on the study results, no impairment of the immunological IgM response was observed after immunization with SRBC of rats receiving trifloxystrobin at dietary doses up to and including 4000 ppm (equivalent to 263 mg/kg bw/day) for 28 days. Therefore, trifloxystrobin was considered not to have an immuno-suppressive potential.

Based on body weight effects at 4000 ppm, the NOAEL in this study was 1000 ppm, corresponding to 70.5 mg/kg bw/day.

Analytical methods

A method for the determination of trifloxystrobin by HPLC analysis in rodent diet was developed. The reference of the study report is presented in the following.

Report: KCA 5.8.2 /23; [redacted]; [redacted];2011;M-411302-01
Title: Trifloxystrobin - Determination by high performance liquid chromatography analysis in ground rodent diet
Report No: SA 11197
Document No: M-411302-01-1
Guidelines: OECD, 1997;
Deviation(s): not specified
GLP/GEP: Yes

CA 5.8.3 Endocrine disrupting properties

In the apical toxicological studies (subchronic, chronic/oncogenicity, reproduction and developmental toxicity) no evidence of any endocrine effect was seen. Therefore, based on a complete toxicological data set, there is no evidence of an endocrine potential. Furthermore, trifloxystrobin does not fall under the interim criteria.

CA 5.9 Medical data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Report:	KCA 5.9.1/02; [redacted]; 2013; M-465980-01-1
Title:	Occupational medical experience with trifloxystrobin
Report No:	Not applicable
Document No:	M-465980-01-1
Guidelines:	**Not applicable;** **Deviation(s): not applicable**
GLP/GEP:	**No**

Findings
Manufacturing employees in Switzerland were medically examined by a company physician from 2000 until 2013. Examinations were done in two year intervals until 2008, and every three years since 2010.
Routine medical examinations included:

- Anamnesis
- Full physical examination
- Laboratory examinations: FBC, urine analysis, FBS, lipid profile, liver enzymes, creatinine, CRP
- Spirometry
- Audiometry

From 2000 until 2013 trifloxystrobin was formulated in the processing plant at Muttenz (Switzerland). The annual production rate was in the range of 291.3 to 3752 metric tons per year. A total 210 (49 per shift) workers were involved in the formulation of CGA 279202. Personal safety measures were standard work clothing, safety shoes, head guard, chemical protective gloves, goggles, protective coverall (Tyvek suit) and a dust mask P3.

The medical examinations revealed no unwanted effects. There were no accidents with trifloxystrobin observed, and no consultations of the Medical Department due to work or contact with trifloxystrobin were necessary.

CA 5.9.2 Data collected on humans

No cases of human poisoning have been reported in literature.

CA 5.9.3 Direct observations

No direct observations have been made.

CA 5.9.4 Epidemiological studies

Not applicable.

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

No human cases have been reported. In animal experiments no specific symptoms have been seen.

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

First Aid:

- Remove patient from exposure/terminate exposure
- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylenglykol 300 followed by water.

Note: Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethyleneglykol 300 is not required.

- Flushing of the eyes with lukewarm water for 15 minutes
- Induction of vomiting does not seem to be required in regard of the low toxicity. It should only be considered if a large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.

Induced vomiting can remove maximum 50% of the ingested substance.

Note: Induction of vomiting is forbidden, if a formulation containing organic solvents has been ingested!

Treatment:

- Gastric lavage does not seem to be required in regard of the low toxicity of the compound.
- The application of activated charcoal and sodium sulphate (or other carthartic) might be considered in significant ingestions.
- As there is no antidote, treatment has to be symptomatic and supportive.

CA 5.9.7 Expected effects of poisoning

With regard to the low toxicity no effects are expected.

Overall summary and conclusion

The following overall summary is taken from the Monograph and amended by the new information of this supplemental dossier. New information is written in bold letters.

The absorption, distribution, metabolism and excretion of trifloxystrobin in the rat was investigated with both [Glyoxyl-phenyl-U-^{14}C] and [Trifluormethyl-Phenyl-U-^{14}C] labelled trifloxystrobin.

After oral administration of trifloxystrobin, the extent of absorption was influenced by the dose level and the sex of the animals. Female rats absorbed about 65% of the low dose (0.5 mg/kg bw) from the GI tract based on urinary and biliary excretion, and the tissues residues, whereas in male rats the extent of absorption was only 56%. At the high dose (100 mg/kg bw), the absorbed portion decreased to about 55% and 45% of the dose in males and females, respectively. Absorption from the GI tract at the high dosed appears to be saturated since the AUC 0-48 h was approximately 129-fold higher than that at the low dose while the dose level ratio was 200:1.

Within 48 hours, 72 - 96% of the dose was eliminated with the urine and faeces independent of the dose level, pretreatment with non-radiolabelled trifloxystrobin, the site of label, and the sex of the animals. However, the routes of elimination were different in male and female rats. Within seven days, male rats eliminated approximately 15% of the dose via the urine while females excreted 33%. Bile-duct cannulated rats demonstrate that the bile (ca 44% of the dose) is the principal route of elimination in both males and females. There was evidence of the involvement of enterohepatic circulation in the excretion process.

Seven days after administration of [glyoxyl-phenyl-UL-^{14}C] trifloxystrobin at the low dose (0.5 mg/kg), the tissue residues were very low (total residues <0.5% of the administered dose). At the high dose residues were about 126 and 108-fold higher than the low dose level, in males and females respectively. Some sex and label-specific differences in tissue residues were observed at the high dose level. Generally tissue residues were higher in females than males. Label-specific differences were noted in the fat, kidneys, liver and plasma.

Based on the half-life values and toxicity data for trifloxystrobin there is no evidence that significant bioaccumulation will occur.

Trifloxystrobin appeared to be extensively metabolised at the low dose level (0.5 mg/kg) 4-7% unchanged parent in faeces, whereas at the high dose level (100 mg/kg bw) 31-47% unchanged parent was found in faeces reflecting the extent of absorption at the two dose levels investigated. The amount of parent found in faeces at the low dose does not correlate well with levels of absorption seen at this dose. This may be an artifact of the relatively low recoveries of radiolabel from the faeces at the low dose.

The metabolite pattern in rats was very complex. About 35 metabolites were isolated from urine, faeces and bile and identified. The major steps in the metabolic pathway include hydrolysis of the methyl ester to the corresponding acid, demethylation of the methoxyimino group yielding a hydroxyimino compound and oxidation of the methyl side chain to a primary alcohol, followed by partial oxidation to the respective carboxylic acid. These are followed by a complex pattern of further, minor reactions. Cleavage between the glyoxyl-phenyl and trifluoromethyl-phenyl moieties accounted for about 10% of dose.

The major metabolic pathways of trifloxystrobin seen in the rat were not significantly influenced by the dose and pretreatment but were by the sex of the animals resulting in sex specific urinary metabolites.

The mode of action of trifloxystrobin in common with other strobilurin fungicides is as a respiration inhibitor by blocking the electron transfer between cytochrome B and cytochrome C in mitochondria of fungi. The effects of trifloxystrobin and its carboxylic acid CGA 321113 on the respiratory chain of mammals were investigated in an in vitro assay on rat liver mitochondria. Trifloxystrobin caused a significant, concentration dependent inhibition of mitochondrial respiration. In contrast CGA 321113 was not inhibiting over a wide range of concentrations indicating a difference of at least two to three orders of magnitude. This could be the explanation for the lack of significant evidence, that the respiratory chain is a major toxicological target in mammals.

Trifloxystrobin is of low acute toxicity via the oral, inhalational and dermal routes. It is a slight skin and moderate eye irritant. Skin sensitization potential was demonstrated in a Maximization study.
The local lymph node assay (LLNA) in mice exhibited no sensitizing potential up to the highest test concentration of 30%. Furthermore, trifloxystrobin does not show a phototoxic potential.

Short-term oral toxicity of trifloxystrobin was assessed in the rat, mouse and dog.
Adverse effects on bodyweight development and food consumption were noted in all three species. In the rat the main organs affected were liver, kidney and pancreas. Major histopathological observations were hepatocellular hypertrophy, acute tubular lesions in kidney and atrophy in the endocrine pancreas.
In the mouse there was evidence of effects on the liver and spleen. Histopathological changes include hepatocellular hypertrophy and necrosis in liver as well as hemosiderosis and extramedullary hematopoiesis in spleen. In female mice markedly increased water consumption was observed at top dose levels.
In the dog effects were seen on the liver (changes in blood chemistry, increased liver weights and hepatocellular hypertrophy), gall bladder (hyperplasia of gall bladder epithelium) and haematopoietic system (including lowered erythrocyte, haemoglobin and haematocrit values, and eosinopenia). There was some evidence of bone marrow toxicity. Vomiting and diarrhoea were noted in all the dog studies.

When tested in vitro, trifloxystrobin was negative in an Ames test, a cytogenetic test in CHO cells and a UDS assay in rat hepatocytes. However an equivocal mutagenic effect was observed in a gene mutation assay in Chinese hamster V79 cells, although this occurred under extreme conditions. There was no evidence of chromosomal damage in an in vivo micronucleus study in the mouse. Overall it is concluded that trifloxystrobin demonstrates no genotoxicity potential.

In lifetime studies in the rat and mouse there was no clear evidence for a carcinogenic potential. Reductions in bodyweight development and diminished food intake were evident in both species. Probably as a consequence of this survival in the rat was significantly improved in high dose animals. Although organ weight changes were noted in the rat there were no clear correlated findings in serum chemistry parameters nor histopathological observations. There were clear treatment related decreased incidences of some neoplastic and non-neoplastic findings, but no clear indication of any tumourogenic potential. In the mouse, liver toxicity was apparent with increases in absolute and relative weights and microscopic changes including single cell necrosis, focal necrosis, fatty change and hepatocellular hypertrophy.

No specific toxic effects on reproduction were found in a rat 2-generation feeding study with trifloxystrobin at any dose level tested. The two higher dose levels caused a reduced body weight gain in dams and in pups of both sexes.
There was no evidence of a teratogenic effect in developmental toxicity studies in rats or rabbits. In both species treatment during gestation resulted in effects on maternal bodyweight gain and food consumption. In rats enlarged thymus was found in fetuses of the high dose group. In rabbits a slightly increased incidence of fused sternebrae in fetuses was noted at the two higher dose levels tested.

The neurotoxic potential of trifloxystrobin was investigated in an acute neurotoxicity study in rats, and as part of a 90 day dietary study also in the rat. There was no evidence of neurotoxicity in either study.

Although a number of changes noted in some studies suggested an immunotoxic potential overall there was no clear consistency of effects to suggest a real concern in this area. **This is supported by a 28-day immunotoxicity study conducted in rats. There was no evidence for an immuno-suppressive potential of trifloxystrobin after dietary exposure.**

Two studies were submitted investigating the potential induction of hepatocellular proliferation in rats and mice dosed with trifloxystrobin for 90 days by PCNA staining. There was no evidence for an induction of hepatocellular proliferation in either species.

In the apical toxicological studies (subchronic, chronic/oncogenicity, reproduction and developmental toxicity) no evidence of any endocrine effect was seen. Therefore, based on a complete toxicological data set, there is no evidence of an endocrine potential.

During the EU review process toxicological data on CGA 340161 (= CA 2446 A) and CGA 289998 were evaluated in order to support the limits of specified impurities (Confidential Information Volume 4, Annex C). Toxicological studies conducted with CA 2249 A are also considered supportive to justify the limits of specified impurities.

The toxicological properties of several plant and/or soil metabolites (CGA 373466, CGA 357261, NOA 414412, NOA 413163 and NOA 413161) were investigated in acute oral toxicity studies and Ames tests. All metabolites were found to be of low acute oral toxicity to rats (LD_{50} >2000 mg/kg bw), and not mutagenic to bacteria.

The genotoxic potential of CGA 357261, CGA 331409, CGA 357262 as well as of the metabolite CGA 321113 and CGA 367619 (phthalic acid) was further investigated. All metabolites were non-mutagenic and overall non-genotoxic.
Also the potential effects of CGA 357261, CGA 331409, CGA 357262 and the metabolites CGA 321113, CGA 373466, NOA 413161 and NOA 413163 on the mitochondrial respiration and on cytotoxicity were investigated *in vitro* in rat hepatocytes. Trifloxystrobin was a potent inhibitor of mitochondrial respiration at nanomolar concentrations in contrast to CGA 357261, CGA 331409, and CGA 357262 which were at least more than one order of magnitude less active in inhibiting mitochondrial respiration. The other metabolites showed actually no effect on mitochondrial respiration. Also in the second *in vitro* test trifloxystrobin revealed the strongest cytotoxic response whereas the CGA 357261 and CGA 331409 were about 35 times less cytotoxic. CGA 357262 and the other metabolites were even less cytotoxic.
Four week oral toxicity studies in rats provided further evidence of the metabolites CGA 373466, NOA 413161 and NOA 413163 being less toxic than trifloxystrobin.

Acceptable daily intake (ADI)

The ADI for trifloxystrobin was derived during the previous evaluation for Annex I listing.

The ADI is based on the NOAEL derived in the 2 year rat study (9.8 mg/kg bw/day) taking into account a 100 fold uncertainty factor.

Therefore, the ADI is 0.1 mg/kg bw/day.

Acute reference dose (ARfD)

During the previous evaluation for Annex I listing of trifloxystrobin no ARfD was derived. Due to the low acute toxicity of trifloxystrobin the allocation of an ARfD is not considered necessary.

Acceptable operator exposure level (AOEL)

The AOEL for trifloxystrobin was derived during the previous evaluation for Annex I listing.

The AOEL based on the NOAEL derived in the 2 year rat study (9.8 mg/kg bw/day) taking into account a 100 fold uncertainty factor, as well as an adjustment for oral absorption of 60%.

Therefore, the AOEL is 0.06 mg/kg bw/day.