Cyprodinil

NOTIFICATION OF AN ACTIVE SUBSTANCE UNDER COMMISSION REGULATION (EU) 844/2012

DOCUMENT M-CA, Section 5 Supplement

TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Version history[1]

Date	Data points containing amendments or additions and brief description	Document identifier and version number
20 May 2016	CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals CA 5.4 Genotoxicity Testing CA 5.6 Reproductive Toxicity CA 5.6 Neurotoxicity Studies CA 5.8.1 Toxicity studies of metabolites CA 5.8.2 Supplementary studies on the active substance CA 5.8.3 Endocrine disrupting properties CA 5.9.2 Data collected on humans (All changes are highlighted in yellow)	CGA219417_11532 8 October 2015 updated 20/5/16
03 February 2017	CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals CA 5.4 Genotoxicity Testing CA 5.8.1 Toxicity studies of metabolites (All changes are highlighted in green)	CGA219417_11532 8 October 2015 updated 20/5/16, 01/02/17
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8 June 2017	CA 5.8.1 Toxicity studies of metabolites (All changes are highlighted in green)	CGA219417_11532 8 October 2015 updated 20/5/16, 01/02/17, 07/04/17, 26/05/17

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

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Introduction

This document supports the application for renewal of the regulatory approval of cyprodinil under Commission Implementing Regulation (EU) 844/2012 of 18 September 2012. This document reviews the toxicological and metabolism studies, including additional data and risk assessments, for cyprodinil.

Cyprodinil was included into Annex I of Council Directive 91/414/EEC (Commission Directive 2006/64/CE of 18 July 2006). This active substance is an approved active substance under Regulation (EC) 1107/2009 (repealing Commission Directive 91/414/EEC) as specified in Commission Implementing Regulation (EU) No. 540/2011 of 25 May 2011.

In accordance with Commission Implementing Regulation (EU) 844/2012, this document summarises new information which are relevant for the renewal of the approval of cyprodinil under Regulation (EC) 1107/2009. Where appropriate this document refers to the Commission Implementing Regulation (EU) No. 540/2011 for cyprodinil and to the EFSA report for cyprodinil (EFSA Scientific Report (2005) 51, 1-78), and in particular the endpoints provided in Appendices I and II thereof.

This document covers data and risk assessments which were not part of the original dossier and which are necessary to reflect changes:

- In requirements under Commission Regulation (EU) No 283/2013, and the associated Annex, which repeals Commission Regulation (EU) No 544/2011 which, under Regulation (EC) 1107/2009, replaced the requirements of Annex II to Directive 91/414/EEC
- In scientific and technical knowledge since the approval or last renewal of the approval
- To representative uses

Where the conclusions of the EU review had specific areas of concern on cyprodinil, new data and/or reviews and/or risk assessments have been provided. Where additional and/or new data on cyprodinil are provided, a justification has been included. Also a justification has been given if new data are required but none were provided.

Details of the literature search undertaken can be found in M-CA Section 9. If a relevant scientifically peer-reviewed open literature reference has been identified for cyprodinil or its major metabolites, it has been discussed within the relevant data point.

CA 5.1 Studies on Absorption, Distribution, Metabolism and Excretion in Mammals

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

Please refer to original EU review. No new data or assessment is provided.

Comparative *in-vitro* metabolism

At the time of submission of the dossier (31 October 2015), there are no guidelines describing the conduct of an *in-vitro* metabolism study comparing key animal test species and humans or describing how to evaluate the data. Therefore, in accordance with the guidance document, SANCO/10181/2013-rev 2.1, no study ~~has been~~ was provided at ~~this~~ that stage. However, Syngenta ~~is currently evaluating how the~~

~~requested data could be provided in the absence of guidance.~~ has now evaluated how to fulfil this data requirement and have provided the following study which is summarised below:-

Report:	K-CA 5.1.1/01 Thibaut R. (2017). Cyprodinil - *In Vitro* Comparative Metabolism of [^{14}C] Cyprodinil in Human and Rat Liver Microsomes. Innovative Environmental Services (IES) Ltd, Benkenstrasse 260, 4108 Witterswil, Switzerland. Laboratory Report No. 20160331. 31 January 2017. Unpublished. Syngenta File No. CGA219417_11769.

GUIDELINES: There are no specific testing regulations or guidelines applicable for this study. The data requirement was based on the Commission Regulation (EU) No 283/2013, 5.1.1, in accordance with Regulation (EC) No 1107/2009.

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

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

EXECUTIVE SUMMARY

In vitro metabolic profiling of cyprodinil was carried out by incubating human and rat liver microsomes (0.5 mg protein/mL) with 10 µM [^{14}C]cyprodinil and a NADPH-regenerating system for 60 minutes at 37°C. The study was conducted with a mixed gender pool of human liver microsomes and separated pools of male and female Wistar rat liver microsomes.

Time zero and negative controls without microsomes and without NADPH-regenerating system were also performed in order to determine the stability of cyprodinil in the incubation medium and to validate NADPH-dependent metabolism.

Positive controls, *i.e.* testosterone 6β-hydroxylation and 7-ethoxycoumarin *O*-dealkylation enzymatic activities, were used to check the metabolic competences of human and rat liver microsomes, respectively.

Positive control enzymatic activities showed that human and rat liver microsomes possessed metabolic competences in agreement with the acceptance criteria. Negative controls indicated that cyprodinil did not degrade in the incubation medium and no NADPH-independent metabolism of cyprodinil occurred.

An extensive NADPH-dependent metabolism of cyprodinil was observed in human and rat liver microsomes after 60 minutes of incubation. Remaining parent compound accounted for 31.6% of the dose in human and for 5.5% and 6.8% in male and female rats, respectively.

Up to 14 radio-HPLC peaks (P1 to P14) were observed in human and rat liver microsome incubates. P12 was assigned to unchanged cyprodinil. P1 to P11 were produced by NADPH-dependent metabolism of cyprodinil in both human and rats. P7 was the main metabolite in human, accounting for 61% of the dose after 60 minutes of incubation. The other metabolic peaks were below 5% of the dose. The metabolic pattern of cyprodinil in rats was similar to human. P7 was the main metabolite, accounting for 59.6% of the dose in male and 71.7% of the dose in female. In male rats two other metabolites accounted for more than 5% of the dose, *i.e.* P4 (12.4%) and P6 (5.9%). In female rats P4 accounted for 8.7% of the dose. The rest of the metabolites were below 5% of the dose.

Cyprodinil was extensively metabolised in human and rat liver microsomes. In a validated *in vitro* test system, all the human metabolites formed were detected in rat. No significant differences were observed between male and female rats.

MATERIALS AND METHODS

Materials:

Test Material:	[^{14}C]cyprodinil
Description:	[Phenyl-U-^{14}C]-CGA219417 (Company code)
Lot/Batch number:	RDR-XXVI-77
Purity:	98.9% as per CoA; 99.3% as determined before use.
CAS#:	121552-61-2
Marker substrate 1:	Testosterone (for human liver microsomes)
Description:	Mixture of [4-14C]Testosterone and unlabelled Testosterone
Lot/Batch number:	130812 for [14C]Testosterone and BCBL3419V for Testosterone
Purity:	Radiochemical purity of 99.4% (determined before used)
CAS#:	58-22-0
Marker substrate 2:	7-Ethoxycoumarin (for rat liver microsomes)
Description:	Mixture of [3-^{14}C]7-Ethoxycoumarin and unlabelled 7-Ethoxycoumarin
Lot/Batch number:	150514 for [3-^{14}C]7-Ethoxycoumarin and 10707DJV for 7-Ethoxycoumarin
Purity:	Radiochemical purity of 100.0% (determined before used)
CAS#:	31005-02-4
Reference item 1:	**6β-Hydroxytestosterone**
Description:	Metabolite of Testosterone
Lot/Batch number:	FN04141413
Purity:	99.3 %
Reference item 2:	**7-Hydroxycoumarin**
Description:	Metabolite of 7-Ethoxycoumarin
Lot/Batch number:	1693700
Purity:	99.5 %

***In vitro* test systems:**

Human liver microsomes: (HLM)	Pool of mixed gender human liver microsomes (100 male and 100 female)
Male rat liver microsomes: (Male RLM)	Pool of male Wistar rat liver microsomes (200 animals)
Female rat liver microsomes: (Female RLM)	Pool of female Wistar rat liver microsomes (100 animals)

Reagents:

Incubation Buffer	100 mM potassium phosphate buffer system (pH 7.4; 3 mM $MgCl_2$)
NADPH-Regenerating System (NADPH-GS)	1 mM NADP, 5 mM G6P and 1 Unit/mL G6PDH as final concentrations in the incubation medium

Study Design and Methods

Study dates:

Study Initiation Date: 22 November 2016

Experimental Starting Date: 25 November 2016

Experimental Completion Date: 09 December 2016

Study Completion Date: 31 January 2017

Preparation of dosing solutions

Cyprodinil dosing solution was prepared at a target concentration of 1 mM by diluting [^{14}C]cyprodinil (Batch No. RDR-XXVI-77) in acetonitrile. Marker substrate dosing solutions (both testosterone and 7-ethoxycoumarin) were prepared by mixing ^{14}C-labelled marker substrate and unlabelled marker substrate in acetonitrile. Target concentrations of [^{14}C]testosterone and [^{14}C]7-ethoxycoumarin dosing solutions were 25 mM and 50 mM, respectively.

In the microsomal assays, a 5 µL aliquot of cyprodinil or marker substrate dosing solution was added to the incubation medium which final volume was 0.5 mL. The final target concentration of cyprodinil in microsomal assays was 10 µM. The final target concentration of testosterone and 7-ethoxycoumarin in microsomal assays was 250 µM and 500 µM, respectively. Final concentration of acetonitrile in microsomal assays was 1%, avoiding inhibition of metabolism enzymes.

Preparation of microsomal suspension

After thawing, HLM and RLM (20 mg protein/mL) were diluted at a ratio of 1:1 with a solution containing cryoprotectant substance (sucrose) to a concentration of 10 mg protein/mL.

In the microsomal assays, an aliquot of 25 µL of diluted microsomal suspension (10 mg protein/mL) was added to the incubation medium (final volume of 0.5 mL) in order to obtain a final microsomal protein concentration of 0.5 mg/mL.

Microsomal assays

Metabolic profiling of cyprodinil

In vitro metabolic profiling of cyprodinil was performed with a target concentration of test item of 10 µM.

HLM and RLM (0.5 mg protein/mL) were preincubated with 10 µM of cyprodinil in 100 mM potassium phosphate buffer (pH 7.4; 3 mM $MgCl_2$) for 3 to 5 minutes in a shaking water bath at 37°C. Incubations were initiated by the addition of NADPH-GS. The samples were then incubated for 60 minutes in a shaking water bath at 37°C.

Time zero controls were performed for HLM and RLM incubated with cyprodinil. Microsomes, potassium phosphate buffer, NADPH-GS and [^{14}C]cyprodinil were added to an equal volume of ice-cold solvent in order to stop the reaction immediately.

Negative controls

Control incubations without microsomes were performed to check the chemical stability of cyprodinil under incubation conditions. Microsomal suspension was replaced by an equal volume of potassium phosphate buffer.

Control incubations without cofactor (NADPH-GS) were performed to validate NADPH-dependent metabolism. Incubations were initiated after addition of potassium phosphate buffer instead of NADPH-GS.

Positive controls

Testosterone 6β-hydroxylation, which is a marker substrate reaction for human CYP3A4/5 activity, was used as positive control to check the metabolic competences of HLM. HLM were incubated with 250 µM of testosterone and NADPH-GS in 100 mM potassium phosphate buffer (pH 7.4; 3 mM $MgCl_2$) for 20 minutes in a shaking water bath at 37°C.

7-Ethoxycoumarin *O*-dealkylation, which is a marker substrate reaction for multiple cytochrome P450 activities, was used as positive controls to check the metabolic competences of RLM. RLM were incubated with 500 µM 7-ethoxycoumarin and NADPH-GS in 100 mM potassium phosphate buffer (pH 7.4; 3 mM $MgCl_2$) for 20 minutes in a shaking water bath at 37°C.

Sample analysis

After termination of incubations by solvent protein precipitation and centrifugation, the radioactivity present in the resulting supernatant was measured by liquid scintillation counting (LSC) of 10 µL aliquots in duplicate. Radioactivity remaining in the microsomal pellet was measured after solubilisation of the pellet. Solubilisation was achieved by incubating the pellet in 1 mL of Solvable (Perkin Elmer, Shelton, CT, USA) overnight at room temperature. The LSC measurement was performed on the whole solubilised pellet.

Supernatants were analysed by radio-HPLC. HPLC peak corresponding to parent [^{14}C]cyprodinil was confirmed by co-chromatography with unlabelled cyprodinil. Radio-HPLC peaks corresponding to 6β-hydroxytestosterone and 7-hydroxycoumarin (marker substrate metabolites used for determination of positive control enzymatic activities) were attributed by co-chromatography with the corresponding reference items.

Data analysis

The radioactivity recovery of each sample incubated with [^{14}C]cyprodinil and marker substrate was determined by the sum of radioactivity measured in the supernatant and the microsomal pellet. Radioactivity recovery was considered valid if ranging between 90-110% of [^{14}C]cyprodinil or marker substrate amount applied to the *in vitro* system.

Radio-HPLC quantification was done by integrating the area under the radio-chromatographic peaks. Quantification was done by using ROI´s (regions of interest), i.e. background regions between radio-chromatographic peaks were not considered in the quantification.

The rates of testosterone 6β-hydroxylation and 7-ethoxycoumarin *O*-dealkylation were calculated by quantifying 6β-hydroxytestosterone and 7-hydroxycoumarin produced in human and rat liver microsomal incubates, respectively. Testosterone 6β-hydroxylation and 7-ethoxycoumarin *O*-dealkylation activities were calculated in units of picomoles of metabolite formed per minute per milligram of microsomal protein (pmol/min/mg). Metabolic competences of liver microsomes were considered acceptable if measured enzymatic rate reached, at least, 80% of the rate given by the supplier for each lot of microsomes.

RESULTS AND DISCUSSION

Radiochemical purity of ^{14}C-labelled cyprodinil and marker substrates

Radiochemical purity of [^{14}C]cyprodinil was determined in the dosing solution prior use. It was 99.3%. Radiochemical purity of ^{14}C-labelled marker substrates was determined in the dosing solution prior to use. The radiochemical purity of [^{14}C]testosterone and [^{14}C]7-ethoxycoumarin was 99.4% and 100.0%, respectively.

Concentration of cyprodinil and marker substrates in microsomal assays

Concentration of cyprodinil and marker substrate was measured in the dosing solutions by counting the radioactivity prior to each test performed. Actual concentration of cyprodinil and marker substrates in microsomal assays was calculated according to those radioactivity measurements. Results are presented in the table below.

Table 5.1.1-1: Concentration of cyprodinil and marker substrates in microsomal assays

Substrate	Concentration	
Cyprodinil	Target Actual	10.0 µM 10.4 µM
Testosterone	Target Actual	250.0 µM 254.5 µM
7-Ethoxycoumarin	Target Actual	500.0 µM 524.5 µM

Positive control enzymatic activities

Testosterone 6β-hydroxylation rates observed in HLM and 7-ethoxycoumarin *O*-dealkylation rates observed in RLM were higher than the rate reported by the supplier for the corresponding lot of microsomes. The positive control enzymatic activities showed that HLM and RLM used in the present study possessed metabolic competences in agreement with the acceptance criteria.

Cyprodinil metabolism in human and rat liver microsomes

Radioactivity recovery

Recovery of radioactivity in HLM and RLM incubated with [^{14}C]cyprodinil ranged between 102.0% and 107.1% in HLM, from 99.8% to 104.2% in male RLM and from 99.3% to 105.4% in female RLM. In all incubates, more than 94% of the recovered radioactivity was located in the supernatant, therefore no further extraction of the microsomal pellet was performed.

Cyprodinil stability in the incubation medium

In time zero controls the percentage of parent compound was slightly lower than the radio-purity determined in the dosing solution. However no significant degradation of cyprodinil was observed.

In negative controls without microsomes, after 60 minutes of incubation in the buffer system no degradation of cyprodinil was observed, the percentage of parent compound remained unchanged compared to time zero. This result indicated that cyprodinil did not degrade in the incubation medium.

Cyprodinil metabolism

In HLM and RLM incubated without NADPH-GS for 60 minutes, the percentage of remaining unchanged parent compound did not significantly differ from time zero. This result showed that no NADPH-independent metabolism of cyprodinil occurred in HLM and RLM.

In both HLM and RLM incubated with NADPH-GS for 60 minutes, cyprodinil was extensively metabolised. In HLM, remaining parent cyprodinil accounted for 31.6% of the dose. In male and female RLM, higher metabolism rates were observed. Remaining parent cyprodinil accounted for 5.5% of the dose in male RLM and for 6.8% of the dose in female RLM. This result also indicated that the metabolism of cyprodinil was NADPH-dependent in both HLM and RLM.

Up to 14 radio-HPLC peaks were observed in HLM and RLM incubated with cyprodinil, namely P1 to P14 (Table 5.1.1-2). P12 was assigned to parent cyprodinil. P13 and P14 were observed in time zero samples, in negative controls and as impurities in the dosing solution. P13 and P14 did not increase significantly after 60 minutes of incubation. On the other hand, P1 to P11 were clearly produced by NADPH-dependent metabolism of cyprodinil in both HLM and RLM.

P7 was the main metabolite observed in HLM. P7 accounted for 61% after 60 minutes of incubation. The other metabolic peaks, P1 to P6 and P8 to P11, were below 5% of the dose.

The metabolic pattern of cyprodinil observed in RLM was similar to HLM. P7 was the main metabolite, accounting for 59.6% of the dose in male and 71.7% of the dose in female. All other metabolites, namely P1 to P6 and P8 to P11, were produced in similar amount or at higher level in RLM. In male RLM two of these metabolites accounted for more than 5% of the dose, P4 for 12.4% and P6 for 5.9%. In female RLM P4 accounted for 8.7% of the dose.

Table 5.1.1-2: Comparison of [^{14}C]cyprodinil metabolic profile in human and rat liver microsomes after 60 minutes of incubation.

% of ROI´s	Human	Male Rat	Female Rat
HPLC Peak	Mean ± SD *(RRT)*	Mean ± SD *(RRT)*	Mean ± SD *(RRT)*
P1	0.8 ± 0.0 *(0.07)*	1.9 ± 0.1 *(0.07)*	1.3 ± 0.2 *(0.07)*
P2	n.d.	2.4 ± 0.2 *(0.46)*	n.d.
P3	0.3 ± 0.4 *(0.60)*	4.3 ± 0.3 *(0.60)*	2.4 ± 0.3 *(0.60)*
P4	1.7 ± 0.1 *(0.62)*	12.4 ± 0.2 *(0.62)*	8.7 ± 0.3 *(0.62)*
P5	n.d.	0.2 ± 0.4 *(0.67)*	n.d.
P6	n.d.	5.9 ± 0.9 *(0.69)*	3.5 ± 0.2 *(0.69)*
P7	61.0 ± 0.4 *(0.80)*	59.6 ± 0.7 *(0.80)*	71.7 ± 0.7 *(0.80)*
P8	1.2 ± 0.2 *(0.83)*	1.6 ± 0.4 *(0.83)*	1.5 ± 0.2 *(0.83)*
P9	1.3 ± 0.2 *(0.86)*	2.6 ± 0.2 *(0.86)*	1.1 ± 0.2 *(0.86)*
P10	2.2 ± 0.2 *(0.88)*	1.9 ± 0.1 *(0.88)*	3.2 ± 0.3 *(0.88)*
P11	n.d.	1.7 ± 0.2 *(0.91)*	n.d.
P12 (Parent)	30.8 ± 0.3 *(1.00)*	5.4 ± 0.1 *(1.00)*	6.7 ± 0.4 *(1.00)*
P13*	0.8 ± 0.1 *(1.02)*	n.d.	n.d.
P14	n.d.	n.d.	n.d.

ROI´s: regions of interest
RRT: relative retention time (mean value)
SD: standard deviation
n.d.: not detected
Results are expressed as mean ± SD of 4 replicates
*P13 was detected in stability controls

CONCLUSIONS

Cyprodinil was extensively metabolised in human and rat liver microsomes. In a validated *in vitro* test system, all the human metabolites formed were detected in rat. No significant differences were observed between male and female rats.

(Thibaut R., 2017)

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

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2 Acute Toxicity

Cyprodinil has a low toxicity in respect to acute oral, dermal and inhalation toxicity and is not irritating to the rabbit skin or eye, however, is a skin sensitiser in the guinea pig. The results are summarised in the following table:

Table 5.2-1: Summary of Acute Toxicity Studies

Study Type	Species	Outcome	Reference
Acute Oral LD_{50}	Rat	> 2000 mg/kg bw	Hartmann, 1990b*
Acute Oral LD_{50}	Mouse	> 5000 mg/kg bw	Winkler, 1995*
Acute Dermal LD_{50}	Rat	> 2000 mg/kg bw	Hartmann, 1990a*
Acute Inhalation LC_{50}	Rat	> 1200 mg/m^3	Hartmann, 1991*
Acute Skin Irritation	Rabbit	non irritant	Schneider, 1990a*
Acute Eye Irritation	Rabbit	non irritant	Schneider, 1990b*
Skin Sensitization - Maximization Test	Guinea Pig	sensitiser	Winkler, 1996*

*Included in original EU review;

CA 5.2.1 Oral

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2.2 Dermal

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2.3 Inhalation

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2.4 Skin irritation

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2.5 Eye irritation

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2.6 Skin sensitisation

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.2.7 Phototoxicity

Table 5.2.7-1: EU Conclusions – Phototoxicity

Property	EU agreed endpoint (Cyprodinil: EFSA Scientific Report (2005) 51, 1-78)	Proposed endpoint
Phototoxicity	-	Not phototoxic

A phototoxicity study is included in response to the new data requirements. Cyprodinil was not phototoxic in the *In Vitro* 3T3 NRU phototoxicity test.

Report:	K-CA 5.2.7/01 Lehmeier D (2014). Cyprodinil: In Vitro 3T3 NRU Phototoxicity Test. BSL Bioservice, Scientific Laboratories GmbH, Behringstrasse 6/8, 82152 Planegg, Germany. Laboratory Report No. 142177. 9 September 2014. Unpublished. Syngenta File No. CGA219417_11555.

GUIDELINES: OECD 432 (2004); OECD 101 (1981); 440/2008/EC (2008).

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

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

EXECUTIVE SUMMARY

The phototoxic potential of cyprodinil technical was determined in an in vitro cytotoxicity assay with the BALB/3T3 mouse fibroblast cell line. The basis of this test is a comparison of the cytotoxicity of the test item when tested in the presence and in the absence of exposure to a non-cytotoxic dose of UVA light and cytotoxicity is expressed as a concentration dependent reduction of the uptake of the vital dye neutral red.

Cyprodinil was dissolved 1% Dimethylsulfoxide (DMSO) in Earle‘s balanced salt solution. BALB/3T3 cells were treated for 1 hour with different concentrations of the test solution at 37±1°C and for a further 50 min in the absence and presence of a non-toxic dose of UVA light. One day after treatment cytotoxicity was analysed as a measure of reduction of neutral red uptake and compared to the controls.

In this study under the given conditions cyprodinil technical showed a cytotoxic effect with and without irradiation. With irradiation the viability of the cells was reduced to 10.8% and without irradiation to -0.1%. Based on this the EC50 values and the PIF were calculated:

- UVA: 21.17 µg/mL
+ UVA: 28.32 µg/mL
PIF = 0.748

In this study under the given circumstances the test item showed no phototoxic effects.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil technical
Description:	light beige solid
Lot/Batch number:	P.012011
Supplied by:	Syngenta Crop Protection
Purity:	99.2%
Contaminants:	Not reported
CAS#:	121552-61-2
Stability of test compound:	31 January 2018 (stored at <30°C)

Media:
Cell culture medium: Dulbecco's Modified Eagle Medium (DMEM, 10x; Biochrom) with 4.5 g/L D-glucose. The medium was diluted 1:10 with A. dest. (Sigma) and supplemented with the following items:
10% New Born Calf Serum (NCS)
1% Penicillin/Streptomycin (final concentration: 100 IU/100 µg/mL)
1% Amphotericin B (final concentration: 2.5 µg/mL)
2% L-glutamine (final concentration: 4 mM)
2% NaHCO3 (final concentration: 1.5 g/L)
1% Na-pyruvate (final concentration: 1 mM)
Neutral Red (NR) Stock Solution
0.4 g neutral red
100 mL H_2O
NR Medium
1 mL NR stock solution
79 mL cell culture medium (without NCS)
NR Desorb
1% acetic acid, glacial
50% ethanol
49% H_2O

Controls: Negative (untreated): 1% Dimethylsulfoxide (DMSO) in Earle's balanced salt solution (EBSS). Positive: Chlorpromazine (2-Chloro-10-[3-dimethylaminopropyl] phenothiazine.

Study Design and Methods:

Experimental dates: Start: 6 May 2014 End: 09 May 2014

Preparation of test item: Cyprodinil technical was dissolved in DMSO by vortexing and diluted in EBSS (Earle's balanced salt solution) in a 1:100 ratio to a highest final concentration of 50 µg/mL (stock solution).

Cells: The test was carried out with BALB/3T3 cells. Cells from frozen stock cultures, tested routinely for mycoplasma, were seeded in culture medium at an appropriate density and subcultured at least once before they were used in the *in vitro* 3T3 NRU phototoxicity test. Cells at passage number 77 were used. Cells were precultured in 75 cm^2 culture flasks in DMEM with 10% new born calf serum at 37 ± 1°C and 5% CO2.

The UVA-sensitivity of the cells was determined and found to be acceptable. Six microtiter plates with cells were irradiated with the UVA-doses 0 (dark control), 3, 5, 7, 9 and 11 J/cm^2. The cells meet

acceptance criteria if their viability after irradiation with 5 J/cm^2 is not less than 80% and after irradiation with 9 J/cm^2 is not less than 50%

Doses: 0; 15.81; 5; 1.58; 0.5; 0.158; 0.05 and 0.0158 µg/mL, plus negative control (1% DMSO in EBSS), blank (EBSS) and positive control (Chlorpromazine 100; 31.6, 10.0; 3.16; 1.00; 0.316; 0.100 and 0.0316 µg/mL without UVA and 10; 3.16; 1.00; 0.316; 0.100, 0.0316, 0.0100, and 0.00316 µg/mL with UVA).

Experimental procedure: A cell suspension of 1× 10^5 cells/mL in culture medium was prepared. 100 µL culture medium were dispensed into the peripheral wells of a 96-well tissue culture microtiter plate (blanks). In the remaining wells, 100 µL of a cell suspension of 1×10^5 cells/mL (1×10^4 cells/well) were dispensed. For each test item two plates were prepared: one for determination of cytotoxicity (without UVA), and the other for determination of photocytotoxicity (with UVA).

The cells were incubated for 24±2 h (5% CO_2, 37±1°C) until they formed a half-confluent monolayer. This incubation period allowed for cell recovery and adherence, and for exponential growth.

After incubation, cells were washed with 150 µL EBSS per well.

The solutions of the test item and the positive control were diluted seven times at a ratio of √10. The positive control was tested in a full scale phototoxicity test on two plates in parallel to the test item. 8 different concentrations were applied to 6 parallel cultures each.

100 µL of the appropriate concentration of test item or solvent only (negative control) were added to the cells. The cells were then incubated in the dark for 60 minutes (5% CO_2, 37±1°C).

The cells were irradiated for 50 min through the lid of the 96-well plate with 1.5-1.8 mW/cm2 UVA (= 4.8-5.4 J/cm2). The positions of the plates were exchanged after half time of the irradiation (25 min.). The distance light source - test system was 64 cm. The temperature during irradiation was 26.0°C (+ UVA) and 24.2°C (- UVA). Duplicate plates (- UVA) were kept at room temperature in a dark box for 50 min (= - UVA exposure time).

After exposition the cells were washed with 150 µL EBSS. EBSS was replaced with culture medium and the plates were incubated (5% CO_2, 37±1°C) overnight (18-22 hours). Following the incubation the cells were washed with 150 µL EBSS. 100 µL neutral red (NR) medium were added and the plates were incubated at 37 ± 1°C, in a humidified atmosphere of 5% CO_2, for 3 hours.

After incubation, the NR medium was removed and the cells were washed with 150 µL EBSS. 150 µL NR desorb solution (freshly prepared ethanol/acetic acid) was added. The microtiter plate was shaken rapidly on a microtiter plate shaker for 10 min, until the NR had been extracted from the cells and had formed a homogeneous solution. Then the optical density of the NR extract was measured at 540 nm in a micro plate auto reader, using blanks as a reference.

Data analysis: Relative cell viability, expressed as percentage of untreated controls, was calculated for each of the eight test concentrations. To predict the phototoxic potential, the concentration responses obtained in the presence (+UVA) and in the absence (-UVA) of irradiation were compared at the EC_{50} level, i.e. at the concentration inhibiting cell viability by 50% in comparison with untreated controls.

If complete concentration response curves are obtained, both in the presence (+UVA) and in the absence (-UVA) of light, a photo-irritation-factor (PIF) is calculated by means of the following formula:

$$PIF = \frac{EC_{50}\ (-UVA)}{EC_{50}\ (+UVA)}$$

Interpretation - PIF < 2: “no phototoxicity”, PIF > 2 and < 5: “probable phototoxicity”, PIF > 5: “phototoxicity”. If both EC_{50} (-UVA) and EC_{50} (+UVA) cannot be calculated due to the fact that a test item does not show any cytotoxicity up to the highest concentration, this indicates no phototoxic potential.

If a test item is only cytotoxic + UVA and is not cytotoxic when tested - UVA, the PIF cannot be calculated, although this result indicates a phototoxic potential of the test item. In such cases the mean photo effect (MPE) is analysed. The MPE is based on comparison of the complete concentration response curves and is defined as the weighted average across a representative set of photo effect values.

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

The photo effect PEC at any concentration C is defined as the product of the response effect REC and the dose effect DEC i.e. PEC = REC x DEC. The response effect REC is the difference between the responses observed in the absence and presence of light, i.e. REC = RC (- UVA) - RC (+ UVA). The dose effect is given by

$$DE_C = \frac{C/C^* - 1}{C/C^* + 1}$$

Where C* represents the equivalence concentration, i.e. the concentration at which the +UVA response equals the - UVA response at concentration C. If C* cannot be determined because the response values of the + UVA curve are systematically higher or lower than RC (- UVA) the dose effect is set to 1. The weighting factors wi are given by the highest response value, i.e. wi = MAX {Ri (+ UVA), Ri (- UVA)}. The concentration grid Ci is chosen such that the same number of points falls into each of the concentration intervals defined by the concentration values used in the experiment. The calculation of MPE is restricted to the maximum concentration value at which at least one of the two curves still exhibits a response value of at least 10%. If this maximum concentration is higher than the highest concentration used in the + UVA experiment the residual part of the + UVA curve is set to the response value “0“.

Interpretation of results:
MPE < 0.1: “no phototoxicity”
MPE > 0.1 and < 0.15: “probable phototoxicity”
MPE > 0.15: “phototoxicity”

Statistics: Data was analysed using the Phototox Version 2.0 Software (***Peters B. and Holzhütter HG, 2002 and Bundesinstitut für Risikobewertung {Federal Institute for Risk Assessment, BfR}***).

RESULTS AND DISCUSSION

The test item showed a cytotoxic effect with and without irradiation. With the highest concentration of the test item (50 µg/mL), viability of the irradiated cells was reduced to --0.1 % and without irradiation to 0.5%. Based on this the EC_{50} values and the PIF were calculated:

- UVA: 21.17 µg/mL
+ UVA: 28.32 µg/mL
PIF = 0.748

The positive control showed cytotoxic and phototoxic effects. With the highest concentration of the positive control in the non-irradiated part of the experiment (100 µg/mL), viability of the cells was reduced to 0.3% relative to the untreated negative controls. The EC50 value was calculated to 16.14 µg/mL. In the irradiated part of the experiment (highest test item concentration: 10 µg/mL), the EC50 value was calculated to 0.45 µg/mL.

Table 5.2.7-2: Mean OD_{550nm} and cell viability (%) with and without UVA irradiation

Compound	Without UVA			With UVA		
	Concentration (µg/mL)	Mean value OD_{550nm}	% viability	Concentration (µg/mL)	Mean value OD_{550nm}	% viability
Cyprodinil	50.00	0.000	-0.1	50.00	0.048	10.8
	15.81	0.346	71.7	15.81	0.387	87.1
	5.00	0.475	98.3	5.00	0.436	98.1
	1.58	0.495	102.4	1.58	0.489	110.0
	0.50	0.570	118.0	0.50	0.487	109.7
	0.16	0.562	116.2	0.16	0.484	109.0
	0.05	0.470	97.3	0.05	0.441	99.4
	0.02	0.483	99.9	0.02	0.459	103.3
Chlorpromazine	100.00	0.002	0.3	10.00	0.005	0.9
	31.62	0.003	0.4	3.16	0.002	0.4
	10.00	0.578	85.7	1.00	0.016	3.2
	3.16	0.692	102.7	0.316	0.363	71.2
	1.00	0.739	109.6	0.100	0.449	88.1
	0.316	0.729	108.1	0.0316	0.533	104.6
	0.100	0.703	104.2	0.0100	0.432	84.9
	0.0316	0.697	103.4	0.00316	0.522	102.6

CONCLUSION: In this study under the given conditions the test item showed no phototoxic effects.

REFERENCES

Peters B. and Holzhütter HG (2002), *In Vitro* Phototoxicity Testing: Development and Validation of a New Concentration Response Analysis Software and Biostatistical Analyses Related to the Use of Various Prediction Models, *ATLA* **30**, pp. 415-432.

Bundesinstitut für Risikobewertung (Federal Institute for Risk Assessment, BfR), Zentralstelle zur Erfassung und Bewertung von Ersatz- und Ergänzungsmethoden zum Tierversuch (Centre for the Documentation and Evaluation of Alternatives to Animal Experiments, ZEBET), Phototox Version 2.0, "How to get started".

(Lehmeier D, 2014)

CA 5.3 Short-Term Toxicity

Following repeated oral administration of high doses of cyprodinil, no evidence for cumulative toxicity was seen in the rat, the mouse and the dog.

In the rat and the dog non-specific effects such as reduced body weight development and/or food consumption were observed. Especially in rodents the liver was the main target organ for cyprodinil. At higher doses increased liver weights as well as hepatocellular hypertrophy, single cell necrosis, cytoplasmatic inclusion bodies or depletion of glycogen was noted. In addition, elevated levels of serum alanine aminotransferase and alkaline phosphatase activities were observed in the rat. In the dog the only indication for the liver as a target organ was the pigmentation of hepatocytes in high dose males.

Changes in haematological and blood chemistry parameters were noted mainly in the rat: increased serum total protein, cholesterol and phospholipid values as well as elevated leucocyte counts. Equivocal results were obtained for the prothrombin time in the rat; in the high dose groups, reduction in the 28-day study and prolongation in the 90-day study. Additionally, in the rat some effects in the kidney (increased weight, inflammatory changes) and – based on a hepatotrophic effect – a functional stimulation of the thyroid (increased weight, hypertrophy) and pituitary gland (hypertrophy) were noted at high doses.

After dermal administration, non-specific symptoms such as piloerection and dyspnoea were observed in the first 28-day study, but no other indications for systemic toxicity in this study or the second 28-day study conducted. In addition, there were no signs for local skin irritation observed at the application site.

The results are summarised in the following table:

Table 5.3-1: Summary of Short-Term Toxicity Studies

Study Type	Dose Levels	NO(A)EL	Major effects at LOAEL	Reference
28 Day Gavage Toxicity Rat	0, 10, 100 and 1000 mg/kg bw/day	NOEL: 10 mg/kg bw/day; NOAEL: 100 mg/kg bw/day.	Reduced body weight development Liver: increased weight, hepatocellular hypertrophy; Thyroid gland: increased weight, hypertrophy of follicular epithelium.	Fankhauser, 1991c*
90 Day Dietary Toxicity Rat	0, 50, 300, 2000 and 12000 ppm (3.14, 19.0, 133.6 and 809.7 mg/kg bw/day in males and 3.24, 19.3, 136.6 and 803.0 mg/kg bw/day in females).	NOEL: 50 ppm (3.14 and 3.24 mg/kg bw/day for males and females, respectively); NOAEL: 300 ppm (19.0 and 19.3 mg/kg bw/day for males and females, respectively).	Liver: increased weight, hepatocellular hypertrophy and necrosis; increased serum ALP and γ-GT; increased serum cholesterol and phospholipid levels. Thyroid: increased weight, hypertrophy of follicular epithelium; Pituitary cell hypertrophy; Kidney: chronic tubular lesion (males only).	Fankhauser, 1991b*

Study Type	Dose Levels	NO(A)EL	Major effects at LOAEL	Reference
90 Day Dietary Toxicity Mouse	0, 500, 2000 and 6000 ppm (73.3, 257.3 and 848.6 mg/kg bw/day in males and 102.5, 349.2 and 1121 mg/kg bw/day in females).	NOEL: 500 ppm (73.3 and 103 mg/kg bw/day for males and females, respectively); NOAEL: 2000 ppm (257 and 349 mg/kg bw/day for males and females, respectively).	Slight tendency towards reduced body weight development; increased liver weight.	Fankhauser, 1991a*
90 Day Dietary Toxicity Dog	0, 200, 1500, 7000 and 20000 ppm (6.07, 45.9, 210.3 and 559.7 mg/kg bw/day in males and 6.79, 52.8, 231.9 and 581.0 mg/kg bw/day in females).	NOEL: 1500 ppm (49.5 and 52.8 mg/kg bw/day for males and females, respectively); NOAEL: 7000 ppm (210 and 232 mg/kg bw/day for males and females, respectively).	Reduced body weight development and food consumption; increased number of blood platelets.	Altmann, 1991*
1 Year Dietary Toxicity Dog	0, 25, 250, 2500 and 15000 ppm (0.72, 6.87, 65.6 and 449 mg/kg bw/day in males and 0.76, 6.80, 68.0 and 446 mg/kg bw/day in females).	NOEL = NOAEL: 2500 ppm (65.6 and 68.0 mg/kg bw/day for males and females, respectively).	Reduced body weight development and food consumption.	Altmann, 1992*
28 Day Dermal Toxicity Rat	0, 5, 25, 125 and 1000 mg/kg bw/day.	NOEL 5 and 125 mg/kg bw/day for females and males, respectively; NOAEL: 125 mg/kg bw/day.	Reduced food consumption ratio (males); clinical signs.	Hagemann, 1991*
28 Day Dermal Toxicity Rat	0, 5, 25, 125 and 1000 mg/kg bw/day.	NOEL = NOAEL: 1000 mg/kg bw/day.	No LOAEL.	Sommer, 2008#

*Included in original EU review;
#Study summary included in **Section 5.3.3**.

CA 5.3.1 Oral 28-day study

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.3.2 Oral 90-day study

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.3.3 Other routes

Table 5.3.3-1: EU Conclusions - Relevant Dermal NOAEL

Property	EU agreed endpoint (Cyprodinil: EFSA Scientific Report (2005) 51, 1-78)	Proposed endpoint
Relevant dermal NOAEL	5 mg/kg bw/day	1000 mg/kg bw/day

A 28 day repeated dose dermal toxicity study in rats has been conducted since the previous EU review and is included below for evaluation. This study was conducted to support regulatory requirements in other regions.

Report:	K-CA 5.3.3/01 Sommer E (2008). CGA219417 Tech - 28 Day Dermal Toxicity (Semi occlusive) Study in the Wistar Rat RCC Ltd, Zelgliweg 1, CH-4452 Itingen, Switzerland. Laboratory Report No. B65485. 31 July 2008. Unpublished. Syngenta File No. CGA219417_10986.

GUIDELINES: OECD 410 (1981); OPPTS 870.3200 (1998).

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

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

EXECUTIVE SUMMARY

Groups of ten male and ten female HanRcc: WIST (SPF) rats were administered under semi-occlusive condition dermal doses of 0 (control), 5, 25, 125 or 1000mg CGA219417 Tech./kg bw/day in 0.5% w/v aqueous carboxymethylcellulose (CMC) in 0.1% w/v polysorbate 80 for 6 hours per day for 5 days a week over a period of 28 days.

General clinical observations, detailed behavioural observations, body weight measurements and food consumption measurements were made once weekly during the acclimatization and treatment periods. A functional observational battery including grip strength and locomotor activity measurements was conducted during the last week of treatment before treatment for that day. Ophthalmoscopic examinations were performed in all animals during acclimatization and during week 4 in animals of the control and high dose groups. Clinical laboratory investigations were conducted with blood samples obtained from fasted animals at the end of the treatment period. At necropsy selected organs were weighed and a range of tissues and organs were examined macro- and microscopically.

One female at 25 mg/kg/day was sacrificed due to poor condition associated with swelling at the left shoulder that occurred as a consequence of an accident. The exact cause of morbidity of this animal could not be determined. All other animals survived their scheduled study period.

No test item-related effects were recorded at the treated skin sites. General and detailed clinical observations, ophthalmoscopical examination and FOB tests and grip strength measurements revealed no test item-related effects. Locomotor activity measurements showed a slight increase in motor activity in males and females treated at 1000 mg/kg/day during the first ten minutes of the test run only. Food consumption and body weight development were not affected by treatment with the test item. Clinical laboratory data gave no indication of a test item-related effect on any investigated parameter in haematology and clinical biochemistry. Histopathological investigation of the treated skin, selected organ and tissue samples revealed no adverse effects from the test item at any dose group in males and females.

Dermal administration of CGA219417 Tech. at doses up to 1000 mg/kg/day during 6 hours a day for 5 days a week over four consecutive weeks resulted in no toxicologically significant findings. The only possible treatment related effect was transient increase in locomotor activity during the first 10 minutes of the assessment period in males and females at 1000 mg/kg/day.

Based on the results of this study a no-observed–adverse effect level (NOAEL) was determined at 1000 mg/kg/day.

MATERIALS AND METHODS

Materials:

Test Material:	CGA219417 Tech.
Description:	Light beige, solid
Lot/Batch number:	P.012011
Purity:	99.2 % a.i (by HPLC)
CAS#	Not reported
Stability of test compound:	Stable at room temperature (20 °C ± 5 °C) in the original container away from direct sunlight.

Vehicle and/or positive control: Carboxymethylcellulose (high viscosity grade)/Polysorbate 80, Lot No. 1140855/ Batch No.1318043, purity 63.1%.

Test Animals:	
Species:	Rat
Strain:	HanRcc: WIST (SPF)
Age/weight at dosing:	7 weeks/males: 224.6 grams; females: 162.9 grams
Source:	RCC Ltd, Laboratory Animal Services, CH-4414 Fuellinsdorf
Housing:	Individually in Makrolon type-3 cages
Acclimatisation period:	7 days
Diet:	Pelleted standard Kliba Nafag 3433 rodent maintenance diet *ad libitum*
Water:	Community tap water from Itingen *ad libitum*
Environmental conditions:	Temperature: 22±3 °C Humidity: 30-70% Air changes: 10-15 air changes per hour Photoperiod: 12-hour fluorescent light / 12-hour dark cycle, with music during the light period.

Study Design and Methods:

Experimental dates: Start: 12 November 2007 End: 18 December 2007

Animal assignment: The animals were assigned to each group following a computer-generated random algorithm. The group identification and animal nos. assigned are stated in the table below:

Test group	Dose Level	# male	# female
Control	0 mg/kg/day	01-10	51-60
Low	5 mg/kg/day	11-20	61-70
Lower mid	25 mg/kg/day	21-30	71-80
Upper mid	125 mg/kg/day	31-40	81-90
High	1000 mg/kg/day	41-50	91-100

Diet preparation and analysis: Dose formulations were prepared daily. The test item was ground and the vehicle, 0.5% (w/v) aqueous carboxymethylcellulose in 0.1% (w/v) polysorbate 80, was added to give the appropriate final concentration of the test item in the dose formulation.

The dosing volume applied was 4 mL/kg bw.

Concentration analysis results: The application formulation consisted of between 89.6% and 116.2% CGA219417 Tech. Analysis of the dose preparations showed that the achieved concentrations were satisfactory throughout the study.

Homogeneity results: The homogeneity of CGA219417 Tech. in the preparations did not deviate more than 11.8% (<15%) from the corresponding mean.

Stability results: The test item was found to be stable in application formulations when kept for 8 hours and 7 days at room temperature.

Preparation and treatment of animal skin: Shortly before the first application and weekly thereafter, the fur of each test animal was clipped from the dorsal area of the trunk over an area of at least 10% of the body surface, exposing an area of approximately 25 cm². The application was semi-occlusive for 6 hours a day for a total of 5 days each week.

After each 6 hour exposure period, the dressing and gauze patch were removed carefully and the treated area was gently rinsed with lukewarm tap water and the skin was dried with a disposable paper towel.

Rats in the control group were exposed to the vehicle using the same procedure as described for the treated rats.

Observations: Animals were observed daily for signs of mortality, general signs of toxicity, and the presence of dermal irritation. The animals were examined for signs of local skin irritation approximately 1 hour after removing the gauze patches and were evaluated using the Draize method. Weekly FOB evaluation including grip strength and locomotor activity were performed. An ophthalmoscopic examination was performed on treatment day 24. Clinical laboratory investigations were performed from blood samples collected after 4 weeks.

Bodyweight: Animals were weighed prior to initiation of the study and at the beginning of each study week.

Food consumption: Food consumption for each animal was determined weekly during acclimatization and treatment periods.

Ophthalmoscopic examination: Eyes were examined in all animals during acclimatization. During week 4, animals of the control and high concentration groups were examined before treatment was applied for that day.

Haematology and clinical chemistry: Blood samples were drawn from the retro-orbital plexus from all animals under light isoflurane anesthesia. The animals were fasted for approximately 18 hours before blood sampling but allowed access to water *ad libitum*. The samples were collected early in the working day to reduce biological variation caused by circadian rhythms.

The following parameters were examined:

Haematology:

haemoglobin
haematocrit

red blood cell count
mean corpuscular volume
red cell volume distribution width
mean corpuscular haemoglobin
mean cell haemoglobin concentration
haemoglobin concentration distribution width
prothrombin time
reticulocyte count
reticulocyte maturity index (low, medium, high fluorescence)
total white cell count
differential white cell count:
neutrophils, eosinophils, basophils, lymphocytes, monocytes, large unstained cells
platelet count
methaemoglobin
Activated partial thromboplastin time

Clinical chemistry:

urea
creatinine
glucose
albumin
total protein
cholesterol
triglycerides
total bilirubin
creatine kinase activity
lactate dehydrogenase activity
glutamate dehydrogenase activity
alkaline phosphatase activity
aspartate aminotransferase activity
alanine aminotransferase activity
gamma-glutamyl transferase activity
calcium
phosphorus (as phosphate)
sodium
potassium
chloride
globulin
albumin/globulin ratio

Investigations *post mortem:*

Macroscopic examination: All animals were examined *post mortem*. This involved an external observation and an internal examination of all organs and structures.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

adrenal glands
brain
epididymides
heart
kidneys
liver
ovaries
spleen
testes
thymus

Paired organs were weighed together.

Tissue submission: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

gross lesions
adrenal gland
aorta
brain (cerebrum, cerebellum and brainstem)
ovary
Peyer's patches
pancreas
parathyroid gland

bone marrow (femur)
caecum
colon
duodenum
epididymis
eyes (retina, optic nerve)
femur (including stifle joint)
Harderian gland
heart
ileum
jejunum
kidney
larynx
liver
lung
lymph node - mandibular
lymph node - mesenteric
mammary gland (females only)
nerve - sciatic
nasal cavity
oesophagus
pharynx
pituitary gland
prostate gland
rectum
salivary gland
seminal vesicle
skeletal muscle
spinal cord (cervical, thoracic, lumbar)
skin (treated and untreated)
spleen
sternum
stomach
testis
thymus
thyroid gland
tongue
trachea
urinary bladder
uterus (with cervix)
vagina

Microscopic examination: All processed tissues were examined by light microscopy.

Statistics: The following statistical approaches have been used in this study:

All analyses were two-tailed for significance levels of 5% and 1%.

All means are presented with standard deviations.

If the variances are clearly heterogeneous, appropriate transformations (e.g. log, square root, double arcsine) have been used in an attempt to stabilise the variances. Any transformations that were utilized have been indicated in the specific results tables and/or the Statistical Methods section.

For quantitative data: Body weights, cumulative body weight gain, food consumption, quantitative FOB measurements (grip strength, landing foot splay, and/or time to tail flick), motor activity data at each measurement interval and overall activity, and absolute organ weights were analyzed initially by a one-way analysis of variance (ANOVA).

Organ weights were analyzed by analysis of covariance (ANCOVA) on final body weight (Shirley, 1977). This statistical analysis provides an Adjusted Organ Weight value, which has been displayed in the results table along with flags for statistical significance.

Summary values of organ to body weight ratios are presented but these have not been analysed statistically. For all of the parameters evaluated initially by ANOVA or ANCOVA, Dunnett's test were used to compare the control and treated groups, based on the error mean square in the ANOVA or ANCOVA. The Dunnett's test was performed for all continuous data parameters, regardless of whether the initial ANOVA or ANCOVA was statistically significant, and statistical flags have been presented in the tables of results in the final report.

Macropathology and micropathology incidence data were analysed using Fisher's Exact Test.

For qualitative data (e.g. possible values of 0, 1, 2 or present/absent): Qualitative functional observational battery parameters or any other parameters not specifically mentioned above that yield qualitative data have been presented as summary data, but have not been analysed statistically.

Individual values have been rounded before printing. All derived values that appear in the report tables represent the rounded results of calculations that are based on the exact (non-rounded) raw data values. Statistical analyses have been carried out on the exact raw data values.

The analyses were performed even if the number of statistical units was low.

RESULTS AND DISCUSSION

Mortality: One female at 25 mg/kg/day was sacrificed due to poor condition associated with swelling at the left shoulder that occurred as a consequence of an accident. The exact cause of morbidity of this animal could not be determined. All remaining animals survived the scheduled study period.

Clinical observations: Daily clinical signs recorded were restricted to general findings such as fissures or reddish sore at the ear auricle, neck or shoulder, scabs at the nose, eye, cervical neck or shoulder region, maculate or focal erythema at the head region, neck and ventral or dorsal thorax, hair loss on the ventral thorax, neck, shoulder and abdomen and were observed in animals of the control and treated groups.

Weekly observations revealed no test item-related findings in any group. Clinical signs were restricted to general findings such as mydriasis, iridic reflex absent and miosis that were observed at a low incidence in one control and 2 treated animals.

The functional observation battery revealed no test item-related effects.

Fore- and hind limbs grip strength measurements showed no test item-related effect. Hind limb grip strength was slightly higher in all male treated groups. However, the differences were considered to reflect biological variation rather than being a test item–related effect as there was no clear dose response relationship.

Locomotor activity measurement revealed a test item-related excitatory effect in males and females of the high dose groups during the first 10 minutes of locomotor activity measurement only.

There were no effects at any other timepoint or in males or females at any other dose level.

Bodyweight and weight gain: Body weight and body weight gain over the full 28-day treatment period were not affected by treatment with the test item.

Food consumption and compound intake: In females, food consumption during week 2 was slightly increased in all treated groups, but was compensated in the following week. At treatment end food consumption did not significantly differ between treated groups and the control group.

Ophthalmoscopic examination: Ophthalmoscopic examination performed on treatment day 24 gave no indication of any test item-related effect.

Haematology: There were no differences in haematological parameters which were considered to be related to treatment.

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

Sacrifice and pathology:

Organ weights: Organ weights were not affected by treatment with the test item.

Macroscopic findings: No macroscopic abnormalities were present in any of the treated male and female groups.

Microscopic findings: Under the conditions of this study, the test substance CGA219417 Tech. produced no microscopic findings at the dermal application site, which distinguished test item-treated animals from control. In addition, there were no further findings of toxicological properties in any other tissue or organ examined.

CONCLUSION: Dermal administration of CGA219417 Tech. at doses up to 1000 mg/kg/day during 6 hours a day for 5 days a week over four consecutive weeks resulted in no toxicologically significant findings. The only possible treatment related effect was transient increase in locomotor activity during the first 10 minutes of the assessment period in males and females at 1000 mg/kg/day.

Based on the results of this study a no-observed-adverse effect level (NOAEL) was determined at 1000 mg/kg/day.

(Sommer E., 2008)

CA 5.4 Genotoxicity Testing

The genotoxic potential of cyprodinil was investigated in six independent studies covering different end points in eukaryotes and prokaryotes *in vivo* and *in vitro.*

Cyprodinil caused no chromosomal damage in mammalian cells *in vitro* and *in vivo*. It was not mutagenic in bacteria or mammalian cells and caused no DNA damage in rat hepatocytes *in vitro*. Cyprodinil is considered to have no genotoxic potential. The results are summarised in the following table:

Table 5.4-1: Summary of Genotoxicity Studies

Study Type	Species	Outcome	Reference
In vitro			
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA 98, TA 100, TA 1535, TA 1537; *Escherichia coli* strain WP2uvrA.	Negative (±S9)	Ogorek, 1990*
Bacterial reverse mutation assay	*Salmonella typhimurium* strains TA 1535, TA 1537, TA 98, and TA 100; *Escherichia coli* strains WP2 uvrA pKM 101, and WP2 pKM 101.	Negative (±S9)	Sokolowski, 2009#
In vitro mammalian cell gene mutation test	Chinese hamster lung fibroblasts V79	Negative (±S9)	Geleick, 1990*
In vitro mammalian cell gene mutation test	Chinese hamster lung fibroblasts V79	Negative (±S9)	Wollny, 2017
In vitro mammalian cytogenetic test	Chinese hamster ovary (CHO) cells	Negative (±S9)	Strasser, 1991*
In vitro mammalian cytogenetic test	Human Lymphocytes	Negative (±S9)	Naumann, 2017
In vitro mammalian cell DNA repair test	Primary rat hepatocytes	Negative	Geleick, 1991*

Study Type	Species	Outcome	Reference
In vivo			
Micronucleus assay	Mouse bone marrow	Negative	Ceresa, 1990*
Proof of Exposure Study to Support Micronucleus assay	CD-1 Mouse	-	Fincher, 2016

*Included in original EU review;
#Study summary included in **Section 5.4.1**.

A new guideline bacterial reverse mutation assay has been conducted with cyprodinil technical material to support the specification. Cyprodinil tech. was considered to be non-mutagenic in this study which is in concordance with data contained within the original EU review. The study is summarised below and details with regards the specification can be found in **Document J**.

CA 5.4.1 *In vitro* studies

Report:	K-CA 5.4.1/01 Sokolowski A. (2009) Cyprodinil tech. - Salmonella Typhimurium and Escherichia Coli Reverse Mutation Assay. Harlan Cytotest Cell Research GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1247000, 16 July 2009. Unpublished. Syngenta File No. CGA219417_11373.

GUIDELINES: OECD 471 (1997); OPPTS 870.5100 (1998); 2008/440/EC B.13/B.14 (2008).

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

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

EXECUTIVE SUMMARY

This study was performed to investigate the potential of Cyprodinil tech. to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II) using the Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strains WP2 uvrA pKM 101, and WP2 pKM 101 over the range; Experiment I and II: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate;

Experiment IIA: 1; 3; 10; 33; 100; and 333µg/plate; and Experiment II A:0.1; 0.3; 1; 3; 10; 33; 100; and 333 µg/plate.

Reduced background growth was observed at the higher concentrations with and without metabolic activation in nearly all strains used in experiments I, II, IIA and IIB.

Distinct toxic effects were observed at the higher concentrations with and without metabolic activation in nearly all strains used in experiments I, II, IIA and IIB.

No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with Cyprodinil tech. at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance. The positive control chemicals induced appropriate responses in the corresponding strains.

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, Cyprodinil tech. is considered to be non-mutagenic in this Salmonella typhimurium and Escherichia coli reverse mutation assay.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil tech.
Description:	Solid, Beige
Lot/Batch number:	SMU9AP001
Purity:	99.9 %
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	Concurrent untreated and solvent controls were performed
Solvent control (final concentration):	100µl/plate
Positive control:	Non-activation: Sodium azide 10 µg/plate TA100, TA1535 4-nitro-o-phenylene-diamine, 50 µg/plate TA 1537, 10 µg/plate TA98 methyl methane sulfonate 3 µL/plate WP2 (pKM101), WP2 *uvrA* (pKM101) Activation: 2-Aminoanthracene 2.5 µg/plate TA 1535, TA 1537, TA100, TA98 10 µg/plate WP2 (pKM101), WP2 *uvrA* (pKM101)

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

Before the experiment an appropriate quantity of S9 supernatant was thawed and mixed with S9 co-factor solution. The amount of S9 supernatant was 10% v/v in the S9 mix. Cofactors are added to the S9 mix to reach the following concentrations in the S9 mix:

8 mM $MgCl_2$
33 mM KCl
5 mM Glucose-6-phosphate
5 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4.
During the experiment the S9 mix was stored in an ice bath.

Test organisms:

S. typhimurium strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

E. coli strains

X	WP2 (pKM101)	X	WP2 *uvrA* (pKM101)						

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	X	Yes		No

Test compound concentrations used

The test item was tested at the following concentrations:
Experiment I and II: 3; 10; 33; 100; 333; 1000; 2500; and 5000 µg/plate
Experiment IIA: 1; 3; 10; 33; 100; and 333µg/plate
Experiment II A: 0.1; 0.3; 1; 3; 10; 33; 100; and 333 µg/plate

Study Design and Methods:

In-life dates: Start: 24 February 2009 End: 27 April 2009

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: Not performed.
Type of Bacterial assay:

- X standard plate test (pre-experiment/experiment I; –S9, +S9)
- X pre-incubation (60 minutes) (second experiment ; –S9, +S9)
- __ "Prival" modification (i.e. azo-reduction method)
- __ spot test
- __ other

Protocol:

For each strain and dose level including the controls, three plates were used.

The following materials were mixed in a test tube and poured onto the selective agar plates:

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

In the pre-incubation assay 100 µL test solution, 500 µL S9 mix / S9 mix substitution buffer and 100 µL bacterial suspensions were mixed in a test tube and shaken at 37° C for 60 minutes. After pre-incubation 2.0 mL overlay agar (45° C) was added to each tube. The mixture was poured on selective agar plates.

After solidification the plates were incubated upside down for at least 48 hours at 37° C in the dark.

Statistical analysis: None – see Evaluation Criteria below.

Evaluation criteria: A test item is considered as a mutagen if a biologically relevant increase in the number of revertants exceeding the threshold of twice the colony count of the corresponding solvent control is observed.

A dose dependent increase is considered biologically relevant if the threshold is exceeded at more than one concentration.

An increase exceeding the threshold at only one concentration is judged as biologically relevant if reproduced in an independent second experiment.

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

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: Not performed.

Mutagenicity assay: The test item Cyprodinil tech. was assessed for its potential to induce gene mutations in the plate incorporation test (experiment I) and the pre-incubation test (experiment II, IIA, IIB) using Salmonella typhimurium strains TA 1535, TA 1537, TA 98, and TA 100, and the Escherichia coli strains WP2 uvrA pKM 101 and WP2 pKM 101.

Reduced background growth was observed at the following concentrations (µg/plate):

Strain	Exp. I		Exp. II		Exp. IIA	Exp. IIB
	without S9 mix	with S9 mix	without S9 mix	with S9 mix	without S9 mix	without S9 mix
TA 1535	333 - 5000	333 - 5000	333 - 5000	333 - 5000	n.p.	n.p.
TA 1537	333 - 5000	333 - 5000	100 - 5000	100 - 5000	33 - 333	100 - 333
TA 98	333 - 5000	333 - 5000	100 - 5000	100 - 5000	n.p.	n.p.
TA 100	333 - 5000	333 - 5000	100 - 5000	100 - 5000	n.p.	n.p.
WP2 uvrA	/	/	333 - 5000	333 - 5000	n.p.	n.p.
WP2 uvrA pKM101	333 - 5000	333 - 5000	333 - 5000	333 - 5000	n.p.	n.p.

/ = no reduced background growth observed; n.p. = not performed

Distinct toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5) were observed at the following concentrations (µg/plate):

Strain	Exp. I		Exp. II		Exp. IIA	Exp. IIB
	without S9 mix	with S9 mix	without S9 mix	with S9 mix	without S9 mix	without S9 mix
TA 1535	333, 5000 (1000, 2500)	333, 5000 (1000, 2500)	333, 1000 (2500, 5000)	333 (1000 - 5000)	n.p.	n.p.
TA 1537	333 - 5000	1000 - 5000	333 - 5000	333 - 5000	33 - 333	100 - 333
TA 98	333 - 5000	1000 - 5000	333, 1000 (2500, 5000)	333, 1000 (2500, 5000)	n.p.	n.p.
TA 100	333, 1000 (2500, 5000)	333, 1000 (2500, 5000)	333 (1000 - 5000)	333, 1000 (2500, 5000)	n.p.	n.p.
WP2 uvrA	/	/	/	5000	n.p.	n.p.
WP2 uvrA pKM101	1000 - 5000	2500, 5000	1000 - 5000	2500, 5000	n.p.	n.p.

/ = no toxic effect observed; in brackets ( ) = not evaluable due to reduced background growth; n.p. = not performed

Precipitation of the test item was observed in the test tubes from 333 - 5000 µg/plate in experiment I and II, and at 333 µg/plate in experiments IIA and IIB. Precipitation of the test item was also observed on the incubated agar plates at 5000 µg/plate without metabolic activation and at 2500 and 5000 µg/plate with metabolic activation in experiment I and II. No visible precipitation on the incubated agar plates was observed in the additional experiments IIA and IIB.

No substantial increase in revertant colony numbers of any of the six tester strains was observed following treatment with Cyprodinil tech. at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

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

CONCLUSION: In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Therefore, Cyprodinil tech. is considered to be non-mutagenic in this Salmonella typhimurium and Escherichia coli reverse mutation assay.

(Sokolowski A., 2009)

Cyprodinil triggers a photomutagenicity study according to the criteria laid down in **Commission Regulation (EU) No 283/2013**. However, such a study has not been conducted with cyprodinil for the following reasons:

- Cyprodinil was not genotoxic when tested in 3 *in vitro* studies and one *in vivo* study, reported in **EFSA Scientific Report (2005) 51, 1-78** and, more recently, the new technical material has been confirmed to be without activity in the Ames assay.

- Results from the phototoxicity study confirm that cyprodinil is not phototoxic. The mechanism for photomutagenicity is assumed to be the same as that for phototoxicity, hence the lack of activity in the phototoxicity study supports the view that cyprodinil would be inactive in a photomutagenicity assay.

    - This position is in agreement of that of a recent report by the International Workshop on Genotoxicity Testing Working Group (IWGT)[1] who concluded that data from photogenotoxicity assays provided no added value over and above that available from phototoxicity studies.

- Exposure of the skin, either directly following accidental spillage of product, or indirectly via potential systemic exposure, would be very low:

- The skin is not a target organ for cyprodinil.

Syngenta believes that, on the basis of the facts presented above, cyprodinil would not present an undue risk to light exposed skin and that a photomutagenicity study is not necessary.

[1] Lynch AM, *et al.* (2013) Considerations on photochemical genotoxicity II: Report of the 2009 International Workshop on Genotoxicity Testing Working Group Mutation Research 723 91-100.

Therefore although cyprodinil would trigger a photomutagenicity study based on the criteria laid down in **Commission Regulation (EU) No 283/2013**, Syngenta believes that such a study is not necessary. In addition, there is no published OECD guideline (or indeed any other internationally recognised guideline) for photomutagenicity testing and in the absence of a clear guideline we believe that it is inappropriate to conduct a study at this time.

The publication listed below have been taken from the open literature which was found as part of the comprehensive literature search (**M-CA Section 9**). This paper presents data from in GreenScreen HC, CellCiphr p53 and CellSensor p53RE-bla high-throughput genotoxicity screens to evaluate the use of such screens as an aid to prioritization of carcinogenicity assessment. Cyprodinil gave a negative response in all screens. Therefore, as such Syngenta does not believe that this publication can be considered to change any genotoxicity endpoint for cyprodinil.

Report:	K-CA 5.4.1/02. Knight AW, Little S, Houck K, Dix D, Judson R, Richard A, McCarroll N, Akerman G, Yang C, Birrell L and Walmsley R. (2009). Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast™ chemicals. Published paper. Regulatory Toxicology and Pharmacology 55 (2009) 188–199. Syngenta File No. NA_13830.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: This project aims to evaluate the use of *in vitro* assays for understanding the types of molecular and pathway perturbations caused by environmental chemicals and to build initial prioritization models of *in vivo* toxicity.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

Three high-throughput screening (HTS) genotoxicity assays-GreenScreen HC GADD45a-GFP (Gentronix Ltd.), CellCiphr p53 (Cellumen Inc.) and CellSensor p53RE-bla (Invitrogen Corp.) were used to analyse the collection of 320 active compounds (predominantly pesticides) being tested in Phase I of US. Environmental Protection Agency's ToxCast™ research project. Between 9% and 12% of compounds were positive for genotoxicity in the assays. However, results of the varied tests only partially overlapped, suggesting a strategy of combining data from a battery of assays. The HTS results were compared to mutagenicity (Ames) and animal tumorigenicity data. Overall, the HTS assays demonstrated low sensitivity for rodent tumorigens, likely due to: screening at a low concentration, coverage of selected genotoxic mechanisms, lack of metabolic activation and difficulty detecting non-genotoxic carcinogens. Conversely, HTS results demonstrated high specificity, >88%. Overall concordance of the HTS assays with tumorigenicity data was low, around 50% for all tumorigens, but increased to 74–78% (vs. 60% for Ames) for those compounds producing tumors in rodents at multiple sites and, thus, more likely genotoxic carcinogens. The aim of the present study was to evaluate the utility of HTS assays to identify potential genotoxicity hazard in the larger context of the ToxCast project, to aid prioritization of environmentally relevant chemicals for further testing and assessment of carcinogenicity risk to humans.

Cyprodinil is not specifically mentioned in this publication. Comparisons to Ames and tumorigenicity data have been useful to assess the relevance and quality of the results produced, positive data from these high-throughput genotoxicity assays alone are not suitable surrogates for

standard regulatory genotoxicity assays; neither can these HTS results be used in isolation as direct predictors of animal tumorigenicity.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	In ToxCast™ Database

Vehicle: Not specified. The compounds were supplied to the assay operators as frozen aliquots dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mM.

Study Design and Methods:

In-life dates: Not reported.

Data sources: The data used to develop the prioritization profiles for the 309 unique chemicals are housed in U.S. EPA's ToxMiner database, an internal repository for assay data from ToxCast.

GreenScreen HC assay: The GreenScreen HC assay uses two genetically modified TK6 cell lines: the GADD45a-GFP reporter strain (GenM-T01) and a control strain (GenM-C01) containing an out-of-frame EGFP gene, such that a functional and fluorescent GFP protein is not produced. A suspension of 2 x 10^6 cells per mL in a proprietary assay medium are added to a dilution series of the test compound, and separately to a standard genotoxicant (methylmethane sulfonate), in 96-well microplates. At 24 and 48 h time-points during incubation of the microplate at 37°C, 5% CO_2, the measurement of the induction in cellular fluorescence was indicative of genotoxicity, while the measurement of the reduction in optical absorbance, proportional to the inhibition of cell proliferation, was used to quantify general cytotoxicity, both with reference to statistically defined thresholds. Cytotoxicity was assessed by the percentage reduction in cell proliferation (relative cell density) compared to that achieved in the vehicle-treated controls.

CellCiphr Cytotox Profiling Panel-p53 endpoint: Assessment of the DNA damage response in human-derived HepG2 cells was determined by measurement of p53 activation via a fluorescent anti-p53 antibody, one of 10 phenotypic endpoints in the Cellumen CellCiphr Cytotox Profiling Panel. 10 concentrations of test compound, from 200 to 0.39 lM. HepG2 cells in log-phase growth were seeded in collagen-coated microplates at 3 cell concentrations corresponding to different exposure times, [4.3 x 10^3 (acute), 2.4 x 10^3 (early), 1.2 x 10^3 (chronic) cells/well], allowed to settle for 30 min at room temperature and incubated at 37°C, 5% CO_2 for 16 h prior to treatment. Following exposure, cells were fixed, the nucleus stained and read on an Arrayscan HCS Reader to quantitate valid objects (cells) defined by Hoescht staining and p53 activity by quantifying the amount of Alexa Fluor 488 antibody fluorescence in the area defined by the nuclear dye. Responses were measured at 3 different time-points (30 min (acute), 24 h (early) and 72 h (chronic)) for each of 10 concentrations of the test compounds (serial dilutions from 200 lM) tested in duplicate along with positive and negative controls, to collect the data todetermine the half-maximal activity (AC50). AC50 values were determined by fitting the data to the Hill equation. A positive result was concluded if the p53 AC50 was < 200 lM, provided the AC50 was lower than the IC50 for cell loss/cytotoxicity at for that time point.

Invitrogen CellSensor p53RE-bla HCT-116 assay: HCT-116 cells with a stably integrated beta-lactamase reporter gene under control of p53 response elements were suspended in OPTI medium including 0.5% dialyzed FBS, and plated onto assay plates at a density of 4000 cells/well. After incubation at 37 °C, 5% CO_2 for 6 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO alone were transferred to the assay plate resulting in a 217-fold dilution. The final compound

concentration in the 5 µL assay volume ranged from 1.2 nM to 92 lM in 15 concentrations. Nultilin-3 was used as a positive control. The plates were then incubated for a further 16 h at 37 °C, 5% CO_2. Subsequently, 1 µL of GeneBLAzerTM B/G FRET substrate was added, the plates incubated at room temperature for 2 h and and fluorescence intensity at 460 and 530 nm emission was measured with excitation at 405 nm. Data were expressed as the ratio of emissions at 460 nm/530 nm. Concentration–response titration points for each compound were fitted to the Hill equation and concentrations of half-maximal activity (AC50) and maximal response (efficacy) values were calculated. A positive result was concluded if the p53 AC50 was calculated to be below 92 lM..

Reference data

Ames test data: In order to provide a link and a fair comparison between the new *in vitro* HTS genotoxicity data generated in this project and the chronic toxicity data from rodent bioassays, results the bacterial Salmonella mutagenesis (Ames) test, were compiled from the ***Leadscope toxicity database (2008)***

Chronic toxicity data: Rodent tumorigenesis data from chronic, two-year bioassays were collated from the ToxRefDB database, as of October 2008 (***Martin et al., 2009; EPA ToxRefDB, 2009***).

RESULTS AND DISCUSSION

Cyprodinil is not specifically mentioned in the main text of this publication. However, an interrogation of the supplementary data files indicated that cyprodinil did not show any evidence of genotoxicity in GreenScreen HC, Invitrogen p53RE-bla HCT-116, or the CellCiphr Cytotox Panel.

There was poor concordance of results of the three HTPS methods. While the GreenScreen HC, CellCiphr p53 and CellSensor p53 assays produced similar numbers of positive results (32, 27 and 36, respectively) the overlap between data sets was relatively small. The number of positive results which were common to CellCiphr p53 and GreenScreen HC was 6. The number of positive results which were common to GreenScreen HC and CellSensor p53 was 9. Eleven positive compounds were detected in both the CellCiphr p53 and CellSensor p53 sets.

CONCLUSION: Comparisons to Ames and tumorigenicity data have been useful to assess the relevance and quality of the results produced, positive data from these high-throughput genotoxicity assays alone are not suitable surrogates for standard regulatory genotoxicity assays; neither can these HTS results be used in isolation as direct predictors of animal tumorigenicity.

REFERENCES

EPA ToxRefDB, 2009. US Environmental Protection Agency's National Center for Computational Toxicology ToxRefDB (Toxicology Reference Database). Available from: <http://www.epa.gov/ncct/toxrefdb>. (accessed by author January 2009).

Martin, M.T., Judson, R.S., Reif, D.M., Kavlock, R.J., Dix, D.J., 2009. Profiling chemicals based on chronic toxicity results from the US EPA ToxRef database. Environ. Health Perspect. 117, 392–399.

(Knight AW, *et al.,* 2009)

In addition, since the original Annex 1 renewal submission in October 2015, two further *in vitro* genotoxicity assays (K-CA 5.4.1/03 and K-CA 5.4.1/04) have been conducted with cyprodinil in accordance with the most current OECD guidances which were adopted in July 2016, and the results of which are presented below. Cyprodinil was considered to be non-mutagenic in these assays which are in concordance with data contained within the original EU review.

Report:	K-CA 5.4.1/03 Wollny H-E. (2017) Cyprodinil - Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1791000, 30 January 2017. Unpublished. Syngenta File No. CGA219417_11767.

~~This study is currently on-going and the final report was not available at the time of submission. The study final report and the summary will be made available as soon as possible.~~

GUIDELINES: *In Vitro* Mammalian Cell Gene Mutation Test (HPRT); OECD 476 (2016).

COMPLIANCE: A signed and dated GLP statement was provided.

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

EXECUTIVE SUMMARY

The test item cyprodinil was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. The assay was performed in one experiment, using two parallel cultures. The experiment was performed with and without liver microsomal activation and a treatment period of 4 hours.

The maximum concentration of the pre-experiment was 2000 µg/mL and was chosen with respect to the current OECD Test Guideline 476 (2016). The concentration range of the main experiment was limited by precipitation and cytotoxicity of the test item.

The main experiment was evaluated at the following concentrations:

without metabolic activation: 2.0; 4.0; 8.0; 16.0; and 24.0 µg/mL
with metabolic activation: 8.0; 16.0; 32.0; 48.0; and 64.0 µg/mL

Relevant cytotoxic effects indicated by an adjusted cloning efficiency I (RS) below 50% (mean value of both parallel cultures) occurred at 24.0 µg/mL without metabolic activation and at 64.0 µg/mL with metabolic activation. In the absence of metabolic activation a mean relative adjusted cloning efficiency I (RS) of 23% was observed. The study should aim to achieve a cytotoxic range of approximately 10% to 20% and this aim was covered without metabolic activation. In culture I, a higher cytotoxicity was not associated with an increase in mutant frequency. In the presence of metabolic activation, the maximum concentration was limited by precipitation of the test item observed at 48.0 µg/mL and above. Additionally, severe cytotoxic effects were noted at the second precipitating concentration of 64.0 µg/mL.

No relevant and reproducible increase in mutant colony numbers/106 cells was observed in the main experiment up to the maximum concentration. The mutation frequency (mean values of both parallel cultures) remained within the 95% control limit with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined with any of the experimental groups.

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

In conclusion it can be stated that under the experimental conditions reported, the test item did not induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster. Therefore, cyprodinil is considered to be non-mutagenic in this HPRT assay.

MATERIALS AND METHODS

Materials:

Test Material: Cyprodinil
Description: Solid, light beige
Lot/Batch number: P.012011
Molecular weight: 225.3 g/mol
Purity: 99.2% w/w
Retest date: 31 January 2018
Stability of test compound: Not indicated by the sponsor

Control Materials:
Negative: -
Solvent control (final concentration): DMSO (0.5 %)
Positive control: Absence of S9 mix: Ethylmethane sulfonate (EMS), 300 µg/mL = 2.4 mM
Presence of S9 mix: DMBA, 2.3 µg/mL = 8.6 µM

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others

Media: RPMI 1640				
Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No
Periodically "cleansed" against high spontaneous background?	X	Yes		No

X indicates those that apply

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ ATPase**
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	2.0; 4.0; 8.0; 16.0; and 24.0 µg/mL
Presence of S9 mix	8.0; 16.0; 32.0; 48.0; and 64.0 µg/mL

Study Design and Methods:

In-life dates: Start: 01 September 2016, End: 15 November 2016

Test performance:

Cell treatment: Cells were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

For seeding and treatment of the cell cultures the complete culture medium was 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. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

Two to three days after subcultivation stock cultures are trypsinized at 37 °C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10 % FBS and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2 % in phosphate buffered saline (PBS).

Prior to the trypsin treatment the cells are were with PBS containing 200 mg/l EDTA. Approximately 7 to 7.5×10^6 and $5x10^2$ cells were seeded in plastic flasks. The cells were grown for 24 hours prior to treatment. With the cell doubling time of approximately 12 h this ensures a population of more than 20×10^6 cells treated with the test substance.

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G".

Immediately after the end of treatment the cells were trypsinised as described above and sub-cultivated. At least 2.0×10^6 cells per experimental point (concentration series plus controls) were subcultured in 175 cm² flasks containing 30 mL medium.

Two additional 25 cm² flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (cloning efficiency I) as measure of test item induced cytotoxicity. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment as described below.

Three or four days after first sub-cultivation approximately 2.0×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium.

Following the expression time of approximately 7 days five 80 cm² cell culture flasks were seeded with about 3 to 5×10^5 cells each in medium containing 6-TG (11 µg/mL). Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

After 7 to 10 days the colonies were stained with 10% methylene blue in 0.01% KOH solution. Colonies with more than 50 cells were counted. If in doubt the colony size was checked with a preparation microscope.

Statistical Methods: A linear regression analysis (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

A t-test was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to evaluate an isolated increase of the mutation frequency at a test point exceeding the 95% control limit. Again a t-test is judged as significant if the p-value (probability value) is below 0.05.

However, both, biological and statistical significance was considered together.

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent solvent control in both parallel cultures,
b) the increase is concentration-related when evaluated with an appropriate trend test,
c) any of the results are outside the distribution of the historical solvent control data.

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

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
b) there is no concentration-related increase when evaluated with an appropriate trend test,
c) all results are inside the distribution of the historical negative control data

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

There is no requirement for verification of a clearly positive or negative response.

In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

RESULTS AND DISCUSSION

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

The main experiment was evaluated at the following concentrations:

without metabolic activation:	2.0; 4.0; 8.0; 16.0; and 24.0 µg/mL
with metabolic activation:	8.0; 16.0; 32.0; 48.0; and 64.0 µg/mL

Relevant cytotoxic effects indicated by an adjusted cloning efficiency I (RS) below 50% (mean value of both parallel cultures) occurred at 24.0 µg/mL without metabolic activation and at 64.0 µg/mL with metabolic activation. In the absence of metabolic activation a mean rel. adjusted cloning efficiency I (RS) of 23.0% (culture I - 20.2% and culture II - 25.9%, respectively) was observed. The study should aim to achieve a cytotoxic range of approximately 10% to 20% RS and this aim was covered without metabolic activation. In culture I, a higher cytotoxicity was not associated with an increase in mutant frequency. In the presence of metabolic activation, the maximum concentration was limited by precipitation of the test item observed at 48.0 µg/mL and above. Additionally, severe cytotoxic effects were noted at the second precipitating concentration of 64.0 µg/mL.

No relevant and reproducible increase in mutant colony numbers/106 cells was observed in the main experiment up to the maximum concentration.

The mutation frequency (mean values of both parallel cultures) remained within the 95% control limit with and without metabolic activation.

A linear regression analysis (least squares) was performed on the mean mutant frequencies to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

In the main experiments of this study (with and without S9 mix) the range of the solvent controls was from 10.2 to 18.6 mutants per 106 cells; the range of the groups treated with the test item was from 11.1 to 29.6 mutants per 106 cells. This range refers to individual cultures.

Based on these results, cyprodinil meets the criteria for being clearly negative in the HPRT assay according to the current OECD guideline 476 (2016).

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

CONCLUSION: In conclusion it can be stated that under the experimental conditions reported, the test item did not induce gene mutations at the HPRT locus in V79 cells of the Chinese hamster. Therefore, cyprodinil is considered to be non-mutagenic in this HPRT assay.

(Wollny H-E, 2017)

Report:	K-CA 5.4.1/04 Naumann S. (2017) Cyprodinil - Micronucleus Test in Human Lymphocytes *In Vitro*. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1790900, 29 March 2017. Unpublished. Syngenta File No. CGA219417_11780.

GUIDELINES: Micronucleus Test in Human Lymphocytes *In Vitro*. OECD 487 (2016).

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

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

EXECUTIVE SUMMARY

The test substance cyprodinil, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in four independent experiments.

In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic damage.

The highest applied concentration in this study (2000 µg/mL of the test substance) was chosen with respect to the current OECD Guideline 487.

Concentration selection of the cytogenetic experiment was performed considering the toxicity and precipitation data in accordance with OECD Guideline 487.

In Experiment IA, in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest concentration that could be evaluated. Higher concentrations could not be evaluated, because an insufficient number of binucleated cells was available for scoring due to strong cytotoxic effects. Additional, in the absence of S9 mix the CBPI could not be determined because an insufficient number of cells was present on the slides. The experiments were repeated with a more refined range of concentrations.

In Experiment IB without S9 mix, again no concentrations showing cytotoxicity could be evaluated because of insufficient numbers of binuclear cells present. The cytotoxicity was so strong that the CBPI could not be determined because an insufficient number of cells was present on the slides. The concentration range was further refined in Experiment IC. In Experiment IC in the absence of S9 mix, the highest concentration was restricted by precipitation at moderate cytotoxic levels.

In Experiment IB with S9 mix and in Experiment IIB in the absence of S9 mix, cytotoxicity was observed at the highest concentration evaluated.

In the absence and presence of S9 mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test substance.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

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

Therefore, cyprodinil is considered to be non-mutagenic in this in vitro micronucleus test, when tested up to cytotoxic or precipitating or the highest evaluable concentrations.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	Solid, light beige
Lot/Batch number:	P.012011
Purity:	99.2% w/w, no correction for purity was made
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: MMC, 1.0 µg/mL (Exp. IA, IB, IC), Absence of S9 mix: Demecolcin, 75 ng/ml (Exp. II) Presence of S9 mix: Cyclophosphamide 15.0 µg/mL (Exp. IA), 12.5 µg/mL (Exp. IB)

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment 1A Experiment 1C Experiment 2B	7.7, 13.5, 23.7, 41.5 µg/mL 45.7, 48.0, 50.4, 52.9 µg/mL 22.8, 45.7, 47.9, 50.3 µg/mL
Presence of S9 mix	Experiment 1A Experiment 1B	13.5, 23.7, 41.5, 72.6 µg/mL 40.4, 52.5, 68.3, 88.8 µg/mL

Study Design and Methods:

In-life dates: Start: 21 September 2016, End: 30 January 2017

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. The pre-test was performed with 11 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

Cytogenetic Assay:

Cell exposure time:		**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix	Experiment 1	4h	4h	4h
+ S9 mix		4h	4h	4h
- S9 mix	Experiment 2	20h	20h	20h

Cytokinesis block:	Cytochalasin B (4 µg/mL)

Cell harvest time after termination of treatment:	**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix (4 hour treatment)	36h	36h	36h
+ S9 mix (4 hour treatment)	36h	36h	36h
- S9 mix (20 hour treatment)	0h	0h	0h

Details of slide preparation

Pulse exposure
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

Preparation and analysis of cells: The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were washed and fixated. The slides were added to a microscope slide and stained with Giemsa. Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area.
1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criteria. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Evaluation criteria: The percentages of micronuclei in binucleate cells were calculated for each treatment scored. The data have been interpreted as follows:

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

– None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
– There is no concentration-related increase
– The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

– At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
– The increase is concentration-related in at least one experimental condition
– The results are outside the range of the laboratory historical solvent control data

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

Statistical analysis: Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using the validated R Script CHI2.Rnw for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: A pre-experiment to evaluate the cytotoxicity of the test item was performed. The pre-experiment is reported as the main Experiment I since the criteria mentioned under Acceptability of the assay were met.

Cytogenetic assay: The test substance cyprodinil, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Five independent experiments were performed. In Experiment IA, IB and IC, the exposure period was 4 hours with and without S9 mix, respectively. In Experiment IIA and IIB, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analysed. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration. To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in Experiment IA, 2000 µg/mL was chosen with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

Precipitation was observed at the end of treatment as follows:
Experiment IA: 72.6 µg/mL (without S9 mix)
127 µg/mL (with S9 mix)
Experiment IB: 52.5 µg/mL (without S9 mix)
115 µg/mL (with S9 mix)
Experiment IC: 52.9 µg/mL (without S9 mix)

In Experiment IIA and IIB, no precipitation was observed.

No relevant influence on the osmolarity and pH was observed.

In Experiment IA, in the absence and presence of S9 mix, no cytotoxicity was observed up to the highest concentration that could be evaluated. Higher concentrations could not be evaluated, because an insufficient number of binucleated cells was available for scoring due to strong cytotoxic effects. Additional, in the absence of S9 mix the CBPI could not be determined because an insufficient number of cells was present on the slides. The experiments were repeated with a more refined range of concentrations.

In Experiment IB without S9 mix, again no concentrations showing cytotoxicity could be evaluated because of insufficient numbers of binuclear cells present. The cytotoxicity was so strong that the CBPI could not be determined because an insufficient number of cells was present on the slides. The concentration range was further refined in Experiment IC. In Experiment IC in the absence of S9 mix, the highest concentration was restricted by precipitation at moderate cytotoxic levels.

In Experiment IB with S9 mix and in Experiment IIB in the absence of S9 mix, cytotoxicity was observed at the highest concentration evaluated.

In this study, in none of the experiments an increase in the number of micronucleated cells outside the 95% control limits was observed after treatment with the test substance. None of the increases were statistically significant. The outcome of the study is clearly negative.

Demecolcin (75 ng/mL), MMC (1.0 µg/mL) or CPA (15.0 or 12.5 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei.

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

Therefore, cyprodinil is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic, precipitating or the highest evaluable concentrations.

(Naumann S, 2017)

CA 5.4.2 *In vivo* studies in somatic cells

Please refer to original EU review. ~~No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.~~

The mouse micronucleus assay with cyprodinil conducted by Ceresa (1990) was conducted prior to the 2014 revision of OECD Test Guideline 474 which implies the requirement to verify exposure of the test item to the bone marrow, the study conducted by Ceresa (1990) was in compliance with the version of the OECD 474 test guideline in place when the study was conducted. It should be noted , "if there is evidence that the test substance(s) or its metabolite(s) will not reach the target tissue it may not be appropriate to use this test", (OECD 474, 2014).

In the Ceresa (1990) study no analytical verification of proof of exposure to the bone marrow was carried out, in addition the PCE/NCE ratio was unaffected by treatment with cyprodinil and no mortality or clinical observations consistent with systemic exposure were observed. Therefore, in order to demonstrate exposure of the test material to the bone marrow in the study conducted by Ceresa (1990) a proof of exposure study in the mouse is currently being conducted by Syngenta.

Report:	K-CA 5.4.2/01 Fincher K. (2016) Cyprodinil - Oral (Gavage) Proof of Exposure in the Mouse. Sequani Limited, Bromyard Road, Ledbury, Herefordshire, HR8 1LH, United Kingdom. Laboratory Report No. BFI0514, 26 September 2016. Unpublished. Syngenta File No. CGA219417_11759.

GUIDELINES: Proof of exposure to support a mouse micronucleus test; OECD 474 (1983).

COMPLIANCE: A signed and dated GLP statement was provided.

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: The mouse is a suitable rodent species for toxicity testing and is one of the species recommended in relevant regulatory guidelines for *in vivo* micronucleus studies and was also the species used in the previously conducted regulatory mouse micronucleus study (Ceresa, 1990).

EXECUTIVE SUMMARY

The purpose of the study was to demonstrate proof of exposure in the mouse after oral (gavage) administration of cyprodinil. A proof of exposure experiment was required to support a previously conducted regulatory mouse micronucleus study in order to demonstrate that the bone marrow was exposed to cyprodinil.

Three male and three female mice of the CRL: CD-1 (ICR) strain were dosed once with 2000 mg/kg cyprodinil, at a dose volume of 20 mL/kg body weight as a suspension in 0.5% carboxymethylcellulose.

All animals were observed from the start of treatment and body weights were recorded on Days 1 and 2. Blood samples were taken from all animals on Day 1 at 1, 4 and 24 hours after dosing for bioanalysis. All animals were killed following their final blood sample and discarded without necropsy.

There were no deaths and no clinical signs were observed throughout the study. Exposure to cyprodinil was confirmed in all blood samples.

A single oral (gavage) administration of cyprodinil at 2000 mg/kg was well tolerated in all animals. Proof of exposure was demonstrated by detectable blood concentrations of cyprodinil in male and female Crl:CD-1 mice.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	Light to beige crumbs
Lot/Batch number:	P.012011
Purity:	99.2 % w/w
CAS#:	Not available
Stability of test compound:	31 January 2018

Control Materials:

Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	0.5 % carboxymethylcellulose	**Final Volume:** 20 mL/kg	**Route:** Oral
Positive control :	N/A	**Final Doses:** N/A	**Route:** N/A

Test Animals:

Species	Mouse
Strain	Crl:CD-1
Age/weight at dosing	Four to five weeks/28 to 33 g (males) and 21 to 23 g (females)
Source	Charles River (UK) Limited, Margate, Kent, CT9 4LT, England.
Housing	3/cage
Acclimatisation period	6 days
Diet	LabDiet® 5L0S EU Rodent Diet, *ad libitum*
Water	Mains water *ad libitum*
Environmental conditions	Temperature: 19-23°C Humidity: 40-70% Photoperiod: 12 hours dark/12 hours light

Test compound administration:

Dose Level	Final Volume	Route
2000 mg/kg bw	20 mL/kg	Oral gavage

Study Design and Methods:

In-life dates: Start: 08 June 2016 End: 09 June 2016

Experimental design:

Group	Number of males	Number of females	Dose level (mg/kg) Cyprodinil	Dose concentration (mg/mL)
1	3	3	2000	100

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

Blood sampling: Blood samples (80 µL) were taken from the lateral tail vein, into tubes containing K_2EDTA anticoagulant. All animals were sampled 1, 4 and 24 hours after dosing on Day 1. Immediately following collection of each sample, 50 µL of whole blood (residual blood was discarded), was accurately measured into a polypropylene tube containing 50 µL of deionised water (for Male 2 at one hour after dosing, 25 µL of whole blood was accurately measured into a polypropylene tube containing 25 µL of deionised water). The samples were thoroughly mixed keeping upright and then placed directly on dry ice and were stored frozen (≤-70 °C) until analysis.

Bioanalysis: Cyprodinil was recovered from mouse blood:water and quantitatively analysed by LC-MS/MS. The presence of cyprodinil was confirmed by analysis of the study samples alongside samples of blank matrix and matrix spiked with cyprodinil.

RESULTS AND DISCUSSION

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

Exposure to cyprodinil was confirmed in all blood samples. Since bone marrow is well perfused, exposure of the marrow to the test item was indirectly demonstrated by the presence of measurable quantities of test item in the blood.

CONCLUSION: A single oral (gavage) administration of cyprodinil at 2000 mg/kg was well tolerated in all animals. Proof of exposure was demonstrated by detectable blood concentrations of cyprodinil in male and female Crl:CD-1 mice.

(Fincher K, 2016)

CA 5.4.3 *In vivo* studies in germ cells

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.5 Long-Term Toxicity and Carcinogenicity

Following repeated oral administration of cyprodinil, no evidence for cumulative toxicity was seen in the rat and the mouse. Non-specific effects such as reduced body weight development and/or food consumption were observed in both species. The liver was the main target organ for cyprodinil. At the highest doses increased liver weights in both species and degenerative changes in the rat were observed. Additionally, a slight increase in kidney weight was also noted.

Neither in the rat nor in the mouse were any changes in the incidence and distribution of neoplastic lesions by cyprodinil treatment detected. Cyprodinil did not induce any carcinogenicity in both species after lifelong exposure. The results are summarised in the following table:

Table 5.5-1: Summary of Long-Term Toxicity and Carcinogenicity Studies

Study Type	Dose Levels	NOEL	Major effects at LOAEL	Reference
2 Year Chronic Toxicity and Carcinogenicity Rat	0, 5, 75, 1000, 2000 ppm (0.177, 2.70, 35.6 and 73.6 mg/kg bw/day in males and 0.204, 3.22, 41.2 and 87.1 mg/kg bw/day in females).	NOEL: 75 ppm (2.70 mg/kg bw/day in males and 3.22 mg/kg bw/day in females).	Liver: increased relative weight and degenerative changes; Kidney: increased relative weight.	Fankhauser, 1994b*
18 Month Carcinogenicity Mouse	0, 10, 150, 2000 and 5000 ppm (1.15, 16.1, 212.4 and 629.9 mg/kg bw/day in males and 1.08, 14.7, 196.3 and 558.1 mg/kg bw/day in females).	NOEL: 2000 ppm (212.4 mg/kg bw/day in males and 196.3 mg/kg bw/day in females).	Reduced body weight development; Liver: increased relative weight; Kidney: increased relative weight (females only).	Fankhauser, 1994a*

*Included in original EU review.

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.6 Reproductive Toxicity

No effects of cyprodinil on reproduction were detected in a 2-generation study in the rat. Unspecific toxic symptoms such as reduced body weight development and/or food consumption were observed among the parental animals and the pups at the highest dose. Additionally increased liver and kidney weight was noted. Histopathological changes in these organs were limited to the high dose animals.

There was no indication of a developmental toxicity potential of cyprodinil detected in the rat or the rabbit. Maternal toxicity was observed in the highest dose levels in both species manifesting mainly as reduced body weight development and food consumption. Reduced foetal weight and some indications for delayed ossification were observed among the high dose rat foetuses, which are considered to be related to the marked maternal toxicity at this dose level. No significant embryotoxic effects were observed in the rabbit study. Cyprodinil was not developmental toxic in the rat and the rabbit and had no adverse effects on the reproduction of rats. The results are summarised in the following table:

Table 5.6-1: Summary of Developmental and Reproductive Toxicity Studies

Study Type	Dose Levels	NO(A)EL	Major effects at LOAEL	Reference
2-Generation Reproductive Toxicity Rat	0, 10, 100, 1000 and 4000 ppm (0.5 to 0.9, 5.2 to 8.4, 51.0 to 144.6 and 217.7 to 377.5 mg/kg bw/day in males and 0.7 to 1.6, 6.9 to 16.0, 70.6 to 153.5 and 292.1 to 633.5 mg/kg bw/day in females)	NOEL: 100 ppm 5.2-8.4 mg/kg bw/day in males 6.9-16 mg/kg bw/day in females NOAEL:1000 ppm 51.0-144.6 mg/kg bw/day in males 70.6-153.5 mg/kg bw/day in females	Paternal: reduced body weight development; increased relative liver and kidney weight (increased tubular basophilia in male kidney). Pups: reduced body weight development.	Khalil, 1993*
Developmental Toxicity Rat	0, 20, 200 and 1000 mg/kg bw/day	Maternal NOEL: 200 mg/kg bw/day Foetal NOEL: 200 mg/kg bw/day	Maternal: reduced body weight development and food consumption; Foetal: decreased body weight and delay of ossification	Marty, 1991a *
Developmental Toxicity Rabbit	0, 5, 30, 150 and 400 mg/kg bw/day	Maternal NOEL: 150 mg/kg bw/day Foetal NOEL: >400 mg/kg bw/day	Maternal: reduced body weight development and food consumption; Foetal: none	Marty, 1991b *

*Included in original EU review.

CA 5.6.1 Generational studies

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.6.2 Developmental toxicity studies

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

The publication listed below has been taken from the open literature which was found as part of the comprehensive literature search (**M-CA Section 9**). This paper describes use of existing *in vivo* data on cyprodinil to help build a predictive model for developmental toxicity. Therefore, as no new data is presented Syngenta does not believe that this publication is relevant for the assessment of cyprodinil. During the RMS review of cyprodinil it was requested that a summary of this publication be presented.

Report:	K-CA 5.6.2/01. Sipes N, Martin M, Reif D, Kleinstreuer N, Judson R, Singh A, Chandler K, Dix D, Kavlock R, and Knudsen T (2011). Predictive models of prenatal developmental toxicity from ToxCast high-throughput screening data. Published paper. *Toxicological Sciences.* 124(1), 109-127. Syngenta File No. NA_13834.

KLIMSCH RELIABILITY SCORE: Not applicable.

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: To test the hypothesis that developmental toxicity in guideline animal studies captured in the ToxRefDB database would correlate with cell-based and cell-free *in vitro* high-throughput screening (HTS) data to reveal meaningful mechanistic relationships and provide models identifying chemicals with the potential to cause developmental toxicity. The work is intended to indicate the utility of HTS assays for developing pathway-level models predictive of developmental toxicity.

EXECUTIVE SUMMARY

EPA's ToxCast project is profiling the *in vitro* bioactivity of chemicals to assess pathway-level and cell-based signatures that correlate with observed *in vivo* toxicity. The developmental toxicity in guideline animal studies captured in the ToxRefDB database was hypothesised to correlate with cell-based and cell-free *in vitro* high-throughput screening (HTS) data to reveal meaningful mechanistic relationships and provide models identifying chemicals with the potential to cause developmental toxicity. To test this hypothesis, a statistical association was built based on HTS and *in vivo* developmental toxicity data from ToxRefDB. Univariate associations were used to filter HTS assays based on statistical correlation with distinct *in vivo* endpoint. This revealed 423 total associations with distinctly different patterns for rat and rabbit across multiple HTS assay platforms. From these associations, linear discriminant analysis with cross-validation was used to build the models. Species-specific models of predicted developmental toxicity revealed strong balanced accuracy (> 70%) and unique correlations between assay targets such as transforming growth factor beta, retinoic acid receptor, and G-protein– coupled receptor signalling in the rat and inflammatory signals, such as interleukins (IL) (IL1a and IL8) and chemokines (CCL2), in the rabbit. Species-specific toxicity endpoints were associated with one another through common Gene Ontology biological processes, such as cleft palate to urogenital defects through placenta and embryonic development. This work indicates the utility of HTS assays for developing pathway-level models predictive of developmental toxicity.

This work indicates the utility of HTS assays for developing pathway-level models predictive of developmental toxicity.

MATERIALS AND METHODS

Test Material:	Cyprodinil (no further information)

Vehicle: Not applicable

Study Design and Methods:

In-life dates: Not reported.

ToxCast Phase I chemical library: Phase I of ToxCast employed a chemical library containing 309 unique structures and some replicates for internal quality control (QC). The rationale for chemical selection was based on several criteria, including available *in vivo* data from a chronic/cancer, multigenerational/reproductive, or prenatal developmental study (95% of compounds meet these criteria); soluble in dimethyl sulfoxide (97.5% meet this criteria); molecular weight range 250–1000 (90% meet this criteria); and commercially available with purity > 90% (98% meet this criteria). These criteria were largely satisfied with pesticide active ingredients that have had guideline *in vivo* toxicology studies conducted as part of their registration process with the U.S. EPA.

Data sources: The data used to link in vivo developmental defects with in vitro chemical profiles are available in ToxCastDB (actor.epa.gov/actor/faces/ToxCastDB/Home.jsp), which contains information from both the ToxRefDB *in vivo* guideline studies and ToxCast in vitro assay results.

Univariate associations for in vivo developmental endpoint categories: To address simple univariate (assay to categorical endpoint) associations, individual ToxRefDB *in vivo* developmental endpoints were analyzed for their association with individual ToxCast in vitro assays.

General rat and rabbit developmental toxicity models: Separate predictive models for rat and rabbit developmental toxicity were built. The first step was the univariate assay set selection and the second step was assay set aggregation and reduction.

Data visualization: For cross-species comparison of developmental toxicity models, the predictive models scored and ranked the chemicals based on their activity in the composite *in vitro* assay set, defined for each species.

Endpoint–GO biological process map: GO biological processes were compared with the *in vivo* categorical endpoints using similar univariate association methods to understand the relationship between endpoints and their biological significance. ToxCast HTS assays were mapped to distinct genes where possible.

RESULTS AND DISCUSSION

Predictive modelling of potential *in vivo* developmental toxicity from *in vitro* data is a complex problem that is confounded by many factors such as chemical perturbations of key developmental pathways, intracellular and tissue-level crosstalk, xenobiotic metabolism, and chemical solubility and partitioning. This study is the first attempt to construct predictive models of developmental toxicity based on broad spectrum profiling of biological activity in HTS assays. Results of this study demonstrate the following findings: (1) individual species-specific models are necessary for predicting developmental toxicity in pregnant rats and rabbits, (2) plausible cellular targets and pathways can be linked to specific endpoint toxicity, (3) toxicity endpoints cluster together based on similar biological process associations indicating potential similarities in developmental stage or processes, (4) xenobiotic metabolism plays a role in developmental toxicity, (5) there is no clear trend between *in vivo* chemical dose and assay characteristics, and (6) this analysis demonstrates the capability of using HTS assays to predict developmental toxicity. Taken together, these data indicate for the first time that ToxCast HTS of a large number of compounds can produce *in vitro* bioactivity profiles that can predict, with a BA of over 70%, *in vivo* developmental toxicity potential.

The Phase I chemical library data set was run through each species model for ranking, and these rankings were then visualized as ToxPi. The model score for each chemical was based on the in vitro activity profile from the HTS assays of the selected composite assay set for each model. Higher scoring chemicals are predicted to be more likely developmentally toxic than chemicals with lower ranking scores. The top left ToxPi is the chemical predicted to most likely be a developmental toxicant for the given species, whereas the bottom most ToxPi is the least likely. Optimizing the model through LDA gave the cutoff values for developmental toxicity in species-specific models, which are shown as black bars in the figure. Red-yellow and blue slices of ToxPi indicate positive and negative assay set predictors, respectively. Chemicals with the highest activity among positive and negative predictors are ranked highest and lowest, respectively. Chemicals in the middle either have little activity for the given assay sets or had near equal activity in both positive and negative predictors.

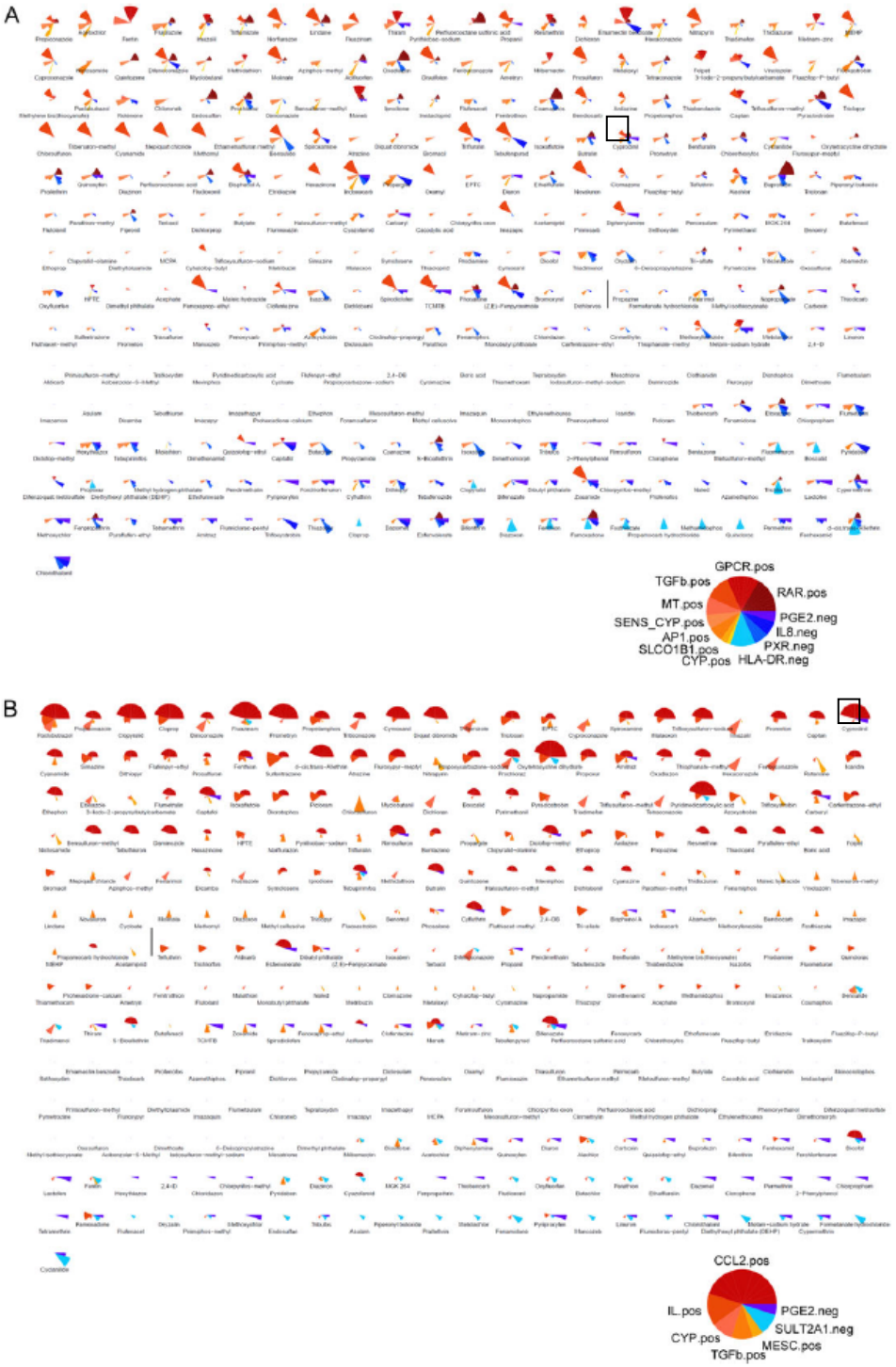

FIG. 6. Cross-species comparison of developmental toxicity predictions. ToxPis (toxicity prioritization index) indicate a graphical representation of predicted developmental toxicity across all 309 unique ToxCast chemicals. (A) The eight positive and four negative predictors (indicated by red-yellow and blue pie slices, respectively) were used as inputs, along with their respective weighting factors for the rat developmental toxicity model. (B) The five positive and two negative predictors (indicated by red-yellow and blue pie slices, respectively) were used as inputs, along with their respective weighting factors for the rabbit developmental toxicity model.

CONCLUSION: This work indicates the utility of HTS assays for developing pathway-level models predictive of developmental toxicity.

(Sipes N, *et al.*, 2011)

CA 5.7 Neurotoxicity Studies

Cyprodinil does not belong to a chemical class, which is suspected to cause delayed neurotoxic effects (organophosphates, carbamates). In addition, no indication for a neurotoxic potential was observed in acute and subchronic neurotoxicity studies performed in agreement with OECD 424 (details see below). Therefore, specific studies on delayed neurotoxicity were not deemed to be necessary.

Three acute neurotoxicity studies were performed with cyprodinil in the rat: one complete study preceded by a range-finding study and followed by a third study with a limited set of parameters to invest whether a slight variation in body temperature observed in the second study was related or unrelated to treatment. In addition, a 90-day neurotoxicity study was performed in the rat.

In the acute neurotoxicity studies at the time of peak effect at app. 2 hours after oral administration of cyprodinil low motor activity and hypothermia was observed along with other signs of systemic toxicity (hunched posture, piloerection) at doses ≥600 mg/kg bw. In one of the acute neurotoxicity studies (Classen, 1997a) hunched posture (5/10 females) and a minimal reduction in body temperature in males were also observed in 200 mg/kg bw treated animals. Hunched posture was not observed in the other acute neurotoxicity studies (Classen, 1996 and 1998) at the same or similar dose levels (200 and 300 mg/kg bw/day). As the occurrence of hunched posture in Classen, 1997a was obviously not reproducible and as clinical signs generally underlie certain subjectivity, it was considered to be incidental and not an adverse effect. The minimal decrease in body temperature in the 200 mg/kg bw males (Classen, 1997a) was also not reproducible in a second study (Classen, 1998). In addition to the only minimal extent of this effect at 200 mg/kg bw in Classen, 1997a, it was therefore not considered to be an adverse effect. The results are summarised in the following table:

Table 5.7-1: Summary of Neurotoxicity Studies

Study Type	Dose Levels	NO(A)EL	Major effects at LOAEL	Reference
Acute Neurotoxicity (Range Finding) Rat	0, 300, 1000 and 2000 mg/kg bw	NOEL (system.): 300 mg/kg bw NOEL (neurotox.): >2000 mg/kg bw	Clinical signs of systemic toxicity.	Classen, 1996*
Acute Neurotoxicity Rat	0, 200, 600 and 2000 mg/kg bw	NOAEL (system.): 200 mg/kg bw NOEL (neurotox.): >2000 mg/kg bw	Clinical signs of systemic toxicity; reduced body temperature.	Classen, 1997a*
Acute Neurotoxicity (Neurobehavioral and Temperature Assessment)	0, 20, 60 and 200 mg/kg bw	NOEL: 200 mg/kg bw	None (no decrease in body temperature).	Classen, 1998*
90 Day Dietary Neurotoxicity Rat	0, 200, 800 and 8000 ppm (5.81, 54.5, 601 mg/kg bw/day for males and 6.34, 58.7, 631 mg/kg bw/day for females).	NOEL (system.): 800 ppm (54.5 and 58.7 mg/kg bw/day for males and females, respectively). NOEL (neurotox.): >8000 ppm (601 and 631 mg/kg bw/day for males and females, respectively).	Reduced body weight development and food consumption; Liver: increased weight, hepatocellular hyper-trophy; Kidney: increased weight (females only), chronic tubular lesion and tubular casts; Thyroid gland: follicular epithelium hypertrophy.	Classen, 1997b*

*Included in original EU review.

The publication listed below has been taken from the open literature. 1 paper (K-CA 5.7/01) was found as part of the comprehensive literature search (**M-CA Section 9**).

Report:	K-CA 5.7/01. Coleman M., O'Neil J., Woehrling E., Ndunge O., Hill E., Menache A. and Reiss C. (2012). A preliminary investigation into the impact of a pesticide combination on human neuronal and glial cell lines *in vitro*. Published paper PLoS ONE 7(8): e42768. doi:10.1371/journal.pone.0042768. Syngenta File No. NA_13832.

KLIMSCH RELIABILITY SCORE: 3 (Not reliable)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: There has been little investigation into the possible synergistic biological effects on human neuronal and glial cellular systems considering the use of many combinations of biocides, their known persistence through foodstuff processing and their potential neurological human impact. In this study, two cell lines have been used, gliomal U251 and neuroblastomal SH-SY5Y cells, which model the basic cell types of the human central nervous system, the astrocytes and neurones. The effects of a combination of three commonly used biocides (pyrimethanil, ciprodinil and fludioxonil) on cell viability, mitochondrial health and generation of oxidative stress, alone and in combination have been investigated.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil and cyprodinil combinations are reported within this summary.

Many pesticides are used increasingly in combinations during crop protection and their stability ensures the presence of such combinations in foodstuffs. The effects of three fungicides, pyrimethanil, cyprodinil and fludioxonil, were investigated together and separately on U251 and SH-SY5Y cells, which can be representative of human CNS glial and neuronal cells respectively.

Over 48h, all three agents showed significant reductions in cellular ATP, at concentrations that were more than tenfold lower than those which significantly impaired cellular viability. The effects on energy metabolism were reflected in their marked toxic effects on mitochondrial membrane potential.

In addition, evidence of oxidative stress was seen in terms of a fall in cellular thiols coupled with increases in the expression of enzymes associated with reactive species formation, such as GSH peroxidase and superoxide dismutase. The glial cell line showed significant responsiveness to the toxin challenge in terms of changes in antioxidant gene expression, although the neuronal SH-SY5Y line exhibited greater vulnerability to toxicity, which was reflected in significant increases in caspase-3 expression, which is indicative of the initiation of apoptosis.

Cyprodinil was the most toxic agent individually, although oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents.

Conclusion: Cyprodinil was the most toxic agent individually, although oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents. The impact of some pesticides, both individually and in combinations, merits further study in terms of their impact on human cellular health.

MATERIALS AND METHODS

Materials:

Test Materials: Cyprodinil (no details reported)
Pyrimethanil (no details reported)
Fludioxonil (no details reported)

Vehicle: Dimethylsulfoxide

Study Design and Methods:

In-life dates: Not reported.

Study design: The pesticides were dissolved completely in stock DMSO solutions and serially diluted in media prior to addition to the cells. Cyprodinil was studied singly in the various assays and in combination with the other pesticides across a concentration range of 0–1 mM. in 100 mL phenol red free DMEM PenStrep solution, in triplicate wells. Cells were incubated with increasingly cytotoxic concentrations of pesticides with the aim of the calculation of IC_{50} values for the various assays. In the combination studies, all three agents were added so that the same net weight of pesticide was present in the incubations as those containing cyprodinil alone. The vehicle controls contained DMSO alone.

Note that all the following experiments were conducted on three separate occasions in quadruplicate.

Cell Viability Assay in the presence of Pesticides: Effects on SHSY5Y and U251 cell viability were evaluated through the CellTiter Blue™ (resazurin assay; Promega), singly and in combinations. Untreated negative controls were run together with the treated cells. Reciprocal absorbance values were calculated and the percent viability was expressed as the value in the presence of toxicant as a percentage of that in the medium only control (set as 100%).

JC-1 Mitochondrial membrane potential assay: Following a 48 hour exposure to the test chemicals, the cell culture medium in each well was completely replaced with phenol red free DMEM containing 5 µM JC-1and incubated. The medium was removed, cells washed three times with PBS and 100 mL PBS added to each well. The red substrate fluorescence intensity was read at 544 nm/590 nm (excitation/emission) and the results were expressed as a percentage of the fluorescence value from the medium only control well (set as 100%).

Total glutathione assay: Following a 48 hour exposure to increasingly cytotoxic concentrations of test chemical, the cell culture medium in each well was completely replaced with 5% (w/v) sulfosalicylic acid (SSA) solution and the plates subjected to three freeze-thawing steps. A 5 U/mL GSR enzyme solution was prepared. Aliquots from each SSA treated well were removed , an NADPH/DTNB working solution added ,followed by incubation at 37°C for 15 min. GSR enzyme solution was added, the plate incubated and the A_{405} measured. The total glutathione content of samples was determined from standard curves generated with known amounts of GSH using the same procedure and expressed as a percentage of the medium only control well (set as 100%).

ATP Assay: Following a 48 hour exposure increasingly cytotoxic concentrations of test chemical, ATP levels were determined using the ATP luminescence assay kit from Invitrogen. The ATP content of samples was determined from standard curves generated with known amounts of ATP using the same procedure and expressed as a percentage of the medium only control well (set as 100%).

Real-Time PCR: Three enzymes that are strongly associated with oxidative stress, (caspase-3, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx), were studied using qRT-PCR.

Total RNA was extracted , reverse transcribed and Real-Time PCR: cDNAs were amplified in a PCR reaction using optimised sequence specific primers for caspase-3, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) , 3 enzymes strongly associated with oxidative stress.

GAPDH was selected as a stably expressed gene for normalisation of test gene expression after GeNorm analysis. The comparative CT method was used to calculate the relative quantification of gene expression.

Statistics: Fold changes in gene expression using the comparative CT method and statistical analysis were determined using the Relative Expression Software Tool (REST 2009, www.qiagen.com).

RESULTS AND DISCUSSION

CellTiter Blue™ results: Both cell lines showed similar levels of sensitivity with regard to the combination of pesticides, as well as to cyprodinil alone. For both cell lines, the combination was the most toxic. With the JC-1 assay, in both cell lines, cyprodinil and the combination were the most toxic ($P<0.001$). Cyprodinil showed an extremely potent reduction in ATP levels, which was approximately ten-fold greater than their effects in the CellTiter Blue™ viability assay ($P<0.001$). ATP reductions for cyprrodinil were significantly different with regard to cell line ($P<0.05$) than those of pyrimethanil in the SHSY5Y cells. The pattern of toxicity of the pesticide combination, with respect to the mitochondrial effects (JC-1 and ATP assays) differed markedly between the neuronal and glial cell lines. Whilst the viability, ATP and JC-1 data assumed an approximately linear increase in toxicity, the U251 cells appeared to demonstrate a more sigmoidal appearance in terms of the ATP and JC-1 data, with the glial lines exhibiting apparently greater resistance to mitochondrial membrane attrition compared with the SH-SY5Y cells ($P<0.001$).

Studies on GSH cellular content indicated that the presence of cyprodinil caused significant reductions in both cell lines at low agent concentrations. However, the pesticide combination showed significant reductions in GSH at low and high agent concentrations in both cell lines ($P<0.001$). Cyprodinil at 62.5 mM appeared to show a net increase in GSH levels with respect to control ($P<0.01$). Overall, with the U251 cells even at the highest concentrations tested, GSH levels did not actually fall to 50% so an IC_{50} could not be calculated. In contrast, in terms of thiol depletion, the SHSY5Y cells were more vulnerable than the glial cell line. IC_{50}'s for thiol depletion for the values for combination (476.7+/234.6 mM) as well as cyprodinil alone (535.8+/254.8 mM) were not significantly different from each other, but they were significantly lower than the values for pyrimethanil and fludioxonil ($P<0.001$).

qPCR gene changes: There was a marked difference between the two cell lines investigated, with the U251 cells demonstrating the most significant responses to the toxicants.

U251 cells: Cyprodinil at both concentrations resulted in a marked decrease in the expression of GSHPx ($P<0.05$). In contrast, SOD expression was elevated to varying degrees by both concentrations, with a 19.93-fold increase with the higher concentration of cyprodinil ($P<0.05$).

Caspase-3 expression was enhanced by 11.53-fold in the presence of 500 mm cyprodinil ($P<0.05$).

The high dose of the compounds in combination resulted in a large increase in the expression of GSHPx, SOD and caspase-3 by 28.54, 18.46 and 13.21-fold, respectively.

SH-SY5Y cells: A 19.79 fold increase was recorded in SOD expression in the neuronal line in the presence of the combination ($P<0.05$). In the neuronal cells, a significant 5.3-fold increase in caspase-3 expression was also recorded ($P<0.05$). GSHPx expression was also found to be elevated in the presence of the higher concentration of the combination (5.12-fold, not statistically significant).

Table 5.7-2: Respective IC_{50} values (mM) after exposure of U251 and SH-SY5Y cell lines to cyprodinil and cyprodinil, fludioxonil and pyrimethanil in combination using three viability assays (CellTiter Blue™, JC-1 and ATP).

Assay	Pesticide	U251 (µM IC_{50})	SH-SY5Y (µM IC_{50})
CellTiter Blue	cyprodinil	332.3 b**, c*** &	304.4 b***, c***
	combination	257.2	269.9 b***
JC-1	cyprodinil	203.7	40.5
	combination	273.6	63.9
ATP	cyprodinil	28	33 @
	combination	30.9	39.3

* P<,0.05, **P<0.001, ***P<,0.001.
b significant difference between respective CellTiter Blue™ and JC-1 IC_{50} values.
c significant difference between respective CellTiter Blue™ and ATP IC_{50} values.
@ P<0.05.
& P<0.001

Pesticide effects on energy metabolism: The toxicity of cyprodinil towards the cell lines appeared to be directly linked with their potent impact on ATP production; this was in turn connected with their detrimental effects on the mitochondrial membrane potential, as indicated by the JC-1 assay. ATP production has a critical effect on cell viability and whether cell death is either necrotic or apoptotic and it is strongly dependent on the level of cellular ATP depletion (***McConkey, 1998***). Cyprodinil is sufficiently lipophilic to penetrate the human blood brain barrier.

In the U251 cell line, cyprodinil directly impaired the expression of a major antioxidant defence enzyme (GSHPx) at low and high concentrations, whilst negatively affecting mitochondrial performance, an effect that should in itself lead to an increase in GSHPx expression. This impact on GSHPx occurred at cyprodinil concentrations when thiol levels were increased and this may be linked with the absence of thiol demand that resulted from the fall in GSHPx expression. Although the pesticides caused some cellular response to increases in reactive species to occur, such as the increase in SOD expression, in both cell lines, the suppression of GSHPx expression in this report suggests that in the U251 line, cell defence could potentially be partially undermined by the pesticides. This effect was not apparent in the neuronal cells.

Pesticide effects on Caspase-3 and detoxification response: Cyprodinil caused caspase-3 activation and the combination of pesticides with the U251 line showed very significant increases in the three key protective enzyme systems, GSHPx, SOD and caspase-3. Whilst this suggests that the combination of these agents was responsible for sufficient oxidative stress generation to elicit appropriate cellular defence gene responses in the U-251 cells, such a response to the combination was not evident in the SH-SY5Y line. At higher pesticide concentrations, it was apparent that thiol levels were also eroded in both cell lines and it is likely that this was a consequence of the combination of oxidative stress, attenuation of the ATP supply and mitochondrial toxicity. Although high consumption of thiols usually triggers a 'thermostatic' increase in thiol synthesis, this requires both reducing power and ATP to recycle GSH through GSSG reductase and *de novo* synthesis. The impact of the pesticides on energy metabolism and antioxidant protection may have amplified the toxic effects of the pesticides alone and in combination.

Context of findings in terms of modes of action and application: The toxicity of cyprodinil and the combination of fungicides seen in this study may be linked with commonality in basic cellular function between mammalian and other target species and this may have significantly eroded these agents' fungal specificity. It is known that concerning mitochondrial structure and function, there are many similarities between mammalian and fungal systems (***Ragan, 1987***). In addition, a recent report has shown cyprodinil

to inhibit human erythrocytic SOD, an effect that may also promote oxidative stress and cellular attrition (***Karadag and Bilgin, 2010***).

CONCLUSIONS: Cyprodinil was the most toxic agent individually, although oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents.

The impact of some pesticides, both individually and in combinations, merits further study in terms of their impact on human cellular health.

REFERENCES:

Karadag H, Bilgin R (2010) Effect of Cyprodinil and Fludioxonil Pesticides on Human Superoxide Dismutase. Asian J Chem 22: 8147–8154.

McConkey DJ (1998) Biochemical determinants of apoptosis and necrosis. Toxicol Lett 99: 157–168

Ragan CI (1987) Structure of NAH-Ubiquinone reductase (complex-1). Curr Top Bioenerget 15: 1–161.

(Coleman M., *et al,* 2012)

CA 5.7.1 Neurotoxicity studies in rodents

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.7.2 Delayed polyneuropathy studies

Data included in the original EU review do not indicate any polyneuropathic effects. No new data or assessment is provided.

CA 5.8 Other Toxicological Studies

Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.

CA 5.8.1 Toxicity studies of metabolites

Several studies to support an assessment of the toxicological relevance of soil and/or plant metabolites CGA249287, CGA275535, NOA422054, CGA321915, ~~and~~ CGA232449, CGA263208, and CGA304075 ~~were~~ have been performed (Tables 5.8.1-02 and Tables 5.8.1-05).

~~At least CGA249287 was detected in rat metabolism studies in an amount of 0.014% of applied cyprodinil dose. CGA232449 was detected in male rat metabolism study at 1.55% of the applied dose, but not determined in females. This metabolite seems to be a transient oxidative metabolite.~~

The metabolites CGA232449, CGA304075 and CGA304076, except CGA249287 were detected in the rat metabolism study as shown below in Table 5.8.1-1.

Table 5.8.1-1: Detected Amounts of Cyprodinil Rat Metabolites [% of Applied Dose]

	Synonyms	Urine	Bile	Faeces
CGA232449	-	-	-	-
CGA232449 glucuronide	L3c	1.5	-	-
CGA249287	-	0.014	-	-
CGA304075	MET2F, M4	0.03	-	2.7-4.5
CGA304075 glucuronide	MET1G	0.47	8.6	-
CGA304075 sulphate	MET3U, M3	2.3-8.4	1.1	-
CGA304076	1F, M1	-	-	4.8-11.4
CGA304076 glucuronide	L3a	0.25	-	-
CGA304076 sulphate	2U, M4	4-8.2	-	-

In urine, 52 % to 63 % of the administered dose was excreted, of which all metabolites are downstream from CGA232449, CGA304075 and CGA304076, except CGA249287, which is a relatively minor metabolite at less than 1 % of the dose in urine. Mono- and di-hydroxylated phenyl/pyrimidinyl ring metabolites, including 1U, 5U and 6U, account for at least 33 % of the 0.5 mg/kg orally administered dose in urine. Metabolites hydroxylated on the phenyl methyl including 4U, 7U and L3C account for 7.6 % to 13 % of the dose in urine.

NOA422054 (and its glucuronide conjugate), CGA321915 and CGA263208 were not detected in the rat metabolism study.

Furthermore, a multi-(Q)SAR genotoxicity assessment of cyprodinil and the aforementioned metabolites has been provided.

For all the ~~five~~ seven metabolites tested the acute oral LD_{50} was >2000 mg/kg bw and no mutagenic potential was detected in a bacterial reverse mutation assay.

For CGA249287 and CGA263208, beside the acute oral toxicity study and bacterial reverse mutation assays further toxicity studies were performed. No evidence for a genotoxic effect was detected in two more *in vitro* genotoxicity tests for CGA249287 and an *in vitro* genotoxicity and *in vivo* genotoxicity test for CGA263208. 90 day repeat dose toxicity studies were conducted for both CGA249287 (NOAEL = 79.5 mg/kg bw/day for males and 90.5 mg/kg bw/day for females, respectively) and CGA263208 (NOAEL = 17.8 mg/kg bw/day for males and 22.1 mg/kg bw/day for females, respectively). In addition, a pre-natal developmental toxicity study in the rat was also conducted on CGA263208 (Maternal NOAEL = 200 mg/kg bw/day and Foetal NOAEL = 200 mg/kg bw/day, respectively).

~~The NOEL of a 90 day toxicity study in the rat was 1000 ppm corresponding to 79.5 and 90.5 mg/kg bw/day for males and females, respectively.~~

None of the tested metabolites were considered to be toxicologically relevant. The results are summarised in Tables 5.8.1-1 and Tables 5.8.1-5. ~~the following tables:~~

Table 5.8.1-~~24~~: Summary of Genotoxicity Studies of Metabolites

Metabolite	Study Type	Species	Outcome	Reference
CGA249287	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA1535, TA1537; *E. coli* WP2 uvrA	Negative (±S9)	Hertner, 1992*
	Gene mutation in mammalian cells	L5178Y TK$^{+/-}$ mouse lymphoma cells	Negative (±S9)	Clay, 2001*
	In vitro cytogenetic test	CCL61 Chinese hamster ovary cells	Negative (±S9)	Ogorek, 2001*
CGA275535	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537; *E. coli* WP2 uvrA	Negative (±S9)	Deparade, 2001*
NOA422054	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537; *E. coli* WP2 uvrA	Negative (±S9)	Deparade, 2000*
	Gene mutation in mammalian cells	Chinese hamster lung fibroblasts V79	Negative (±S9)	Chang, 2017
	In vitro micronucleus assay	Human lymphocytes	Negative (±S9)	Naumann, 2017a
CGA321915	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA102, TA1535, TA1537; *E. coli* WP2 uvrA	Negative (±S9)	Ogorek, 1996*
	Gene mutation in mammalian cells	Chinese hamster lung fibroblasts V79	Negative (±S9)	Chang, 2017a
	In vitro micronucleus assay	Human lymphocytes	Negative (±S9)	Naumann, 2017b
CGA232449	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA 102, TA1535, TA1537; *E.coli* WP2 uvrA	Negative (±S9)	Hertner, 1994*
CGA263208	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA 102, TA1535, TA1537; *E.coli* WP2 uvrA	Negative (±S9)	Hertner, 1994a
	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA 102, TA1535, TA1537; *E.coli* WP2 uvrA	Negative (±S9)	Hertner, 1992a
	Gene mutation in mammalian cells	Chinese hamster lung fibroblasts V79	Negative (±S9)	Chang, 2017b
	In vitro cytogenetic test	CCL61 Chinese hamster ovary cells	Negative (±S9)	Hertner, 1992b
	Micronucleus Assay in Bone Marrow Cells of the Rat	Crl:WI Rat	Negative	Dony, 2015
CGA304075	Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA 102, TA1535, TA1537; *E.coli* WP2 uvrA	Negative (±S9)	Hertner, 1994b
	Gene mutation in mammalian cells	Chinese hamster lung fibroblasts V79	Negative (±S9)	Chang, 2017c
	In vitro micronucleus assay	Human lymphocytes	Negative (±S9)	Naumann, 2017c

*Included in original EU review.

~~TBD: to be determined. Data generation is on-going.~~

~~Please refer to original EU review. No new data or assessment is provided. There are no new requirements or guidance and therefore the original endpoints and assessment are still valid.~~

Report:	K-CA 5.8.1/01 Manton, J.C. and Booth, E. (2016). Multi-(Q)SAR Genotoxicity Assessment of Cyprodinil and Dietary Metabolites. Syngenta Limited, Jealotts Hill International Research Centre, Bracknell, RG42 6EY, United Kingdom. Laboratory Report No. PI0007395. 23 May 2016. Unpublished. (Syngenta File No. CGA219417_11750).

Four (Q)SAR programs were selected working on different basis of expert knowledge rules and statistical methods (DEREK Nexus, Vega suite, ToxTree and the OECD QSAR Toolbox). Using these tools genotoxicity endpoints of *in vitro* and *in vivo* mutagenicity, chromosome damage and DNA binding were considered.

CGA321915 and CGA263208 are not predicted to be of genotoxic concern.

CGA304075 is considered to be potentially clastogenic *in vitro* and therefore of potential genotoxic concern, therefore it is considered that further *in vitro* genotoxicity testing is required.

Metabolites CGA304076 and CGA232449 are considered potentially of low *in vivo* genotoxic concern, due to a non-DNA reactive mode of action; These metabolites triggered no structural alerts in the other (Q)SAR and profiling systems, and therefore it is considered that in vitro genotoxicity testing is not required.

CGA249287 is considered to be of genotoxic concern (Ames mutagenicity). However, existing studies have demonstrated CGA249287 to be non-genotoxic in both bacterial and mammalian gene mutation assays and chromosome aberration assays, therefore this alert is superseded by the negative experimental data.

NOA422054 is considered to be of genotoxic concern based on the presence of two structurally and functionally different alerts, therefore it is considered that further *in vitro* genotoxicity testing is required.

Genotoxicity Studies on NOA422054

Report:	K-CA 5.8.1/02 Chang, S. (2017). NOA422054 - Cell Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1780100, 30 March 2017. Unpublished. Syngenta File No. NOA422054_10002.

GUIDELINES: OECD 476 (2016).

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

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

EXECUTIVE SUMMARY

The test item NOA422054 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. The assay was performed in one experiment, using two parallel cultures. The experiment was performed with and without liver microsomal activation and a treatment period of 4 hours.

The maximum concentration was 1703.0 µg/mL or 10 mM following the current OECD guideline 476 (2016) regarding the purity of the test item.

The main experiments were evaluated at the following concentrations:

Experiment I with and without metabolic activation: 106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL

Experiment II with metabolic activation: 106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL

No relevant cytotoxic effect indicated by an adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred in Experiment I in the absence or presence of S9.

In Experiment I without S9, there was no increase in mutant colonies outside the historical control data 95% control limits. A t-test evaluating the mean data of both parallel cultures only showed a statistically significant increase in mutant frequency (MF) at 106.4 µg/ml. A linear regression analysis (least squares) was performed to assess a possible concentration dependent increase of MF. No significant concentration dependent trend of the MF as indicated by a probability value of <0.05 was determined in any of the experimental groups. Hence the test substance was considered non mutagenic.

In Experiment I in the presence of S9, the 95% control limit was exceeded at 851.5 µg/mL and 1703.0 µg/mL and those increases were statistically significant. However, analyzing the results in detail, these increases were only observed in one culture, but not reproduced in the parallel culture (see Tables 7 and 10). A linear regression analysis (least squares) was performed to assess a possible dose dependency. A statistically significant trend was determined in Experiment I with metabolic activation. The calculation was based on the mean values of both cultures. Because of the notable inter-culture variability in MF and to clarify the results of Experiment I with metabolic activation, a second experiment with S9 was performed.

In Experiment II, a borderline cytotoxic effect indicated by an adjusted relative cloning efficiency I just below 50% with the mean value of both parallel cultures occurred at the maximum concentration of the second experiment with metabolic activation.

In Experiment II, the statistical analysis showed a statistically significant increase in MF when assessed with a t-test at three intermediate concentrations (106.4 µg/mL, 425.8 µg/ml, and 851.5 µg/mL). However, when a linear regression analysis (least squares) was performed to assess a possible concentration dependent increase of MF, no significant concentration dependent trend of the MF was observed. None of the mean MF were outside the 95% control limit of the historical control data. Experiment II in the presence of metabolic activation was concluded to show no mutagenic effect of the test substance.

Because the increases in mutant frequency in Experiment I in the presence of S9 were not consistent between cultures and not reproduced in Experiment II, they are not considered to be of biological relevance. NOA422054 is considered negative in this HPRT assay.

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

In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, NOA422054 is considered to be non-mutagenic in this HPRT assay.

MATERIALS AND METHODS

Materials:

Test Material: NOA422054
- **Description:** Off-white solid
- **Lot/Batch number:** MES 489/1
- **Molecular weight:** 165.2 g/mol
- **Purity:** 97% w/w (± 2%)
- **Retest date:** 30 September 2018
- **Stability of test compound:** Not indicated by the sponsor

Control Materials:
- **Negative:** -
- **Solvent control (final concentration):** DMSO (0.5 %)
- **Positive control:** Absence of S9 mix: Ethylmethane sulfonate (EMS), 300 µg/mL = 2.4 mM
  Presence of S9 mix: DMBA, 2.3 µg/mL = 8.9 µM

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others

Media: RPMI 1640				
Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No
Periodically "cleansed" against high spontaneous background?	X	Yes		No

X indicates those that apply

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ ATPase**
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment I Experiment II	106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL 106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL
Presence of S9 mix	Experiment I	106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL

Study Design and Methods:

In-life dates: Start: 27 October 2016, End: 18 January 2017

Test performance:

Cell treatment: Cells were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

For seeding and treatment of the cell cultures the complete culture medium was 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. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

Two to three days after subcultivation stock cultures are trypsinized at 37 °C for 5 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10 % FBS and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2 % in phosphate buffered saline (PBS).

Prior to the trypsin treatment the cells are were with PBS containing 200 mg/l EDTA. Approximately 0.7 to 1.2×10^7 cells were seeded in plastic flasks. The cells were grown for 24 hours prior to treatment. With the cell doubling time of approximately 12 h this ensures a population of more than 20×10^6 cells treated with the test substance.

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G".

Immediately after the end of treatment the cells were trypsinised as described above and sub-cultivated. At least 2.0×10^6 cells per experimental point (concentration series plus controls) were subcultured in 175 cm² flasks containing 30 mL medium.

Two additional 25 cm² flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (cloning efficiency I) as measure of test item induced cytotoxicity. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment as described below.

Three days after first sub-cultivation approximately 2.0×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium.

Following the expression time of approximately 7 days five 80 cm² cell culture flasks were seeded with about 3 to 5×10^5 cells each in medium containing 6-TG (11 µg/mL). Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

After 7 to 10 days the colonies were stained with 10% methylene blue in 0.01% KOH solution. Colonies with more than 50 cells were counted. If in doubt the colony size was checked with a preparation microscope.

Statistical Methods: A linear regression analysis (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

A t-test was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to evaluate an isolated increase of the mutation frequency at a test point exceeding the 95% control limit. Again a t-test is judged as significant if the p-value (probability value) is below 0.05.

However, both, biological and statistical significance was considered together.

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent solvent control in both parallel cultures,
b) the increase is concentration-related when evaluated with an appropriate trend test,
c) any of the results are outside the distribution of the historical solvent control data.

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

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
b) there is no concentration-related increase when evaluated with an appropriate trend test,
c) all results are inside the distribution of the historical negative control data

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

There is no requirement for verification of a clearly positive or negative response.

In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert

judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

RESULTS AND DISCUSSION

The test item NOA422054 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. Two parallel cultures were used throughout the assay. The treatment period was 4 hours with and without metabolic activation.

The first experiment was performed with and without metabolic activation. The second experiment was performed with metabolic activation only.

The main experiments were evaluated at the following concentrations:

Experiment I with and without metabolic activation: 106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL

Experiment II with metabolic activation: 106.4; 212.9; 425.8; 851.5; and 1703.0 µg/mL

No relevant cytotoxic effect indicated by an adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred in Experiment I in the absence or presence of S9.

In Experiment I without S9, there was no increase in mutant colonies outside the historical control data 95% control limits. A t-test evaluating the mean data of both parallel cultures only showed a statistically significant increase in mutant frequency (MF) at 106.4 µg/ml. A linear regression analysis (least squares) was performed to assess a possible concentration dependent increase of MF. No significant concentration dependent trend of the MF as indicated by a probability value of <0.05 was determined in any of the experimental groups. Hence the test substance was considered non mutagenic.

In Experiment I in the presence of S9, the 95% control limit was exceeded at 851.5 µg/mL and 1703.0 µg/mL and those increases were statistically significant. However, analyzing the results in detail, these increases were only observed in one culture, but not reproduced in the parallel culture (see Tables 7 and 10). A linear regression analysis (least squares) was performed to assess a possible dose dependency. A statistically significant trend was determined in Experiment I with metabolic activation. The calculation was based on the mean values of both cultures. Because of the notable inter-culture variability in MF and to clarify the results of Experiment I with metabolic activation, a second experiment with S9 was performed.

In Experiment II, a borderline cytotoxic effect indicated by an adjusted relative cloning efficiency I just below 50% with the mean value of both parallel cultures occurred at the maximum concentration of the second experiment with metabolic activation.

In Experiment II, the statistical analysis showed a statistically significant increase in MF when assessed with a t-test at three intermediate concentrations (106.4 µg/mL, 425.8 µg/ml, and 851.5 µg/mL). However, when a linear regression analysis (least squares) was performed to assess a possible concentration dependent increase of MF, no significant concentration dependent trend of the MF was observed. None of the mean MF were outside the 95% control limit of the historical control data. Experiment II in the presence of metabolic activation was concluded to show no mutagenic effect of the test substance.

Because the increases in mutant frequency in Experiment I in the presence of S9 were not consistent between cultures and not reproduced in Experiment II, they are not considered to be of biological relevance. NOA422054 is considered negative in this HPRT assay.

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

CONCLUSION: In conclusion it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, NOA422054 is considered to be non-mutagenic in this HPRT assay.

(Chang S, 2017)

Report:	K-CA 5.8.1/03 Naumann, S. (2017a). NOA422054 - Micronucleus test in human lymphocytes *in vitro*. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1780200, 29 March 2017. Unpublished. Syngenta File No. NOA422054_10000.

GUIDELINES: Micronucleus Test in Human Lymphocytes In vitro; OECD 487 (2016).

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

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

EXECUTIVE SUMMARY

The test substance NOA422054, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in four independent experiments.

In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic damage, except for the positive controls in Experiment IA and IB in the absence of S9 mix, where only 500 binucleated cells per culture were evaluated.

The highest applied concentration in this study (1703 µg/mL of the test substance, approx. 10 mM) was chosen with regard to the molecular weight and the purity (97%) of the test substance and with respect to the current OECD Guideline 487.

The dose concentration selection of the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487.

In Experiment IA in the absence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration. Experiment IA was repeated to investigate the increase of micronuclei under these cytotoxic conditions. No cytotoxicity was observed in Experiment IB. Due to the shift in cytotoxicity observed between Experiment IA and IB, a third experiment, Experiment IC, was performed. No cytotoxicity was observed in Experiment IC, confirming the observations of Experiment IB. The same blood donor was used for Experiments IA and IC, but the blood donor was different for Experiment IB. Thus donor variability does not explain the differences in cytotoxicity observed. All other parameters were the same between the experiments and hence there was no obvious explanation for the differential cytotoxicity noted in Experiment IA in the absence of S9 mix.

In Experiment IA in the presence of S9 mix and in experiments IB, IC and II in the absence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In Experiment IA in the absence of S9 mix, one statistically significant increase in the number of micronucleated cells (1.20 %) was observed after treatment with 973 µg/mL. The value also exceeded the 95 % control limits of the historical solvent control data (0.07 –1.15 % micronucleated cells). This increase in micronuclei was not reproducible in Experiment IB and IC and is therefore considered to be a cytotoxicity related artefact and not biologically relevant.

In Experiment IA in presence of S9 mix and in Experiment IB, IC and II in the absence of S9 mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test item.

The overall outcome of the study was considered negative, because the increase in micronuclei observed in Experiment IA without S9 was not reproduced in two repeat experiments, Experiments IIB and IIC, and no other statistically significant increase or increase outside the 95% control limits was observed.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

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

Therefore, NOA422054 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

MATERIALS AND METHODS

Materials:

Test Material:	NOA422054
Description:	Off white solid
Lot/Batch number:	MES 489/1
Purity:	97% w/w (+/- 2%), correction for purity was made.
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:

Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: MMC, 1.0 µg/mL Absence of S9 mix: Demecolcin, 75 ng/ml Presence of S9 mix: Cyclophosphamide 15.0 µg/mL

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl

(33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment 1A Experiment 1B Experiment 1C Experiment 2	182, 318, 556, 973 µg/mL 391, 986, 1419, 1703 µg/mL 986, 1183, 1419, 1703 µg/mL 505, 757, 1135, 1703 µg/mL
Presence of S9 mix	Experiment 1A	318, 556, 973, 1703 µg/mL

Study Design and Methods:

In-life dates: Start: 16 November 2016, End: 10 February 2017

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. The pre-test was performed with 11 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

Cytogenetic Assay:

Cell exposure time:		**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix + S9 mix	Experiment 1	4h 4h	4h 4h	4h 4h
- S9 mix	Experiment 2	20h	20h	20h

Cytokinesis block:	Cytochalasin B (4 µg/mL)

Cell harvest time after termination of treatment:	**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix (4 hour treatment)	36h	36h	36h
+ S9 mix (4 hour treatment)	36h	36h	36h
- S9 mix (20 hour treatment)	0h	0h	0h

Details of slide preparation

Pulse exposure

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

Preparation and analysis of cells: The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were washed and fixated. The slides were added to a microscope slide and stained with Giemsa. Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides, except for the positive controls in Experiment IA and IB in the absence of S9 mix, where only 500 binucleated cells per culture were evaluated. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criteria. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Evaluation criteria: The percentages of micronuclei in binucleate cells were calculated for each treatment scored. The data have been interpreted as follows:

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

– None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control

– There is no concentration-related increase

– The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

– At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control

– The increase is concentration-related in at least one experimental condition

– The results are outside the range of the laboratory historical solvent control data

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

Statistical analysis: Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using the validated R Script CHI2.Rnw for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: A pre-experiment to evaluate the cytotoxicity of the test item was performed. The pre-experiment is reported as the main Experiment I since the criteria mentioned under Acceptability of the assay were met.

Cytogenetic assay: The test substance NOA422054, dissolved in DMSO, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Four independent experiments were performed. In Experiment IA, the exposure period was 4 hours with and without S9 mix. In the experiments IB and IC, the exposure periods were 4 hours without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analysed. 1000 binucleated cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration. For the positive controls in Experiment IA and IB in the absence of S9 mix, only 500 binucleated cells per culture were evaluated due to strong mutagenic effects. To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 binucleated cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in this study, 1703 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the purity (97%) of the test substance and with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

In this study, no precipitation was observed.

No relevant influence on the osmolarity and pH was observed.

In Experiment IA in the absence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentration. Experiment IA was repeated to investigate the increase of micronuclei under these cytotoxic conditions. No cytotoxicity was observed in Experiment IB. Due to the shift in cytotoxicity observed between Experiment IA and IB, a third experiment, Experiment IC, was performed. No cytotoxicity was observed in Experiment IC, confirming the observations of Experiment IB. The same blood donor was used for Experiments IA and IC, but the blood donor was different for Experiment IB. Thus donor variability does not explain the differences in cytotoxicity observed. All other parameters were the same between the experiments and hence there was no obvious explanation for the differential cytotoxicity noted in Experiment IA in the absence of S9 mix.

In Experiment IA in the presence of S9 mix and in experiments IB, IC and II in the absence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In Experiment IA in the absence of S9 mix, one statistically significant increase in the number of micronucleated cells (1.20 %) was observed after treatment with 973 µg/mL. The value also exceeded the 95 % control limits of the historical solvent control data (0.07 –1.15 % micronucleated cells). This increase in micronuclei was not reproducible in Experiment IB and IC and is therefore considered to be a cytotoxicity related artefact and not biologically relevant.

In Experiment IA in presence of S9 mix and in Experiment IB, IC and II in the absence of S9 mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test item.

The overall outcome of the study was considered negative, because the increase in micronuclei observed in Experiment IA without S9 was not reproduced in two repeat experiments, Experiments IIB and IIC, and no other statistically significant increase or increase outside the 95% control limits was observed.

Demecolcin (75 ng/mL), MMC (1.0 µg/mL) or CPA (15.0 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei.

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

Therefore, NOA422054 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

(Naumann S., 2017a)

Genotoxicity Studies on CGA321915

Report:	K-CA 5.8.1/04 Chang, S. (2017a). CGA321915 - Cell Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1780600, 04 April 2017. Unpublished. Syngenta File No. CGA321915_10023.

GUIDELINES: OECD 476 (2016).

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

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

EXECUTIVE SUMMARY

The test item CGA321915 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. The assay was performed in one experiment, using two parallel cultures. The experiment was performed with and without liver microsomal activation and a treatment period of 4 hours.

The maximum concentration of the test item in the pre-experiment and the main experiments was 1548 µg/mL, equal to a molar concentration of about 10 mM.

The main experiments were evaluated at the following concentrations:

Experiment I with and without metabolic activation: 96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL

Experiment II without metabolic activation: 96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL

No relevant cytotoxic effect indicated by an adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred in both main experiments up to the highest concentration with and without metabolic activation.

No biologically relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

Considering the mean values of the mutation frequency the 95% control limit was not exceeded at any experimental point and a *t*-test was not performed.

In the main experiment with and without S9 mix the range of the solvent controls was from 12.3 up to 17.1 mutants per 10^6 cells; the range of the groups treated with the test item was from 10.1 up to 28.4 mutants per 10^6 cells. This range refers to mean values of both cultures.

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

In conclusion, it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CGA321915 is considered to be non-mutagenic in this HPRT assay.

MATERIALS AND METHODS

Materials:

Test Material:	CGA321915
Description:	Beige solid
Lot/Batch number:	MES 356/2
Molecular weight:	150.2 g/mol
Purity:	97% w/w (± 2%)
Retest date:	30 September 2019
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: Ethylmethane sulfonate (EMS), 300 µg/mL = 2.4 mM Presence of S9 mix: DMBA, 2.3 µg/mL = 8.9 µM

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others

Media: RPMI 1640				
Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No
Periodically "cleansed" against high spontaneous background?	X	Yes		No

X indicates those that apply

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ ATPase**
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment I Experiment II	96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL 96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL
Presence of S9 mix	Experiment I	96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL

Study Design and Methods:

In-life dates: Start: 20 October 2016, End: 16 February 2017

Test performance:

Cell treatment: Cells were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

For seeding and treatment of the cell cultures the complete culture medium was 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. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

Two to three days after subcultivation stock cultures are trypsinized at 37 °C for 5 to 10 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10 % FBS and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2 % in phosphate buffered saline (PBS).

Prior to the trypsin treatment the cells are were with PBS containing 200 mg/l EDTA. Approximately 0.7 to 1.2×10^7 cells were seeded in plastic flasks. The cells were grown for 24 hours prior to treatment. With the cell doubling time of approximately 12 h this ensures a population of more than 20×10^6 cells treated with the test substance.

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G".

Immediately after the end of treatment the cells were trypsinised as described above and sub-cultivated. At least 2.0×10^6 cells per experimental point (concentration series plus controls) were subcultured in 175 cm² flasks containing 30 mL medium.

Two additional 25 cm² flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (cloning efficiency I) as measure of test item induced cytotoxicity. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment as described below.

Three or four days after first sub-cultivation approximately 2.0×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium.

Following the expression time of approximately 7 days five 80 cm² cell culture flasks were seeded with about 3 to 5×10^5 cells each in medium containing 6-TG (11 µg/mL). Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

After 7 to 10 days the colonies were stained with 10% methylene blue in 0.01% KOH solution. Colonies with more than 50 cells were counted. If in doubt the colony size was checked with a preparation microscope.

Statistical Methods: A linear regression analysis (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

A t-test was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to evaluate an isolated increase of the mutation frequency at a test point exceeding the 95% control limit. Again a t-test is judged as significant if the p-value (probability value) is below 0.05.

However, both, biological and statistical significance was considered together.

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent solvent control in both parallel cultures,
b) the increase is concentration-related when evaluated with an appropriate trend test,
c) any of the results are outside the distribution of the historical solvent control data.

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

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
b) there is no concentration-related increase when evaluated with an appropriate trend test,
c) all results are inside the distribution of the historical negative control data

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

There is no requirement for verification of a clearly positive or negative response.

In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

RESULTS AND DISCUSSION

The test item CGA321915 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster.

The assay was performed in two experiments, using two parallel cultures. The experiments were performed with and without liver microsomal activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations:

Experiment I with and without metabolic activation: 96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL

Experiment II without metabolic activation: 96.8; 193.5; 387.0; 774.0; and 1548.0 µg/mL

No relevant cytotoxic effect indicated by an adjusted cloning efficiency I below 50% with the mean value of both parallel cultures occurred up to the maximum concentration with and without metabolic activation.

Taken into consideration the calculated mean values no biologically relevant and reproducible increase of the mutation frequency was observed in the main experiments up to the maximum concentration with and without metabolic activation.

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. No significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in any of the experimental groups.

Considering the mean values of the mutation frequency the 95% control limit was not exceeded at any experimental point and consequently no t-test was performed.

In the main experiment with and without S9 mix the range of the solvent controls was from 12.3 up to 17.1 mutants per 10^6 cells; the range of the groups treated with the test item was from 10.1 up to 28.4 mutants per 10^6 cells. This range refers to mean values of both cultures.

Based on these results, CGA321915 meets the criteria for being clearly negative in the HPRT assay (OECD 476, 2016).

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

CONCLUSIONS: In conclusion, it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CGA321915 is considered to be non-mutagenic in this HPRT assay.

(Chang S, 2017a)

Report:	K-CA 5.8.1/05 Naumann, S. (2017b). CGA321915 - Micronucleus Test in Human Lymphocytes *In Vitro*. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1779900, 21 March 2017. Unpublished. Syngenta File No. CGA321915_10021.

GUIDELINES: OECD 487 (2016).

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

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

EXECUTIVE SUMMARY

The test substance CGA321915, dissolved in deionized water, was assessed for its potential to induce micronuclei in human lymphocytes in vitro in two independent experiments. In each experimental group two parallel cultures were analysed. Per culture at least 1000 binucleated cells were evaluated for cytogenetic damage, except for the positive control in Experiment I without S9 mix, where only 500 binucleated cells were evaluated.

The highest applied concentration in this study (1548 µg/mL of the test substance, approx. 10 mM) was chosen with regard to the molecular weight and the purity (97%) of the test substance and with respect to

the current OECD Guideline 487. Dose selection of the cytogenetic experiment was performed considering the toxicity data in accordance with OECD Guideline 487.

In the absence and presence of S9 mix, no cytotoxicity or precipitation was observed up to the highest applied concentration. In the absence and presence of S9 mix, no relevant increase in the number of micronucleated cells was observed after treatment with the test substance when tested up to the maximum required concentration of 10 mM. The percentage micronucleated cells in all evaluated concentrations was within the 95% control limit of the historical control data, therefore the study meets the criteria for being clearly negative in this *in vitro* micronucleus assay.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

In conclusion, it can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes. Therefore, CGA321915 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

MATERIALS AND METHODS

Materials:

Test Material:	CGA321915
Description:	off-white solid
Lot/Batch number:	MES 356/2
Purity:	97% ww/ (+/- 2%), correction for purity was made.
CAS#:	-
Stability of test compound:	Not indicated by the sponsor

Control Materials:	
Negative:	-
Solvent control (final concentration):	Deionised water (10 %)
Positive control:	Absence of S9 mix: MMC, 1.5 µg/mL (Experiment I), Absence of S9 mix: Demecolcin, 100.0 ng/ml (Experiment II) Presence of S9 mix: Cyclophosphamide 17.5 µg/mL

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment 1 Experiment 2	289, 505, 885, 1548 µg/mL 289, 505, 885, 1548 µg/mL
Presence of S9 mix	Experiment 1	289, 505, 885, 1548 µg/mL

Study Design and Methods:

In-life dates: Start: 09 November 2016, End: 10 January 2017

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. The pre-test was performed with 11 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

Cytogenetic Assay:

Cell exposure time:		**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix + S9 mix	Experiment 1	4h 4h	4h 4h	4h 4h
- S9 mix	Experiment 2	20h	20h	20h

Cytokinesis block:	Cytochalasin B (4 µg/mL)

Cell harvest time after termination of treatment:	**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix (4 hour treatment)	36h	36h	36h
+ S9 mix (4 hour treatment)	36h	36h	36h
- S9 mix (20 hour treatment)	0h	0h	0h

Details of slide preparation

Pulse exposure

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium

containing the test item. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)
About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with 10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

Preparation and analysis of cells: The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were washed and fixated. The slides were added to a microscope slide and stained with Giemsa. Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criteria. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Evaluation criteria: The percentages of micronuclei in binucleate cells were calculated for each treatment scored. The data have been interpreted as follows:

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

– None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
– There is no concentration-related increase
– The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval)

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

– At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
– The increase is concentration-related in at least one experimental condition
– The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval)

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

Statistical analysis: Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using a validated R Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: A pre-experiment to evaluate the cytotoxicity of the test item was performed. The pre-experiment is reported as the main Experiment I since the criteria mentioned under 'Acceptability of the assay' were met.

Cytogenetic assay: The test substance CGA321915, dissolved in deionized water, was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Two independent experiments were performed. In Experiment I, the exposure period was 4 hours with and without S9 mix. In Experiment II, the exposure period was 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analysed. At least 1000 binucleated cells per culture were scored for cytogenetic damage on coded slides giving a total of 2000 binucleated cells per test substance concentration, except for the positive control in Experiment I without S9 mix, where only 500 binucleated cells per culture were evaluated due to strong clastogenic effects.

To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in this study, 1548 µg/mL (approx. 10 mM) was chosen with regard to the molecular weight and the purity (97%) of the test substance and with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

No precipitation or phase separation was observed.

		Concentration [µg/mL]	Osmolarity [mOsm]	pH
Exp. I	Solvent control	-	291	7.5
	CGA321915	1548	304	7.5
Exp. II	Solvent control	-	n.d.	7.6
	CGA321915	1548	n.d.	7.6

n.d. Not determined

No relevant influence on the osmolarity or pH was observed.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest applied concentration.

In this study, no biologically relevant increase in the number of micronucleated cells was observed after treatment with the test substance. The percentage micronucleated cells in all evaluated concentrations was within the 95% control limit of the historical control data, therefore the study meets the criteria for being clearly negative in this *in vitro* micronucleus assay.

Demecolcin (100.0 ng/mL), MMC (1.5 µg/mL) or CPA (17.5 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei.

CONCLUSIONS: It can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.

Therefore, CGA321915 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

(Naumann S, 2017b)

Genotoxicity Studies on CGA263208

CGA263208 is the carbonate salt of phenylguanidine. In some study reports, this material is identified as CA1139A or CA1139. CGA263208, CA1139A and CA1139 represent the same test material, that is, the carbonate salt of phenylguanidine. In addition, chemical purity analyses were conducted so as to report both the "free" phenylguanidine and phenylguanidine carbonate content. In study reports where the test material is referenced as CA1139A, the chemical purity reported represents the "free" phenylguanidine. In study reports where the test material is referenced CGA263208, the chemical purity reported represents phenylguanidine carbonate. In study reports where the test material is referenced CA1139, the chemical purities reported represent both the "free" phenylguanidine and phenylguanidine carbonate.

Report:	K-CA 5.8.1/06 Hertner T. (1994). CGA263208 tech. (plant metabolite of CGA219417): Salmonella and Escherichia/mammalian-microsome mutagenicity test. Genetic Toxicology, Ciba-Geigy Limited, Basel, Switzerland. Report No. 943052, 18 July 1994. Unpublished. Syngenta File No. CGA263208/0001.

GUIDELINES: OECD 471 (1983); 92/69/EEC (1992); Ministry of Health and Welfare, Japan, Notification No 118 (1984); EPA § 798.5265 (1987).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: This test permits the detection of gene mutations induced by the test material or its metabolites in histidine-requiring strains of *Salmonella typhimurium* and in a tryptophan-requiring strain of *Escherichia coli*. When the *Salmonella* strains are exposed to a mutagen, some of the bacteria in the treated population, through chemical interaction with the test material or its metabolites, undergo genetic changes which cause them to revert to a non-histidine-requiring state and thus to grow in the absence of exogenous histidine. Similarly, after mutation, the *Escherichia coli* bacteria are able to grow in tryptophan-free medium. Mutagenic effects of the test material are demonstrable on comparison of the number of bacteria in the treated and control cultures that have undergone reverse-mutation to histidine prototrophism or tryptophan prototrophism, respectively. Different tester strains are used because of differing sensitivities to known mutagens.

EXECUTIVE SUMMARY

In a mutation assay in bacteria, *S. typhimurium* histidine-requiring strains TA98, TA100, TA102, TA1535, TA1537 and *E.coli* tryptophan-requiring strain WP2 uvrA were exposed to CGA263208 tech. (plant metabolite of CGA219417) (purity 95.2%). The test was performed with and without the addition of rat-liver post mitochondrial supernatant (S9 fraction) as an extrinsic metabolic activation system. The test material was dissolved in DMSO and tested at five concentrations in the range of 61.73 µg/plate to

5000.0 µg/plate in the presence and absence of a metabolic activation system. In order to confirm the results, the experiments were repeated with and without metabolic activation at five concentrations in the range of 61.73 µg/plate to 5000.0 µg/plate. Each strain was additionally tested in the presence and in the absence of a metabolic activation system with a suitable, known mutagen as positive control.

In both experiments, performed with and without metabolic activation, none of the tested concentrations of CGA263208 tech. led to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants by comparison with the negative control.

Conclusion: Based on the results of these experiments and on standard evaluation criteria, it is concluded that CGA263208 tech. (plant metabolite of CGA219417) and its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

MATERIALS AND METHODS

Materials:

Test Material: CGA263208 tech. (plant metabolite of CGA219417)
Description: Technical, solid
Lot/Batch number: EA168376
Purity: 95.2%
CAS#: Not reported
Stability of test compound: Reanalysis date June 1995

Control Materials:
Negative:
Solvent control (final concentration): Dimethylsulfoxide (DMSO) 10 µL/plate
Positive control: Nonactivation:
Sodium azide 5.0 µg/plate TA100, TA1535
4-Nitroquinoline (4-NQO) 2.0 µg/plate WP2 uvrA
Mitomycin C 2.0 µg/plate TA102
2-Nitrofluorene 20.0 µg/plate TA98
9-Aminoacridine 150 µg/plate TA1537
Activation:
2-Aminoanthracene
2.5 µg/plate TA98, TA100, TA1537
20.0 µg/plate TA102
50 µg/plate WP2 uvrA
Cyclophosphamide
400 µg/plate TA1535

Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Rat-liver post mitochondrial supernatant (S9 fraction) was prepared in advance from male RAI rats (Tif: RAIf [SPF]). The animals were treated with Aroclor 1254 (500 mg/kg, i.p.) 5 days prior to sacrifice. The livers were homogenized with 3 volumes of 150 mM KCI. The homogenate was centrifuged for 15 minutes at 9000x g and the resulting supernatant (S9 fraction) was stored at approximately -80°C for no longer than one year. The protein content of the S9 fraction was 32.22 mg/mL.

The activation mixture contained:

Rat liver S9 fraction	100.0 µL/mL
NADP	4.0 µmol/mL
$MgCl_2$	8.0 µmol/mL
KCl	33.0 µmol/mL
Na-phosphate-buffer, pH 7.4	100.0 µmol/mL
Glucose-6-phosphate	5.0 µmol/mL

Test organisms:

S. typhimurium strains

	TA97	X	TA98	X	TA100	X	TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

E. coli strains

	WP2 (pKM101)	X	WP2 *uvrA*						

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	X	Yes		No

Study Design and Methods:

Experimental dates: Start: 15 June 1994, End: 18 July 1994.

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: Six concentrations of CGA263208 tech. were tested with *Salmonella typhimurium* strain TA100 and *Escherichia coli* strain WP2 uvrA to determine the highest concentration to be used in the mutagenicity assay. The highest concentration applied was 5000 µg/plate. The five lower concentrations decreased by a factor of three. The plates were inverted and incubated for about 48 hours at 37±1.5°C in darkness. Thereafter, they were evaluated by counting the colonies and determining the background lawn. One plate per test material concentration and negative control was used.

Mutagenicity Assay: This was performed with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 and with *Escherichia coli* strain WP2 uvrA with and without metabolic activation. Each of five concentrations of the test material, a negative and a positive control were tested using three plates per test material concentration and controls. The highest concentration applied was determined in the preliminary range finding test and the four lower concentrations decreased by a factor of three. The plates were inverted and incubated for about 48 hours at 37±1.5°C in darkness. Thereafter, they were evaluated by counting the number of colonies and determining the background lawn.

Type of Bacterial assay:

- X standard plate test (both experiments –S9, +S9)
- __ pre-incubation
- __ "Prival" modification (i.e. azo-reduction method)
- __ spot test
- __ other

Test material concentrations used:

Preliminary range finding test:

Non-activated conditions: 5000, 1666.67, 555.56, 185.19, 61.73 and 20.58 µg/plate
Activated conditions: 5000, 1666.67, 555.56, 185.19, 61.73 and 20.58 µg/plate

Original and confirmatory mutagenicity experiments:

Non-activated conditions: 5000, 1666.67, 555.56, 185.19 and 61.73 µg/plate
Activated conditions: 5000, 1666.67, 555.56, 185.19 and 61.73 µg/plate

CGA263208 tech. was dissolved in DMSO at slightly elevated temperature. CGA263208 tech. was soluble up to the concentration of 50 mg/mL. Lower concentrations of the test material were obtained by appropriate dilution of the stock solution with DMSO. No precipitates or aggregates were noted.

Protocol: 0.1 mL of the overnight cultures was mixed with 2 mL of top agar, either 0.5 mL of 100 mM sodium phosphate buffer (experiments without activation) or 0.5 mL of the activation mixture (experiments with activation) and 0.1 mL of a solution of the test material, the positive control or the solvent as a negative control and poured on minimal agar in Petri dishes. Each Petri dish contained about 20.0 mL of minimal agar (1.5% agar supplemented with 2% salts of the Vogel-Bonner Medium E and 2% glucose). The top agar was composed of 0.6% agar and 0.6% NaCl. In the experiment with *Salmonella* the top agar was supplemented with 10% of 0.5 mM L-histidine and 0.5 mM (+)biotin dissolved in water. In the experiment with *E.coli* it was supplemented with 10% of 0.5 mM L-tryptophan dissolved in water.

Colony counting and scoring of the plates: Colonies were counted electronically or manually where agar damage or test material minor precipitates or strong colouration of the agar plates might have interfered with automating counting. Observations indicating precipitates of the test material in the top agar or a reduced or absent bacterial background lawn were registered additionally. Means for all mutagenicity assays were calculated.

Statistical analysis: None – see Evaluation Criteria below.

Evaluation criteria: The test substance will be considered to be positive in the test system if one or both of the following conditions are met:

- At least a reproducible doubling of the mean number of revertants per plate above that of the negative control at any concentration for one or more of the following strains: TA98, TA1535, TA1537, *E.coli* WP2 uvrA.
- A reproducible increase of the mean number of revertants per plate for any concentration above that of the negative control by at least a factor of 1.5 for strains TA 100 or TA 102.

Generally a concentration-related effect should be demonstrable.

RESULTS AND DISCUSSION

Preliminary range finding test: Background growth was reduced with strain TA100 at the highest concentration. The numbers of revertant colonies were not reduced except with strain TA100 in the presence of metabolic activation at the highest concentration. From the results obtained, the highest concentration suitable for the mutagenicity test was selected to be 5000.0 µg/plate, with and without metabolic activation.

Mutagenicity assay, original experiment: In the experiments performed with and without metabolic activation, treatment of strains TA98, TA100, TA102, TA1535, TA1537 and WP2 uvrA with

CGA263208 tech. did not lead to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants in comparison with the negative control.

Mutagenicity test, confirmatory experiment: In the experiments performed with and without metabolic activation, again after treatment of strains TA98, TA100, TA102, TA1535, TA1537 and WP2 uvrA with CGA263208 tech. no increase in the incidence of either histidine- or tryptophan-prototrophic mutants was observed in comparison with the negative control.

In the mutagenicity tests normal background growth was observed with most strains at all concentrations. Only strain TA100 showed reduced background growth at the highest concentration. The numbers of revertant colonies were not reduced with increasing concentration. Therefore, the test substance exerted no substantial toxic effect on the growth of the bacteria.

CONCLUSIONS: Based on the results of these experiments and on standard evaluation criteria, it is concluded that CGA263208 tech. (plant metabolite of CGA219417) and its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

(Hertner T, 1994)

Report:	K-CA 5.8.1/07 Hertner T. (1992). CA1139A: *Salmonella and Escherichia*/liver-microsome test. Genetic Toxicology, Ciba-Geigy Limited, Basel, Switzerland. Report No. 923050, 20 August 1992. Unpublished. Syngenta File No. CA1139/0006.

GUIDELINES: OECD 471 (1983); OJEC L251/143-145 Part B 14 (1984); Ministry of Health and Welfare, Japan, Notification No 118 (1984); EPA § 798.5265 (1987).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: This test permits the detection of gene mutations induced by the test material or its metabolites in histidine-requiring strains of *Salmonella typhimurium* and in a tryptophan-requiring strain of *Escherichia coli*. When the Salmonella strains are exposed to a mutagen, some of the bacteria in the treated population, through chemical interaction with the compound or its metabolites, undergo genetic changes which cause them to revert to a non-histidine-requiring state and thus to grow in the absence of exogenous histidine. Similarly, after mutation, the *Escherichia coli* bacteria are able to grow in tryptophan-free medium. Mutagenic effects of the test substance are demonstrable on comparison of the number of bacteria in the treated and control cultures that have undergone reverse-mutation to histidine prototrophism or tryptophan prototrophism, respectively. Different tester strains are used because of differing sensitivities to known mutagens.

EXECUTIVE SUMMARY

In a mutation assay in bacteria, *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and *E.coli* strain WP2uvrA were exposed to CA1139A (purity 76.2%). The test was performed with and without the addition of rat-liver post mitochondrial supernatant (S9 fraction) as an extrinsic metabolic activation system. The compound was dissolved in DMSO and tested at five concentrations in the range of 312.5 µg/plate to 5000. 0 µg/plate in the presence and absence of a metabolic activation system. In order to confirm the results, the experiments were repeated with and without metabolic activation at the same concentrations. Each strain was additionally tested in the presence and in the absence of a metabolic activation system with a suitable, known mutagen as positive control.

In the original and confirmatory experiments performed with and without metabolic activation, none of the tested concentrations of CA1139A led to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants by comparison with the negative control.

Conclusion: Based on the results of these experiments and on standard evaluation criteria, it is concluded that CA1139A and its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

MATERIALS AND METHODS

Materials:

Test Material: CA1139A
- **Description:** Not reported
- **Lot/Batch number:** P.201025
- **Purity:** 76.2%
- **CAS#:** Not reported
- **Stability of test compound:** Reanalysis date February 1993

Control Materials:
- **Negative:**
- **Solvent control (final concentration):** Dimethylsulfoxide (DMSO) 10 µL/plate
- **Positive control:**

Nonactivation:
- Sodium azide 5.0 µg/plate TA100, TA1535
- 4-Nitroquinoline-N-oxide 2.0 µg/plate WP2 uvrA
- 2-Nitrofluorene 20.0 µg/plate TA98
- 9(5)-Aminoacridine 150 µg/plate TA1537

Activation:
- 2-Aminoanthracene
  - 2.5 µg/plate TA98, TA100, TA1537
  - 50 µg/plate WP2 uvrA
- Cyclophosphamide H_2O
  - 400 µg/plate TA1535

Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

Rat-liver post mitochondrial supernatant (S9 fraction) was prepared in advance from male RAI rats (Tif: RAif [SPF]). The animals were treated with Aroclor 1254 (500 mg/kg, i.p.) 5 days prior to sacrifice. The livers were homogenized with 3 volumes of 150 mM KCI. The homogenate was centrifuged for 15 minutes at 9000x g and the resulting supernatant (S9 fraction) was stored at approximately -80°C for no longer than one year. The protein content of the S9 fraction was 35.39 and 43.06 mg/mL.

The activation mixture contained:

Rat liver S9 fraction	100.0 µL/mL
NADP	4.0 µmol/mL
$MgCl_2$	8.0 µmol/mL
KCl	33.0 µmol/mL
Na-phosphate-buffer, pH 7.4	100.0 µmol/mL

Glucose-6-phosphate 5.0 µmol/mL

Test organisms:

S. typhimurium strains

	TA97	X	TA98	X	TA100		TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

E. coli strains

	WP2 (pKM101)	X	WP2 *uvrA*						

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	X	Yes		No

Study Design and Methods:

Experimental dates: Start: 26 June 1992, End: 17 July 1992.

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: Six concentrations of CA1139A were tested with *Salmonella typhimurium* strain TA100 and *Escherichia coli* strain WP2 uvrA to determine the highest concentration to be used in the mutagenicity assay. The experiments were performed with and without metabolic activation.

Type of Bacterial assay:

- X standard plate test (both experiments –S9, +S9)
- __ pre-incubation
- __ "Prival" modification (i.e. azo-reduction method)
- __ spot test
- __ other

Test compound concentrations used:

Preliminary cytotoxicity test:

Non-activated conditions: 5000, 1666.6667, 555.5556, 185.1852, 61.7284 and 20.5761 µg/plate
Activated conditions: 5000, 1666.6667, 555.5556, 185.1852, 61.7284 and 20.5761 µg/plate

Original and confirmatory mutagenicity experiments:

Non-activated conditions: 5000.0, 2500.0, 1250.0, 625.0 and 312.5 µg/plate
Activated conditions: 5000.0, 2500.0, 1250.0, 625.0 and 312.5 µg/plate

CA1139A was dissolved in DMSO at room temperature. CA1139A was soluble up to the concentration of 50 mg/mL. Lower concentrations of the test substance were obtained by appropriate dilution of the stock solution with DMSO. No precipitates or aggregates were noted.

Analysis: The lowest serial dilution of the test material was analysed and the concentration was found to be in agreement with the intended concentration.

Protocol: 0.1 mL of the overnight cultures were mixed with 2 mL of top agar, either 0. *5* mL of 100 mM sodium phosphate buffer (experiments without activation) or 0. *5* mL of the activation mixture (experiments with activation) and 0 1 mL of a solution of the test substance, the positive control or the solvent as a negative control and poured on minimal agar in Petri dishes. Each Petri dish contained about 20. 0 mL of minimal agar (1.5% agar supplemented with 2% salts of the Vogel-Bonner Medium E and 2% glucose). The top agar was composed of 0.6% agar and 0.6% NaCl. In the experiment with Salmonella the top agar was supplemented with 10% of 0.5 mM L-histidine and 0.5 mM (+)biotin dissolved in water. In the experiment with *E.coli* it was supplemented with 10% of 0.5 mM L-tryptophan dissolved in water.

Each of the five concentrations of the test substance, a negative and a positive control were tested, using three plates per test substance concentration and controls. The highest concentration applied was determined in the preliminary range finding test and the four lower concentrations decreased by a factor of two. The plates were inverted and incubated for about 48 hours at 37±1.5°C in darkness. Thereafter, they were evaluated by counting the number of colonies and determining the background lawn.

Colony counting and scoring of the plates: Colonies were counted electronically using an Artek Colony Counter (Fisher Scientific). Observations indicating precipitates of the test substance in the top agar or a reduced or absent bacterial background lawn were registered additionally. Means for all mutagenicity assays were calculated.

Statistical analysis: None – see Evaluation Criteria below.

Evaluation criteria: The test substance was considered to be positive in the test system if one or both of the following conditions are met:

- At least a reproducible doubling of the mean number of revertants per plate above that of the negative control at any concentration for one or more of the following strains:TA98, TA1535, TA1537, *E.coli* WP2 uvrA.
- A reproducible increase of the mean number of revertants per plate for any concentration above that of the negative control by at least a factor of 1.5 for strain TA100.

Generally a concentration-related effect should be demonstrable.

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: Normal background growth was observed with both strains. The numbers of revertant colonies were not reduced. From the results obtained, the highest concentration suitable for the mutagenicity test was selected to be 5000.0 µg/plate with and without metabolic activation.

Mutagenicity assay, original experiment: In the experiments performed with and without metabolic activation, treatment of strains TA98, TA100, TA1535, TA1537 and WP2 uvrA with CA1139A did not lead to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants in comparison with the negative control.

Mutagenicity test, confirmatory experiment: In the experiments performed with and without metabolic activation, again after treatment of strains TA98, TA100, TA1535, TA1537 and WP2 uvrA with CA1139A no increase in the incidence of either histidine- or tryptophan-prototrophic mutants was observed in comparison with the negative control.

In the mutagenicity tests normal background growth was observed with most strains at all concentrations. Only strain TA100 showed reduced background growth at the highest concentration. The numbers of

revertant colonies were not reduced with increasing concentration. Therefore, the test substance exerted no substantial toxic effect on the growth of the bacteria.

CONCLUSIONS: Based on the results of these experiments and on standard evaluation criteria, it is concluded that CA1139A and its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

(Hertner T, 1992)

Report:	K-CA 5.8.1/08 Chang, S. (2017b). CA1139 - Cell Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1781800, 31 May 2017. Unpublished. Syngenta File No. CA1139_10008.

GUIDELINES: OECD 476 (2016).

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

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

EXECUTIVE SUMMARY

The maximum concentration of the pre-experiment and the main experiments of 2600 µg/mL was chosen with respect to the current OECD Guideline 476 (2016) and a correction factor of 1.3 was used to correct for purity and the ratio of phenylguanidine to carbonate counter ion in the test substance.

Experiment I
without metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL
with metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

Experiment II
with metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

Experiment III
without metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

In Experiment I, relevant cytotoxic effects indicated by an relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred in Experiment I at 2166.6 µg/mL and above without metabolic activation and at 2600.0 µg/mL with metabolic activation. The recommended toxic range of approximately 10-20% relative adjusted cloning efficiency 1 was covered with and without metabolic activation.

In Experiment I without metabolic activation, no increase outside the 95% control limits of the solvent historical control data was observed when the mean data set was evaluated. The positive t-test at 2166.6 µg/mL and 2600 µg/mL is due to the mutation frequency being lower than the concurrent negative control, not to an increase in mutant frequency and hence is not relevant. The linear regression analysis for this experiment was negative. Due to the heterogeneous response between Culture I and Culture II at the three highest concentrations and an increase in mutant frequency (MF) in Culture I at an intermediate concentration (1733.3 µg/mL) a repeat experiment was performed (Experiment III).

In the repeat experiment with metabolic activation (Experiment III), a relevant cytotoxic effect occurred at 2600.0 µg/mL. However, a shift in the cytotoxicity profile compared to Experiment I was observed and the recommended toxic range of approximately 10-20% relative adjusted cloning efficiency I (RS) was not reached in this repeat experiment.

No increase in MF outside the 95% control limits was observed on individual culture level or the mean data. The positive t-test at the highest concentration tested was again due to the mutant frequency in the treated cells being lower than the concurrent negative control and therefore is not relevant. The linear regression analysis was negative. Overall, in the absence of metabolic activation in Experiment I and III no relavent increase in MF were observed.

In Experiment I with metabolic activation, the 95% control limit was exceeded for both the solvent control and 2600.0 µg/mL with metabolic activation. However, the t-test indicated no significant increase at this concentration and the linear regression analysis (trend test) was negative. This observed isolated increase in MF was mainly due to an increase in MF in Culture I, which was not reproduced in Culture II. At the individual culture level an increase in MF outside the 95% control limits was also seen at the intermediate concentration of 650.0 µg/mL in Culture I. Due to the heterogeneous response between Culture I and Culture II at several concentrations and to investigate the observed increases in MF, the experiment was repeated.

In the repeat experiment with metabolic activation (Experiment II) a relevant cytotoxic effect occurred at the highest concentration of 2600.0 µg/mL.

The 95% control limit was solely exceeded at an intermediate concentration of 650.0 µg/mL with metabolic activation. Again this increase was mainly due to an increase mainly in one test culture only (Culture I). The t-test showed no significant trend at this concentration. The linear regression analysis was statistical significant, however, it showed a reciprocal tendency and thus is not of biological significance.

In summary, the increases in MF observed in the presence of metabolic activation were inconsistent between parallel cultures of the experiments and caused by increases in one culture only. None of those increases in MF were statistically significant increases and there was no relevant dose dependent trend. Based on these results, CA1139 meets the criteria for being negative in the HPRT assay (OECD 476, 2016).

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

In conclusion, it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CA1139 is considered to be non-mutagenic in this HPRT assay.

MATERIALS AND METHODS

Materials:

Test Material: CA1139
- **Description:** Light beige, crystalline powder
- **Lot/Batch number:** WRS 1233/1
- **Molecular weight:** 332.4 g/mol (phenylguanidine carbonate); 135.17 g/mol (phenylguanidine)
- **Purity:** 93.8% w/w (estimated error ± 0.5%) (phenylguanidine carbonate); 76.6% w/w (phenylguanidine)
- **Retest date:** 30 September 2022
- **Stability of test compound:** Not indicated by the sponsor

Control Materials:
- **Negative:** -
- **Solvent control (final concentration):** DMSO (0.5 %)
- **Positive control:** Absence of S9 mix: Ethylmethane sulfonate (EMS), 300 µg/mL = 2.4 mM
  Presence of S9 mix: DMBA, 2.3 µg/mL = 8.9 µM

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others

Media: RPMI 1640				
Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No
Periodically "cleansed" against high spontaneous background?	X	Yes		No

X indicates those that apply

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ ATPase**
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment I Experiment III	650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL
Presence of S9 mix	Experiment I Experiment II	650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

Study Design and Methods:

In-life dates: Start: 27 October 2016, End: 18 April 2017

Test performance:

Cell treatment: Cells were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

For seeding and treatment of the cell cultures the complete culture medium was 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. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

Two to three days after subcultivation stock cultures are trypsinized at 37 °C for 5 to 10 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10 % FBS and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2 % in phosphate buffered saline (PBS).

Prior to the trypsin treatment the cells are were with PBS containing 200 mg/l EDTA. Approximately 0.7 to 1.2×10^7 cells were seeded in plastic flasks. The cells were grown for 24 hours prior to treatment. With the cell doubling time of approximately 12 h this ensures a population of more than 20×10^6 cells treated with the test substance.

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G".

Immediately after the end of treatment the cells were trypsinised as described above and sub-cultivated. At least 2.0×10^6 cells per experimental point (concentration series plus controls) were subcultured in 175 cm² flasks containing 30 mL medium.

Two additional 25 cm² flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (cloning efficiency I) as measure of test item induced cytotoxicity. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment as described below.

Three or four days after first sub-cultivation approximately 2.0×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium.

Following the expression time of approximately 7 days five 80 cm² cell culture flasks were seeded with about 3 to 5×10^5 cells each in medium containing 6-TG (11 µg/mL). Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

After 7 to 10 days the colonies were stained with 10% methylene blue in 0.01% KOH solution. Colonies with more than 50 cells were counted. If in doubt the colony size was checked with a preparation microscope.

Statistical Methods: A linear regression analysis (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

A t-test was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to evaluate an isolated increase of the mutation frequency at a test point exceeding the 95% control limit. Again a t-test is judged as significant if the p-value (probability value) is below 0.05.

However, both, biological and statistical significance was considered together.

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent solvent control in both parallel cultures,
b) the increase is concentration-related when evaluated with an appropriate trend test,
c) any of the results are outside the distribution of the historical solvent control data.

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

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
b) there is no concentration-related increase when evaluated with an appropriate trend test,
c) all results are inside the distribution of the historical negative control data

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

There is no requirement for verification of a clearly positive or negative response.

In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert

judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

RESULTS AND DISCUSSION

The test item CA1139 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster.

The assay was performed in three independent experiments, using two parallel cultures each. The first experiment was performed with and without liver microsomal activation and a treatment period of 4 hours. The second experiment was performed with metabolic activation and a treatment period of 4 hours. Experiment III was performed without metabolic activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations:

Experiment I
without metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

with metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

Experiment II
with metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

Experiment III
without metabolic activation: 650.0; 1300.0; 1733.3; 2166.6; and 2600.0 µg/mL

In Experiment I, relevant cytotoxic effects indicated by an relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred in Experiment I at 2166.6 µg/mL and above without metabolic activation and at 2600.0 µg/mL with metabolic activation. The recommended toxic range of approximately 10-20% relative adjusted cloning efficiency 1 was covered with and without metabolic activation.

In Experiment I without metabolic activation, no increase outside the 95% control limits of the solvent historical control data was observed when the mean data set was evaluated. The positive t-test at 2166.6 µg/mL and 2600 µg/mL is due to the mutation frequency being lower than the concurrent negative control, not to an increase in mutant frequency and hence is not relevant. The linear regression analysis for this experiment was negative. Due to the heterogeneous response between Culture I and Culture II at the three highest concentrations and an increase in mutant frequency (MF) in Culture I at an intermediate concentration (1733.3 µg/mL) a repeat experiment was performed (Experiment III).

In the repeat experiment with metabolic activation (Experiment III), a relevant cytotoxic effect occurred at 2600.0 µg/mL. However, a shift in the cytotoxicity profile compared to Experiment I was observed and the recommended toxic range of approximately 10-20% relative adjusted cloning efficiency I (RS) was not reached in this repeat experiment.

No increase in MF outside the 95% control limits was observed on individual culture level or the mean data. The positive t-test at the highest concentration tested was again due to the mutant frequency in the treated cells being lower than the concurrent negative control and therefore is not relevant. The linear regression analysis was negative. Overall, in the absence of metabolic activation in Experiment I and III no relavent increase in MF were observed.

In Experiment I with metabolic activation, the 95% control limit was exceeded for both the solvent control and 2600.0 µg/mL with metabolic activation. However, the t-test indicated no significant increase at this concentration and the linear regression analysis (trend test) was negative. This observed isolated increase in MF was mainly due to an increase in MF in Culture I, which was not reproduced in Culture II. At the individual culture level an increase in MF outside the 95% control limits was also seen at the intermediate concentration of 650.0 µg/mL in Culture I. Due to the heterogeneous response between Culture I and Culture II at several concentrations and to investigate the observed increases in MF, the experiment was repeated.

In the repeat experiment with metabolic activation (Experiment II) a relevant cytotoxic effect occurred at the highest concentration of 2600.0 µg/mL.

The 95% control limit was solely exceeded at an intermediate concentration of 650.0 µg/mL with metabolic activation. Again this increase was mainly due to an increase mainly in one test culture only (Culture I). The t-test showed no significant trend at this concentration. The linear regression analysis was statistical significant, however, it showed a reciprocal tendency and thus is not of biological significance.

In summary, the increases in MF observed in the presence of metabolic activation were inconsistent between parallel cultures of the experiments and caused by increases in one culture only. None of those increases in MF were statistically significant increases and there was no relevant dose dependent trend. Based on these results, CA1139 meets the criteria for being negative in the HPRT assay (OECD 476, 2016).

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

CONCLUSIONS: In conclusion, it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CA1139 is considered to be non-mutagenic in this HPRT assay.

(Chang S, 2017b)

Report:	K-CA 5.8.1/09 Hertner T. (1992a). CA1139A: Cytogenetic Test on Chinese hamster cells in vitro. Genetic Toxicology, Ciba-Geigy Limited, Basle, Switzerland. Report No. 923051, 07 October 1992. Unpublished. Syngenta File No. CA1139/0007.

GUIDELINES: OECD 473 (1983); EPA § 798.5375 (1987); EEC Directive 79/831 B.10 (1984); Japanese MAFF (1985).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: This test system permits the detection of structural chromosome aberrations in Chinese hamster ovary cells *in vitro* induced by the test substance or its metabolites. To ensure that any clastogenic effects of metabolites of the test substance formed in mammals are also detected, an experiment is performed, in which the metabolic turnover of the test material is simulated *in vitro* by the addition of an activation mixture containing rat liver post mitochondrial fraction S9 and co-factors to the cell culture. Metaphase cells were examined from the cultures treated with the test substance for the presence of structural chromosomal aberrations.

EXECUTIVE SUMMARY

In a mammalian cell cytogenetics assay, CA1139A (intermediate of CGA219417, purity 76.2%), was investigated for clastogenic effects on Chinese hamster ovary cells *in vitro* at the following concentrations:

Without extrinsic metabolic activation (S9)

- 18 hour incubation: (original experiment 1and confirmatory experiment 1) 0 (solvent control), 103.13, 206.25, 412.5 µg/mL
- 42 hour incubation: (confirmatory experiment 3) 0 (solvent control), 51.56, 103.13, 206.25 µg/mL
- Mitomycin C 0.2 µg/mL) was used as a positive control in the 18 hour experiments

Without extrinsic metabolic activation (S9)

- 3 hours incubation followed by 15 hours recovery: (original experiment 2 and confirmatory experiment 2) 0 (solvent control), 412.5, 825 and 1650 µg/mL
- 3 hours incubation followed by 39 hours recovery: (confirmatory experiment 4) 0 (solvent control), 825, 1650 and 3300 µg/mL
- Cyclophosphamide (20 µg/mL) was used as a positive control in the 3 hour/15 hour recovery experiments.

Final concentrations (-S9) greater than 412.5 or 206.25 µg/mL of culture medium could not be scored due to cytotoxicity. Final concentrations (+S9) greater than 3300 µg/mL of culture medium could not be achieved due to solubility limitations and in the 3 hour/15 hour recovery experiments, concentrations greater than 1650 µg/mL of culture medium could not be scored due to cytotoxicity.

In all the experiments performed with or without metabolic activation, CA1139A did not induce a biologically significant increase in the number of metaphases containing specific chromosomal aberrations. Positive controls induced the appropriate response.

Conclusion: Under the given experimental conditions no evidence of clastogenic effects was obtained in Chinese hamster ovary cells treated with CA1139A *in vitro*.

MATERIALS AND METHODS

Materials:

Test Material:	CA1139A
Description:	Intermediate of CGA219417, light beige, crystalline powder
Lot/Batch number:	P.201025
Purity:	76.2%
CAS#:	Not reported
Stability of test compound:	Reanalysis date February 1993
Control Materials:	
Negative:	
Solvent control	Culture medium (-S9 mix)
	Nutrient mixture F-12 (+S9 mix)
Positive control:	Without activation: Mitomycin C, 0.2 µg/mL (18 hour treatment)
	With activation: Cyclophosphamide 20 µg/mL (3 hour/15 hour recovery)

Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other		Other		

The metabolic activation system (S9 mix) was prepared immediately before use to give the following concentrations:

Rat liver S9 fraction	15 µL/mL (1.5%)
NADP	3.14 µmol/mL
Isocitric acid (trisodium salt)	15.3 µmol/mL

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. Equal volumes of blood from 2 donors (female for Experiment 1 and male for Experiment 2) were pooled together for each experiment. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
X	Chinese hamster ovary (CHO) cells cell line CCL6

Media: Nutrient mixture F-12 supplemented with 10% foetal calf serum plus penicillin/streptomycin 100 units/mL/100 µg/mL (Gibco AG, Basle, Switzerland)				
Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No

Test compound concentrations used and exposure duration:

Without extrinsic metabolic activation (S9)

- 18 hour incubation: (original experiment 1and confirmatory experiment 1) 0 (solvent control), 103.13, 206.25, 412.5 µg/mL
- 42 hour incubation: (confirmatory experiment 3) 0 (solvent control), 51.56, 103.13, 206.25 µg/mL
- Mitomycin C 0.2 µg/mL) was used as a positive control in the 18 hour experiments

Without extrinsic metabolic activation (S9)

- 3 hours incubation followed by 15 hours recovery: (original experiment 2 and confirmatory experiment 2) 0 (solvent control), 412.5, 825 and 1650 µg/mL
- 3 hours incubation followed by 39 hours recovery: (confirmatory experiment 4) 0 (solvent control), 825, 1650 and 3300 µg/mL
- Cyclophosphamide (20 µg/mL) was used as a positive control in the 3 hour/15 hour recovery experiments.

Study Design and Methods:

Experimental dates: Start: 18 May 1992, End: 14 September 1992

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: Not performed.

Cytotoxicity/mutagenicity assay: The cytotoxicity test was performed as an integral part of the mutagenicity test. A series of glass slides in quadruple culture dishes was seeded with Chinese hamster ovary cells at a density of at least 1 x 10^4 cells/mL. The preincubation time before treatment was 29 hours. The substance in nutrient mixture F-12 was added to the cells in culture medium. In experiments with activation, 0.5 mL of activation mixture was added to 4.5 mL of Nutrient Mixture F-12. Quadruplicate cultures were prepared for each group in each assay.

Two hours prior to harvesting, the cultures were treated with Colcemide 0.4 µg/mL to arrest cells in metaphase. The experiment was terminated by hypotonic treatment (0.075 M KCl solution) of the cells, followed by fixation (methanol:acetic acid, 3:1) . Slides were air-dried and stained with orcein.

The highest concentration used or the lowest concentration which suppresses mitotic activity by approximately 50-80% compared to the control group was selected as the highest for the analysis of chromosome aberrations together with two lower concentrations in succession. For the determination of the mitotic index (M.I.) the preparations from the various cultures were examined first, uncoded. The percentages of mitotic suppression in comparison with the controls were evaluated by counting at least 2000 cells from one slide each of the treatment groups and the negative control group. The determination of the mitotic coefficient was performed for the two experiments of the original study and for the third and fourth experiment of the confirmatory study separately. From the results of corresponding original run, five suitable concentrations were determined for the first and second experiment of the confirmatory study

Scoring of slides: Prior to analysis the selected slides were coded, likewise the cultures treated with the vehicle alone as well as the positive control. Whenever possible two hundred well spread metaphase figures with 19 to 21 centromeres from two cultures (100 metaphases per replicate culture) in the vehicle control and in the treated groups were scored. At least fifty metaphases were scored in the positive controls (25 per replicate culture). The slides were examined for the following aberrations:

a) specific aberrations: breaks, exchanges, fragments,
b) unspecific aberrations: gaps and chromosome decay,
c) numerical alterations (metaphases with >21 centromeres) were registered, but reported only in case of deviations.

Using the Vernier scale on the microscope stage, the coordinates of all metaphases with specific aberrations were recorded.

Evaluation criteria:

Criteria for a positive response

The test substance is generally considered to be active in the Chinese Hamster cells if the following conditions are met:

- The percentage of metaphases containing specific aberrations in a treatment group is higher than 6.0 and differs statistically significantly from the respective value of the negative control.
- A concentration-related response should be demonstrable.

Criteria for a negative response

The test substance is generally considered to be inactive in the Chinese Hamster cells if the following conditions are met:

- The percentage of metaphases containing specific aberrations in all treatment groups is less than or equal to 6.0 and does not differ statistically significant from the respective value of the negative control.

Exceptions

At the limits of the criteria for a positive or for a negative response or if the criteria for a positive response are only partially fulfilled or if effects are obtained at extremely high concentrations or in the toxic range of the test substance only, the Study Director will decide by experience about the interpretation of the results.

Flow cytometry: The DNA distribution of cell cultures was determined by flow cytometry. Cultures treated with the test substance or with the vehicle alone were fixed and stained with DAPI (4',6-diamino-2-phenylindole). Fluorescence of DAPI stained DNA was measured with a Partec PAS-II flow cytophotometer. A substantial shift in the DNA distribution pattern of cell cultures in comparison with the pattern of the vehicle control would indicate a disturbance of the cell cycle induced by the test substance.

Analytical control: To confirm that the cells were actually exposed to the intended test concentrations and to confirm the stability of the test substance in the vehicle used, determination of the concentration of the test substance in solution was performed with the lowest concentration of the stock solution used in the first and last segment of the mutagenicity test. The resulting concentrations were 104.3 and 111.6% of the intended values demonstrating stability of the test substance in solution.

Statistics: The evaluated numbers of specific aberrations were subjected to statistical analysis. In the preliminary test the data were assessed for flask effects (dependence of cells within each culture) using a chi-square test. The nonsignificant result of this test meant there was no substantial evidence to conclude a flask effect (although a flask effect still might exist). Accordingly a chi-square test for trend was performed modelling all cells in a given experiment as independent. That is, the individual cell is taken as the experimental unit and consequently the power of the test was substantially increased. Tests were performed based upon the presence of any specific aberration.

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: Not performed.

Cytotoxicity: The highest concentration of 412.5 µg/mL selected for analysis in the first experiment of the original study caused 77.4% suppression of mitotic activity. The highest concentration of 1650 µg/mL selected for analysis in the second experiment of the original study caused 38.5% suppression of mitotic activity. At the next higher concentration only few cells remained on the slides due to toxicity. In the third experiment of the confirmatory study with a 42 hours treatment period the highest concentration of 206.25 µg/mL selected for analysis caused 47.4% suppression of mitotic activity.At the next higher concentration mitotic activity was suppressed by 92.3%. In the fourth experiment (3 hours treatment 39 hours recovery) the highest concentration of 3300 µg/mL selected for analysis caused 5.9% suppression of mitotic activity. Higher concentrations were not tested due to solubility limitations.

Original mutagenicity study:

1st experiment without S9 mix: In the negative control 1% of metaphases with specific chromosomal aberrations were detected. At the concentration of 103.13 µg/mL 0.5%, at 206.25 µg/mL 0% and at 412.5 µg/mL 2.5% of cells with specific chromosomal aberrations were found.

2nd experiment with metabolic activation: In the negative control 1% of metaphases with specific chromosomal aberrations were seen. At the concentration of 412.5 µg/mL 1%, at 825 µg/mL 1% and at 1650 µg/mL 0.5% of cells with specific chromosomal aberrations were found.

Confirmatory mutagenicity study:

1st experiment without S9 mix: In the negative control 0% of metaphases with specific chromosomal aberrations were detected. At the concentration of 103.13 µg/mL 2%, at 206.25 µg/mL 1.5% and at 412.5 µg/mL 3.5% of cells with specific chromosomal aberrations were registered. The value obtained at the highest concentration showed a statistically significant difference when compared with the respective negative control.

2nd experiment with S9 mix: In the negative control 1% of metaphases with specific chromosomal aberrations were seen. At the concentration of 412.5 µg/mL 2%, at 825 µg/mL 2% and at 1650 µg/mL 5% of cells with specific chromosomal aberrations were found. The value obtained at the highest concentration showed a statistically significant difference when compared with the respective negative control.

3rd experiment without S9 mix: In the negative control cultures 1.5% of metaphases with specific chromosomal aberrations were detected. At the concentration of 51.56 µg/mL 1%, at 103.13µg/mL 4% and at 206.25 µg/mL 1% of cells with specific chromosomal aberrations were observed. The value obtained at the intermediate concentration showed a statistically significant difference when compared with the respective negative control.

4th experiment with S9 mix: In the negative control cultures 2.5% of metaphases with specific chromosomal aberrations were registered. At the concentration of 825 µg/mL 0.5%, at 1650 µg/mL 0.5% and at 3300 µg/mL 6.5% of cells with specific chromosomal aberrations were found. The value obtained at the highest concentration showed a statistically significant difference when compared with the respective negative control.

Positive controls: The treatment of the cultures with mitomycin-C, 0.2 µg/mL and cyclophosphamide, 20.0 µg/mL, respectively, was followed by a high incidence of specific chromosomal aberrations in the experiments one and two of the original study (44% and 32%) and in the experiments one and two of the confirmatory study (28% and 24%).

In all the experiments performed with or without metabolic activation, CA1139A did not induce a biologically significant increase in the number of metaphases containing specific chromosomal aberrations. Positive controls induced the appropriate response.

Table 5.8.1-3: Percentage of metaphases with specific aberrations

Treatment	Expt 1, original study (18 h treatment)	Expt 2, original study (3 h treatment, 15 h recovery)	Expt 1, confirmatory study (18h treatment)	Expt 2, confirmatory study (3 h treatment, 15 h recovery)	Expt 3, confirmatory study (42 h treatment)	Expt 4, confirmatory study (3 h treatment, 39 h recovery)
vehicle control	1	1	0	1	1.5	2.5
51.56 µg/mL					1	
103.13 µg/mL	0.5		2		4*	
206.25 µg/mL	0		1.5		1	
412.5 µg/mL	2.5	1	3.5**	2		
825 µg/mL		1		2		0.5
1650 µg/mL		0.5		5**		0.5
3300 µg/mL						6.5**
Mitomycin-C 0.2 µg/mL (+ve control)	44***		28***			
Cyclophosphamide 20 µg/mL (+ve control)		32***		24***		

Statistically significant difference from vehicle control * p≤0.05; ** p≤0.01; *** p≤0.001;

CONCLUSIONS: Under the given experimental conditions no evidence of clastogenic effects was obtained in Chinese hamster ovary cells treated with CA1139A *in vitro*.

(Hertner T, 1992a)

Report:	K-CA 5.8.1/10 Dony, E. (2015). Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt: Micronucleus Assay in Bone Marrow Cells of the Rat. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Laboratory Report No. 1718800, 30 October 2015. Unpublished. Syngenta File No. CGA263208_10011.

Report:	K-CA 5.8.1/11 Grauert, M. and Kamp, H. (2015) Reg.No. 4182909 (metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt - Plasma analysis for external studies. BASF SE, Experimental Toxicology and Ecology, 67056 Ludwigshafen, Germany. Laboratory Report No. 06Y0319/15Y023, 23 October 2015. Unpublished. Syngenta File No. CGA263208_10010.

GUIDELINES: OECD 474 (2014); OPPTS 870.5395 (1998); 2000/32/EC 440/2008 B.12 (2008).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: The rat was selected as the test species as it is recognized by international guidelines as a standard test species.

EXECUTIVE SUMMARY

This study was performed to investigate the potential of Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt to induce micronuclei in polychromatic erythrocytes (PCE) in the bone marrow of the rat.

The test item was formulated in DMSO / PEG 400 (3/7), which was also used as vehicle control. The volume administered orally was 20 mL/kg b.w. The volume of the positive control administered was 10 mL/kg. 24 h and 48 h after a single administration of the test item the bone marrow cells were collected for micronuclei analysis.

Seven males per test group were evaluated for the occurrence of micronuclei except for the negative and positive control groups with five animals each. At least 4000 polychromatic erythrocytes (PCEs) per animal were scored for micronuclei.

To investigate a cytotoxic effect due to the treatment with the test item the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 500 erythrocytes.

The following dose levels of the test item were investigated:

24 h preparation interval: 125, 250, and 500 mg/kg bw
48 h preparation interval: 500 mg/kg bw

The animals treated with the vehicle control did not exhibit any clinical symptoms.

Clinical symptoms in the main experiment included abdominal posture, partially closed eyes, slightly reduced spontaneous activity, salivation, as well as sleepiness. The animals of all dose levels were affected.

The observed systemic toxicity at the tested doses is indicative for a systemic distribution of the test item. Thus, bioavailability of the test item under the tested conditions is assumed. This was additionally confirmed by analytical detection of the test item in plasma (performed by the Sponsor, see separate report BASF study code 06Y0319/15Y023) (included as K-CA 5.8.1/11).

The highest dose was estimated by two pre-experiments to be suitable. A correction factor of 1.36 was applied based on test item purity data provided by the sponsor.

After treatment with the test item the number of PCEs was not substantially decreased as compared to the mean value of PCEs of the vehicle control thus indicating that Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt did not exert any cytotoxic effects in the bone marrow.

In comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei at any preparation interval after administration of the test item and with any dose level used.

20 mg/kg bw cyclophosphamide administered orally was used as positive control which showed a substantial increase of induced micronucleus frequency.

In conclusion, it can be stated that under the experimental conditions reported, the test item Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt did not induce micronuclei as determined by the micronucleus test with bone marrow cells of the rat. Therefore, Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt is considered to be non-mutagenic in this micronucleus assay.

MATERIALS AND METHODS

Materials:

Test Material:	Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt
Description:	solid, white
Lot/Batch number:	L85-142
Purity:	73.7 %
Stability of test compound:	Retest date : 01 August 2017

Control Materials:

Negative control (if not vehicle) :	N/A	**Final Volume:** N/A	**Route:** N/A
Vehicle:	30% Dimethyl sulphoxide, 70 % Polyethylene glycol	**Final Volume:** 20 mL/kg	**Route:** oral
Positive control :	Cyclophosphamide	**Final Doses:** 20 mg/kg bw	**Route:** oral

Test Animals:

Species	Rat
Strain	Wistar
Age/weight at dosing	Pre-test 1: 8 – 9 weeks pre-test 2: 9 – 10 weeks main study: 6 - 7 weeks (beginning of treatment), Main study: mean weight 185.1±8.1 g
Source	Charles River (UK) Ltd., Sandhofer Weg 7, 97633 Sulzfeld, Germany
Housing	Single
Acclimatisation period	At least 5 days
Diet	Pelleted standard diet, *ad libitum*
Water	Tap water, *ad libitum*
Environmental conditions	Temperature: 22±2 °C Humidity: 45 % to 65 % Photoperiod: 12 hours dark/12 hours light

Test compound administration:

	Dose Levels	**Final Volume**	**Route**
Preliminary:	1st pre-experiment: 100 mg/kg bw 2nd pre-experiment: 500 mg/kg bw	20 mL/kg bw	oral
Main Study:	125, 250, 500 mg/kg bw males only	20 mL/kg bw	oral

Study Design and Methods:

In-life dates: Start: 03 September 2015, End: 06 August 2015

Preliminary Toxicity Assay: The animals were treated once orally with the test item and examined for acute toxic symptoms at intervals of approx. 0-1 h, 2-4 h, 5-6 h, 24 h, 30 h, and 48 h after administration of the test item.

Since bone marrow is well perfused, exposure of the bone marrow to the test item was indirectly assessed in the blood of 3 satellite animals. For this purpose, blood sampling was performed 1 h and 4 h after a single oral application of the highest test item dose (0.5 mL each). Additionally, 3 animals were dosed with the negative control item once and their blood was withdrawn 1 h 13 min. The blood of the animals was collected in tubes containing K_3-EDTA. The blood samples were centrifuged at 10'000 rpm for about 5 minutes to obtain plasma samples. The plasma samples were stored at ≤ - 80°C at Envigo CRS GmbH and were sent to the sponsor for further analysis. Presence of the test material in the blood was confirmed. Results were reported in a separate report BASF study code 06Y0319/15Y023 (included as K-CA 5.8.1/11).

Micronucleus Test: Animals in Groups 1 to 7 were dosed once by oral gavage, with vehicle alone (negative control), the test substance Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) or a 20 mg/kg bw dose of cyclophosphamide at a dose volume of 20 mL/kg. Bone marrow was harvested after 24 h for Groups 1 to 5 and 48 h for Group 6 and 7.

The animals of all dose groups, except the positive control, were examined for acute toxic symptoms at intervals of around 0-1 h, 2-4 h, 5-6 h (except for the 24 h high dose group, which was examined at about 4.5 h after application), 24 h, and/or 48 h after administration of the test item.

Table 5.8.1-4: Experimental Design

Group number	Number of animals	Treatment time (h)	Dose level Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) (mg/kg bw)
1	5	24	Negative Control
2	7	24	125
3	7	24	250
4	7	24	500
5	5	24	Positive Control
6	7	48	Negative Control
7	7	48	500

Animals in Groups 1 to 7 were dosed once by oral gavage, with vehicle alone (negative control), the test substance Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) or a 20 mg/kg bw dose of cyclophosphamide at a dose volume of 20 mL/kg. Bone marrow was harvested after 24 h for Groups 1 to 5 and 48 h for Group 6 and 7.

The animals of all dose groups, except the positive control, were examined for acute toxic symptoms at intervals of around 0-1 h, 2-4 h, 5-6 h (except for the 24 h high dose group, which was examined at about 4.5 h after application), 24 h, and/or 48 h after administration of the test item.

Slide Preparation: The animals were sacrificed using CO2 followed by bleeding. The femora were removed, the epiphyses were cut off and the marrow was flushed out with foetal calf serum (FBS) using a syringe. The nucleated cells were separated from the erythrocytes by using the method of Romagna. Briefly, the cell suspensions were passed through a column consisting of α-Cellulose and Cellulose. The columns were then washed with Hank's buffered saline. The cell suspension was centrifuged at 1500 rpm (390 x g) for 10 minutes and the supernatant was discarded. A small drop of the re-suspended cell pellet was spread on a slide. The smear was air-dried and then stained with May-Grünwald/Giemsa. Cover slips were mounted with EUKITT. At least one slide was made from each bone marrow sample.

Slide Analysis: Evaluation of the slides was performed using NIKON microscopes with 100x oil immersion objectives. At least per animal 4000 polychromatic erythrocytes (PCE) were analysed for micronuclei. To describe a cytotoxic effect the ratio between polychromatic and normochromatic

erythrocytes was determined in the same sample and expressed in polychromatic erythrocytes per 500 erythrocytes. The analysis was performed with coded slides.

RESULTS AND DISCUSSION

Preliminary toxicity assay: 1st pre-experiment: At 1000 mg/kg bw in males and females, clinical observations included abdominal posture, sunken flanks, porphyrine stains and ruffled fur. Other clinical observations were reduced activity, apathy, salivation, disorientation, hyperaemic ears and slight dyspnoea. One male had to be euthanized in moribund condition (lateral posture, coma, salivation, hind legs and testis cyanotic).

2nd pre-experiment: At 500 mg/kg bw in males and females, clinical signs included abdominal posture, ruffled fur, reduced activity, salivation, diarrhoea, lateral posture and thirst.

On the basis of these data 500 mg/kg bw were estimated to be suitable as highest possible dose in either sex. No substantial differences between sexes in toxicity were observed, so that only male animals were used in the main experiment.

Micronucleus test: 24 h and 48 h after a single administration of the test item the bone marrow cells were collected for micronuclei analysis.

Seven males per test group were evaluated for the occurrence of micronuclei except for the negative and positive control groups with five animals each. At least per animal 4000 polychromatic erythrocytes (PCEs) were scored for micronuclei.

To investigate a cytotoxic effect due to the treatment with the test item the ratio between polychromatic and normochromatic erythrocytes was determined in the same sample and reported as the number of PCEs per 500 erythrocytes.

The following dose levels of the test item were investigated:

24 h preparation interval: 125, 250, and 500 mg/kg bw
48 h preparation interval: 500 mg/kg bw

As estimated by two pre-experiments 500 mg Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt per kg bw was suitable as highest treatment dose. Clinical symptoms in the main experiment included abdominal posture, partially closed eyes, slightly reduced spontaneous activity, salivation, as well as sleepiness. The animals of all dose levels were affected.

The observed systemic toxicity at the tested doses is indicative for a systemic distribution of the test item. Thus, bioavailability of the test item under the tested conditions is assumed.

This was additionally confirmed by analytical detection of the test item in plasma (performed by the Sponsor, see separate report BASF study code 06Y0319/15Y023 (included as K-CA 5.8.1/11).

The mean number of polychromatic erythrocytes was not substantially decreased after treatment with the test item as compared to the mean value of PCEs of the vehicle control indicating that Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt did not have any cytotoxic properties in the bone marrow.

In comparison to the corresponding vehicle controls there was no statistically significant or biologically relevant enhancement in the frequency of the detected micronuclei at any preparation interval and dose level after administration of the test item. The mean values of micronuclei observed after treatment with

Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt were below or equal to the value of the respective vehicle control group.

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

CONCLUSIONS: It can be stated that during the study described and under the experimental conditions reported, the test item Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt did not induce micronuclei as determined by the micronucleus test in the bone marrow cells of the rat.

Therefore, Reg.No. 4182909 (Metabolite of BAS 605 F, Pyrimethanil) tested as carbonate salt is considered to be non-mutagenic in this micronucleus assay.

(Dony E, 2015)

Genotoxicity Studies on CGA304075

Report:	K-CA 5.8.1/~~11~~12 Hertner T. (1994a). CGA304075 tech. (plant metabolite of CGA219417): Salmonella and Escherichia/Mammalian-Microsome Mutagenicity Test. Ciba-Geigy Limited, Basle, Switzerland. Report No. 943054, 18 July 1994 . Unpublished. Syngenta File No. CGA304075/0002.

GUIDELINES: OECD 471 (1983); 92/69/EEC (1992); Ministry of Health and Welfare, Japan, Notification No 118 (1984); EPA § 798.5265 (1987).

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

No pre-incubation experiment was performed. Otherwise, there were no deviations from the current regulatory guideline considered to compromise the scientific validity of the study.

JUSTIFICATION FOR TEST SYSTEM SELECTION: This test permits the detection of gene mutations induced by the test material or its metabolites in histidine-requiring strains of *Salmonella typhimurium* and in a tryptophan-requiring strain of *Escherichia coli*. When the Salmonella strains are exposed to a mutagen, some of the bacteria in the treated population, through chemical interaction with the compound or its metabolites, undergo genetic changes which cause them to revert to a non-histidine-requiring state and thus to grow in the absence of exogenous histidine. Similarly, after mutation, the *Escherichia coli* bacteria are able to grow in tryptophan-free medium. Mutagenic effects of the test substance are demonstrable on comparison of the number of bacteria in the treated and control cultures that have undergone reverse-mutation to histidine prototrophism or tryptophan prototrophism, respectively. Different tester strains are used because of differing sensitivities to known mutagens.

EXECUTIVE SUMMARY

CGA304075 tech. was tested for mutagenic effects *in vitro* in histidine-requiring strains of *Salmonella typhimurium* and tryptophan-requiring strains of *Escherichia coli*. The following strains were used: *S. typhimurium* TA98, TA100, TA102, TA1535, TA 1537 and *E. coli* WP2 *uvr*A. The test was performed with and without the addition of rat-liver post mitochondrial supernatant (S9 fraction) as an extrinsic metabolic activation system. The compound was dissolved in DMSO and tested at five concentrations in the range of 312.5 to 5000.0 µg/plate in the presence and absence of a metabolic activation system. In order to confirm the results, the experiments were repeated with and without metabolic activation at five concentrations in the range of 78.13 to 1250.0 µg/plate. Each strain was additionally tested in the presence and in the absence of a metabolic activation system with a suitable, known mutagen as positive control.

In both experiments, performed with and without metabolic activation, none of the tested concentrations of CGA304075 tech. led to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants by comparison with the negative control.

In conclusion, based on the results of these experiments and on standard evaluation criteria, it is concluded that CGA304075 tech. (plant metabolite of CGA219417) and its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

MATERIALS AND METHODS

Materials:

Test Material: CGA304075 tech.
Description: solid
Lot/Batch number: MU-4421/6
Purity: 99 %
CAS#: N/A
Stability of test compound: stable, reanalysis date July 1995

Control Materials:
Negative:
Solvent control (final concentration): Dimethylsulfoxide (DMSO) 10 µL/plate
Positive control: Nonactivation:
Sodium azide 5.0 µg/plate TA100, TA1535
4-Nitroquinoline (4-NQO) 2.0 µg/plate WP2 uvrA
Mitomycin C 2.0 µg/plate TA102
2-Nitrofluorene 20.0 µg/plate TA98
9-Aminoacridine 150 µg/plate TA1537
Activation:
2-Aminoanthracene
2.5 µg/plate TA98, TA100, TA1537
20.0 µg/plate TA102
50 µg/plate WP2 uvrA
Cyclophosphamide
400 µg/plate TA1535

Mammalian metabolic system: S9 derived

X	Induced	X	Aroclor 1254	X	Rat	X	Liver
	Non-induced		Phenobarbitol		Mouse		Lung
			None		Hamster		Other
			Other β-naphthoflavone		Other		

Rat-liver post mitochondrial supernatant (S9 fraction) was prepared in advance from male RAI rats (Tif: RAIf [SPF]). The animals were treated with Aroclor 1254 (500 mg/kg, i.p.) 5 days prior to sacrifice. The livers were homogenized with 3 volumes of 150 mM KCI. The homogenate was centrifuged for 15 minutes at 9000x g and the resulting supernatant (S9 fraction) was stored at approximately -80°C for no longer than one year. The protein content of the S9 fraction was 32.22 mg/mL.

The activation mixture contained:

Rat liver S9 fraction	100.0 µL/mL
NADP	4.0 µmol/mL
$MgCl_2$	8.0 µmol/mL
KCl	33.0 µmol/mL
Na-phosphate-buffer, pH 7.4	100.0 µmol/mL
Glucose-6-phosphate	5.0 µmol/mL

Test organisms:

S. typhimurium strains

	TA97	X	TA98	X	TA100	X	TA102		TA104
X	TA1535	X	TA1537		TA1538		list any others		

E. coli strains

	WP2 (pKM101)	X	WP2 *uvrA* (pKM101)						

Properly maintained?	X	Yes		No
Checked for appropriate genetic markers (*rfa* mutation, R factor)?	X	Yes		No

Test compound concentrations used:

Preliminary cytotoxicity assay:

Non-activated conditions: 5000, 1666.67, 555.56, 186.19, 61.73 and 20.58 µg/plate
Activated conditions: 5000, 1666.67, 555.56, 186.19, 61.73 and 20.58 µg/plate

Mutagenicity assay:

Non-activated conditions: 5000, 2500, 1250, 625 and 312.50 µg/plate
Activated conditions: 5000, 2500, 1250, 625 and 312.50 µg/plate

Confirmatory experiment:

Non-activated conditions: 1250, 625, 312.50, 156.25 and 78.13 µg/plate
Activated conditions: 1250, 625, 312.50, 156.25 and 78.13 µg/plate

For all strains triplicate plates were used for all test substance and positive control treatments. For solvent controls 5 plates were used.

Study Design and Methods:

In-life dates: Start: 15 June 1994 End: 04 July 1994

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: Six concentrations of CGA304075 tech. were tested with *Salmonella typhimurium* strain TA100 and *Escherichia coli* strain WP2 uvrA to determine the highest concentration to be used in the mutagenicity assay. The highest concentration applied was 5000 µg/plate. The five lower concentrations decreased by a factor of three. The plates were inverted and incubated for about 48 hours

at 37±1.5°C in darkness. Thereafter, they were evaluated by counting the colonies and determining the background lawn. One plate per test material concentration and negative control was used.

Type of Bacterial assay:

- X standard plate test (both experiments –S9, initial experiment +S9)
- __ pre-incubation (60 minutes) (second experiment +S9)
- __ "Prival" modification (i.e. azo-reduction method)
- __ spot test
- __ other

Mutagenicity assay: This was performed with *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, TA1537 and with *Escherichia coli* strain WP2 *uvr*A with and without metabolic activation. Each of five concentrations of the test material, a negative and a positive control were tested using three plates per test material concentration and controls. The highest concentration applied was determined in the preliminary range finding test and the four lower concentrations decreased by a factor of three. The plates were inverted and incubated for about 48 hours at 37±1.5°C in darkness. Thereafter, they were evaluated by counting the number of colonies and determining the background lawn.

Protocol: 0.1 mL of the overnight cultures was mixed with 2 mL of top agar, either 0.5 mL of 100 mM sodium phosphate buffer (experiments without activation) or 0.5 mL of the activation mixture (experiments with activation) and 0.1 mL of a solution of the test material, the positive control or the solvent as a negative control and poured on minimal agar in Petri dishes. Each Petri dish contained about 20.0 mL of minimal agar (1.5% agar supplemented with 2% salts of the Vogel-Bonner Medium E and 2% glucose). The top agar was composed of 0.6% agar and 0.6% NaCl. In the experiment with *Salmonella* the top agar was supplemented with 10% of 0.5 mM L-histidine and 0.5 mM (+)biotin dissolved in water. In the experiment with *E.coli* it was supplemented with 10% of 0.5 mM L-tryptophan dissolved in water.

Colony counting and scoring of the plates: Colonies were counted electronically or manually where minor agar damage or test material precipitates or strong colouration of the agar plates might have interfered with automating counting. Observations indicating precipitates of the test material in the top agar or a reduced or absent bacterial background lawn were registered additionally. Means for all mutagenicity assays were calculated.

Statistical analysis: A statistical analysis was not performed. At present the use of statistical methods concerning this particular test is not generally recommended.

Evaluation criteria: The test substance will be considered to be positive in the test system if one or both of the following conditions are met:

- At least a reproducible doubling of the mean number of revertants per plate above that of the negative control at any concentration for one or more of the following strains: TA98, TA1535, TA1537, *E.coli* WP2 *uvrA*.

- A reproducible increase of the mean number of revertants per plate for any concentration above that of the negative control by at least a factor of 1.5 for strains TA 100 or TA 102.

Generally a concentration-related effect should be demonstrable.

RESULTS AND DISCUSSION

Preliminary range finding test: Background growth was inhibited or reduced down to the concentration of 1666.7 µg/plate with both strains tested. The numbers of revertant colonies were reduced at the highest

concentration. From the results obtained, the highest concentration suitable for the mutagenicity test was selected to be 5000.0 µg/plate, with and without metabolic activation.

Mutagenicity assay, original experiment: In the experiments performed with and without metabolic activation the treatment of strains TA98, TA100, TA102, TA1535, TA1537 and WP2 *uvrA* with CGA304075 tech. did not lead to an increase in the incidence of either histidine- or tryptophan-prototrophic mutants in comparison with the negative control.

The test substance exerted cytotoxic effects on the growth of the bacteria at concentrations from 1250 to 2500 µg/plate and higher. With strain TA102 toxicity was noted down to 312.5 to 625 µg/plate.

Precipitates of the compound were visible on the plates at the concentration of 5000 µg/plate.

Mutagenicity test, confirmatory experiment: Due to toxic effects observed in the original experiment lower concentrations were tested in the confirmatory experiment. The highest concentration selected was 1250 µg/plate for all strains. In the experiments performed with and without metabolic activation, again after treatment of strains TA98, TA100, TA102, TA1535, TA1537 and WP2 *uvrA* with GCGA304075 tech. no increase in the incidence of either histidine- or tryptophan-prototrophic mutants was observed in comparison with the negative control.

Normal background growth was observed with all strains at all concentrations. The numbers of revertant colonies were reduced with some strains at the highest concentration.

CONCLUSION: Based on the results of these experiments and on standard evaluation criteria, it is concluded that CGA304075 tech. (plant metabolite of CGA219417) and its metabolites did not induce gene mutations in the strains of *S. typhimurium* and *E. coli* used.

(Hertner T, 1994a)

Report:	K-CA 5.8.1/~~12~~13 Chang S. (2017c). CGA304075 - Cell Gene Mutation Assay in Chinese Hamster V79 Cells *in vitro* (V79/HPRT). Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1781600, 02 June 2017. Unpublished. Syngenta File No. CGA304075_10005.

GUIDELINES: OECD 476 (2016).

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

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

EXECUTIVE SUMMARY

The test item CGA304075 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster. The assay was performed in one experiment, using two parallel cultures. The experiment was performed with and without liver microsomal activation and a treatment period of 4 hours.

The maximum concentration of the test item in the pre-experiment and the main experiments was 2600 µg/mL, equal to a molar concentration of about 10 mM.

The main experiments were evaluated at the following concentrations:

Experiment I
without metabolic activation: 32.0; 42.7; 53.4; 64.0; and 128.0 µg/mL
with metabolic activation: 16.0; 32.0; 64.0; and 85.5µg/mL

Experiment II
without metabolic activation: 32.0; 64.0; 96.0; and 128.0 µg/mL
with metabolic activation: 32.0; 64.0; 80.0; and 96.0 µg/mL

Experiment III
without metabolic activation: 12.5; 25.0; 50.0; and 60.0 µg/mL
with metabolic activation: 30.0; 60.0, 70.0; and 80.0 µg/mL

Experiment I: Absence of metabolic activation

In Experiment I without metabolic activation a relevant cytotoxic effect indicated by a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 64.0 µg/mL. The data generated at the next higher concentration of 128.0 µg/mL in the first experiment without metabolic activation are not considered acceptable as the relative adjusted cloning efficiency I dropped below 1%. No relevant and reproducible increase in mutant colony numbers/106 cells was observed up to the maximum concentration tested. Although none of the MF of the mean data exceeded the 95% control limits of the historical control data, the MF at 32.0, 42.7 and 64.0 µg/mL were statistically significant in the t-test. The analysis of the individual cultures showed a very heterogeneous response and those increases were caused by increases in MF in one culture only.

Experiment I: Presence of metabolic activation

In Experiment I in the presence of metabolic activation strong cytotoxicity below 10% was observed at 85.5 µg/mL. Due to a very steep gradient of cytotoxicity the recommended relative adjusted cloning efficiency I range of approximately 10-20% was not reached. No relevant and reproducible increase in mutant colony numbers/106 cells was observed up to the maximum concentration tested. The 95% control limit of the solvent historical control data was slightly exceeded at 16.0 and 85.5 µg/mL with metabolic activation. However, the t-test indicated no significant increase at these concentrations. The linear regression analysis indicated no significant trend as well.

Due to the heterogeneous response between Culture I and Culture II in Experiment I with and without metabolic activation and the increases in mutant frequency (MF) observed in the individual cultures, a repeat experiment was performed. Therefore, in order to identify a more appropriate range for cytotoxicity, the concentration range was modified for Experiment II.

Experiment II: Absence of metabolic activation

A relevant cytotoxic effect indicated as a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at ≥64.0 µmg/mL and above in the experiments without metabolic activation. At the next higher concentration a very steep increase in cytotoxicity occurred. The data generated at ≥112.0 µg/mL are considered to be unacceptable as the relative adjusted cloning efficiency I dropped below 1%. The recommended relative adjusted cloning efficiency I of approximately 10-20% was not reached. The 95% control limit of the historical control data was not exceeded up to the highest concentrations tested and the linear regression analysis indicated no statistical significant increased trend in MF.

Experiment II: Presence of metabolic activation

A relevant cytotoxic effect indicated as a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at ≥80.0 µg/mL and above. At the next higher concentration a very steep

increase in cytotoxicity occurred. The data generated at ≥112.0 µg/mL are considered to be unacceptable as the relative adjusted cloning efficiency I dropped below 1%. The recommended relative adjusted cloning efficiency I of approximately 10-20% was not reached. The 95% control limit of the historical control data was not exceeded up to the highest concentrations tested and linear regression analysis indicated no statistical significant increased trend in MF. Although none of the MF of the mean data exceeded the 95% control limits of the historical control data, the MF at all analysed concentrations were statistically significant in a t-test.

Because less than four test concentrations were available that had acceptable levels of cytotoxicity and the recommended range of cytotoxicity was not attained in Experiment II, a third experiment (Experiment III) was performed with and without metabolic activation with a further modified concentration range.

Experiment III: Absence of metabolic activation

A relevant cytotoxic effect indicated by a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 50.0 µg/mL. The recommended relative adjusted cloning efficiency I of approximately 10-20% was not achieved where again a very steep increase in cytotoxicity was observed. Therefore, all higher concentrations were not continued due to the strong cytotoxicity observed. There were no relevant and reproducible increases in mutant colony numbers/106 cells observed up to the maximum concentrations tested. The 95% control limit was not exceeded. No statistical significance was observed in the t-test and the linear regression analysis.

Experiment III: Presence of metabolic activation

A relevant cytotoxic effect indicated by a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 70.0 µg/mL. The recommended a relative adjusted cloning efficiency I of approximately 10-20% was achieved. There were no relevant and reproducible increases in mutant colony numbers/106 cells observed up to the maximum concentrations tested. The 95% control limit was not exceeded at any concentration analysed. Although none of the MF of the mean data exceeded the 95% control limits of the historical control data, the MF at the two lowest concentrations tested (30.0 and 60.0 µg/mL) were statistically significant in a t-test. The linear regression analysis indicated no significant trend.

In summary, for the experiments with metabolic activation, increases in MF outside the 95% control limits were only observed in Experiment I with metabolic activation. These increases were not reproduced in the two repeated experiments (Experiments II and III) and were not statistically significant in a t-test and were negative in the linear regression analysis trend test. The increases in MF observed on individual culture levels were inconsistent between parallel cultures of the experiments and are therefore considered not to be of biological relevance. Furthermore, in Experiment III, CGA304075 was tested in the recommended cytotoxicity range of a relative adjusted cloning efficiency of approximately 10-20% and no increase in MF was observed.

Although the recommended cytotoxicity level was not achieved for the conditions without metabolic activation due to the steep increase in cytotoxicity, even when very narrow concentration spacing was utilized, no increases in MF outside the 95% control limits were observed at very cytotoxic levels in Experiment I and II or at any other concentration tested when the mean data were evaluated. Statistical significance in a t-test was only observed for some concentration in Experiment I and not reproduced in the two repeat experiments. The linear regression analysis showed no statistically significant trend in any of the experiments for the conditions without metabolic activation. The increases in MF observed on an individual culture level were inconsistent between parallel cultures of the experiments and are therefore considered not to be of biological relevance.

In the main experiments of this study (with and without S9 mix) the range of the solvent controls was from 12.2 up to 23.0 mutants per 106 cells; the range of the groups treated with the test item was from 15.3 up to 30.2 mutants per 106 cells. These data refer to the mean values of culture I and II.

Based on these results, CGA304075 meets the criteria for being negative in the HPRT assay (OECD 476, 2016).

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

In conclusion, it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CGA304075 is considered to be non-mutagenic in this HPRT assay.

MATERIALS AND METHODS

Materials:

Test Material:	CGA304075
Description:	Yellowish solid
Lot/Batch number:	MES 500/1
Molecular weight:	241.3 g/mol
Purity:	98% w/w (estimated error ± 2%) correction for purity was made.
Retest date:	30 November 2018
Stability of test compound:	Not indicated by the sponsor
Control Materials:	
Negative:	-
Solvent control (final concentration):	DMSO (0.5 %)
Positive control:	Absence of S9 mix: Ethylmethane sulfonate (EMS), 300 µg/mL = 2.4 mM Presence of S9 mix: DMBA, 2.3 µg/mL = 8.9 µM

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to give a final protein concentration of 0.75 mg/mL in the cultures. Cofactors were added to the S9 mix to reach the following concentrations:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP

in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. The concentration in the final test medium was 5 % (v/v).

Test cells: mammalian cells in culture

	Mouse lymphoma L5178Y cells	X	V79 cells (Chinese hamster lung fibroblasts)
	Chinese hamster ovary (CHO) cells		List any others

Media: RPMI 1640				
Properly maintained?	X	Yes		No
Periodically checked for Mycoplasma contamination?	X	Yes		No
Periodically checked for karyotype stability?	X	Yes		No
Periodically "cleansed" against high spontaneous background?	X	Yes		No

X indicates those that apply

Locus Examined:		**Thymidine kinase (TK)**		**Hypoxanthine-guanine-phosphoribosyl transferase (HGPRT)**		**Na+/K+ ATPase**
Selection agent:		Bromodeoxyuridine (BrdU)		8-azaguanine (8-AG)		ouabain
		Fluorodeoxyuridine (FdU)	X	6-thioguanine (6-TG)		
		Trifluorothymidine (TFT)				

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment I Experiment II Experiment III	32.0; 42.7; 53.4; 64.0; and 128.0 µg/mL 32.0; 64.0; 96.0; 112.0; and 128.0 µg/mL 12.5; 25.0; 50.0; and 60.0 µg/mL
Presence of S9 mix	Experiment I Experiment II Experiment III	16.0; 32.0; 64.0; and 85.5µg/mL 32.0; 64.0; 80.0; and 96.0 µg/mL 30.0; 60.0, 70.0; and 80.0 µg/mL

Study Design and Methods:

In-life dates: Start: 08 December 2016, End: 16 May 2017

Test performance:

Cell treatment: Cells were exposed to test compound, negative/solvent or positive controls for 4 hours in both the presence and absence of S9 mix.

For seeding and treatment of the cell cultures the complete culture medium was 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. All cultures were incubated at 37 °C in a humidified atmosphere with 1.5 % CO_2 (98.5 % air).

Two to three days after subcultivation stock cultures are trypsinized at 37 °C for 5 to 10 minutes. Then the enzymatic digestion was stopped by adding complete culture medium with 10 % FBS and a single cell suspension was prepared. The trypsin concentration for all subculturing steps was 0.2 % in phosphate buffered saline (PBS).

Prior to the trypsin treatment the cells are were with PBS containing 200 mg/l EDTA. Approximately 0.7 to 1.2×10^7 cells were seeded in plastic flasks. The cells were grown for 24 hours prior to treatment. With the cell doubling time of approximately 12 h this ensures a population of more than 20×10^6 cells treated with the test substance.

After 24 hours the medium was replaced with serum-free medium containing the test item, either without S9 mix or with 50 µl/mL S9 mix. Concurrent solvent and positive controls were treated in parallel. After 4 hours this medium was replaced with complete medium following two washing steps with "saline G".

Immediately after the end of treatment the cells were trypsinised as described above and sub-cultivated. At least 2.0×10^6 cells per experimental point (concentration series plus controls) were subcultured in 175 cm² flasks containing 30 mL medium.

Two additional 25 cm² flasks were seeded per experimental point with approx. 500 cells each to determine the relative survival (cloning efficiency I) as measure of test item induced cytotoxicity. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

The colonies used to determine the cloning efficiency I were fixed and stained 6 to 8 days after treatment as described below.

Three or four days after first sub-cultivation approximately 2.0×10^6 cells per experimental point were sub-cultivated in 175 cm² flasks containing 30 mL medium.

Following the expression time of approximately 7 days five 80 cm² cell culture flasks were seeded with about 3 to 5×10^5 cells each in medium containing 6-TG (11 µg/mL). Two additional 25 cm² flasks were seeded with approx. 500 cells each in non-selective medium to determine the viability. The cultures were incubated at 37 °C in a humidified atmosphere with 1.5% CO_2.

After 7 to 10 days the colonies were stained with 10% methylene blue in 0.01% KOH solution. Colonies with more than 50 cells were counted. If in doubt the colony size was checked with a preparation microscope.

Statistical Methods: A linear regression analysis (least squares, calculated using a validated excel spreadsheet) was performed to assess a possible dose dependent increase of mutant frequencies. The numbers of mutant colonies generated with the test item were compared to the solvent control groups. A trend is judged as significant whenever the p-value (probability value) is below 0.05.

A t-test was performed using a validated test script of "R", a language and environment for statistical computing and graphics, to evaluate an isolated increase of the mutation frequency at a test point exceeding the 95% control limit. Again a t-test is judged as significant if the p-value (probability value) is below 0.05.

However, both, biological and statistical significance was considered together.

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent solvent control in both parallel cultures,
b) the increase is concentration-related when evaluated with an appropriate trend test,
c) any of the results are outside the distribution of the historical solvent control data.

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

Providing that all acceptability criteria are fulfilled, a test chemical is considered clearly negative if, in all experimental conditions examined:

a) none of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control,
b) there is no concentration-related increase when evaluated with an appropriate trend test,
c) all results are inside the distribution of the historical negative control data

The test chemical is then considered unable to induce gene mutations in cultured mammalian cells in this test system.

There is no requirement for verification of a clearly positive or negative response.

In cases when the response is neither clearly negative nor clearly positive as described above, or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. Performing a repeat experiment possibly using modified experimental conditions (e.g. concentration spacing) could be useful.

In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results. Therefore the test chemical response should be concluded to be equivocal (interpreted as equally likely to be positive or negative).

RESULTS AND DISCUSSION

The test item CGA304075 was assessed for its potential to induce gene mutations at the HPRT locus using V79 cells of the Chinese hamster.

The assay was performed in two experiments, using two parallel cultures. The experiments were performed with and without liver microsomal activation and a treatment period of 4 hours.

The main experiments were evaluated at the following concentrations:

Experiment I
without metabolic activation: 32.0; 42.7; 53.4; 64.0; and 128.0 µg/mL
with metabolic activation: 16.0; 32.0; 64.0; and 85.5µg/mL

Experiment II
without metabolic activation: 32.0; 64.0; 96.0; and 128.0 µg/mL
with metabolic activation: 32.0; 64.0; 80.0; and 96.0 µg/mL

Experiment III
without metabolic activation: 12.5; 25.0; 50.0; and 60.0 µg/mL
with metabolic activation: 30.0; 60.0, 70.0; and 80.0 µg/mL

Experiment I: Absence of metabolic activation

In Experiment I without metabolic activation a relevant cytotoxic effect indicated by a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 64.0 µg/mL. The data generated at the next higher concentration of 128.0 µg/mL in the first experiment without metabolic activation are not considered acceptable as the relative adjusted cloning efficiency I dropped below 1%. No relevant and reproducible increase in mutant colony numbers/106 cells was observed up to the maximum concentration tested. Although none of the MF of the mean data exceeded the 95% control limits of the historical control data, the MF at 32.0, 42.7 and 64.0 µg/mL were statistically significant in the t-test. The analysis of the individual cultures showed a very heterogeneous response and those increases were caused by increases in MF in one culture only.

Experiment I: Presence of metabolic activation

In Experiment I in the presence of metabolic activation strong cytotoxicity below 10% was observed at 85.5 µg/mL. Due to a very steep gradient of cytotoxicity the recommended relative adjusted cloning efficiency I range of approximately 10-20% was not reached. No relevant and reproducible increase in mutant colony numbers/106 cells was observed up to the maximum concentration tested. The 95% control limit of the solvent historical control data was slightly exceeded at 16.0 and 85.5 µg/mL with metabolic activation. However, the t-test indicated no significant increase at these concentrations. The linear regression analysis indicated no significant trend as well.

Due to the heterogeneous response between Culture I and Culture II in Experiment I with and without metabolic activation and the increases in mutant frequency (MF) observed in the individual cultures, a repeat experiment was performed. Therefore, in order to identify a more appropriate range for cytotoxicity, the concentration range was modified for Experiment II.

Experiment II: Absence of metabolic activation

A relevant cytotoxic effect indicated as a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at ≥64.0 µmg/mL and above in the experiments without metabolic activation. At the next higher concentration a very steep increase in cytotoxicity occurred. The data generated at ≥112.0 µg/mL are considered to be unacceptable as the relative adjusted cloning efficiency I dropped below 1%. The recommended relative adjusted cloning efficiency I of approximately 10-20% was not reached. The 95% control limit of the historical control data was not exceeded up to the highest concentrations tested and the linear regression analysis indicated no statistical significant increased trend in MF.

Experiment II: Presence of metabolic activation

A relevant cytotoxic effect indicated as a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at ≥80.0 µg/mL and above. At the next higher concentration a very steep increase in cytotoxicity occurred. The data generated at ≥112.0 µg/mL are considered to be unacceptable as the relative adjusted cloning efficiency I dropped below 1%. The recommended relative adjusted cloning efficiency I of approximately 10-20% was not reached. The 95% control limit of the historical control data was not exceeded up to the highest concentrations tested and linear regression analysis indicated no statistical significant increased trend in MF. Although none of the MF of the mean data exceeded the 95% control limits of the historical control data, the MF at all analysed concentrations were statistically significant in a t-test.

Because less than four test concentrations were available that had acceptable levels of cytotoxicity and the recommended range of cytotoxicity was not attained in Experiment II, a third experiment (Experiment III) was performed with and without metabolic activation with a further modified concentration range.

Experiment III: Absence of metabolic activation

A relevant cytotoxic effect indicated by a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 50.0 µg/mL. The recommended relative adjusted cloning efficiency I of approximately 10-20% was not achieved where again a very steep increase in cytotoxicity was observed. Therefore, all higher concentrations were not continued due to the strong cytotoxicity observed. There were no relevant and reproducible increases in mutant colony numbers/106 cells observed up to the maximum concentrations tested. The 95% control limit was not exceeded. No statistical significance was observed in the t-test and the linear regression analysis.

Experiment III: Presence of metabolic activation

A relevant cytotoxic effect indicated by a relative adjusted cloning efficiency I below 50% (mean value of both parallel cultures) occurred at 70.0 µg/mL. The recommended a relative adjusted cloning efficiency I of approximately 10-20% was achieved. There were no relevant and reproducible increases in mutant colony numbers/106 cells observed up to the maximum concentrations tested. The 95% control limit was not exceeded at any concentration analysed. Although none of the MF of the mean data exceeded the 95% control limits of the historical control data, the MF at the two lowest concentrations tested (30.0 and 60.0 µg/mL) were statistically significant in a t-test. The linear regression analysis indicated no significant trend.

In summary, for the experiments with metabolic activation, increases in MF outside the 95% control limits were only observed in Experiment I with metabolic activation. These increases were not reproduced in the two repeated experiments (Experiments II and III) and were not statistically significant in a t-test and were negative in the linear regression analysis trend test. The increases in MF observed on individual culture levels were inconsistent between parallel cultures of the experiments and are therefore considered not to be of biological relevance. Furthermore, in Experiment III, CGA304075 was tested in the recommended cytotoxicity range of a relative adjusted cloning efficiency of approximately 10-20% and no increase in MF was observed.

Although the recommended cytotoxicity level was not achieved for the conditions without metabolic activation due to the steep increase in cytotoxicity, even when very narrow concentration spacing was utilized, no increases in MF outside the 95% control limits were observed at very cytotoxic levels in Experiment I and II or at any other concentration tested when the mean data were evaluated. Statistical significance in a t-test was only observed for some concentration in Experiment I and not reproduced in the two repeat experiments. The linear regression analysis showed no statistically significant trend in any of the experiments for the conditions without metabolic activation. The increases in MF observed on an individual culture level were inconsistent between parallel cultures of the experiments and are therefore considered not to be of biological relevance.

In the main experiments of this study (with and without S9 mix) the range of the solvent controls was from 12.2 up to 23.0 mutants per 106 cells; the range of the groups treated with the test item was from 15.3 up to 30.2 mutants per 106 cells. These data refer to the mean values of culture I and II.

Based on these results, CGA304075 meets the criteria for being negative in the HPRT assay (OECD 476, 2016).

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

CONCLUSIONS: In conclusion, it can be stated that under the experimental conditions reported the test item did not induce gene mutations at the HPRT locus in V79 cells.

Therefore, CGA304075 is considered to be non-mutagenic in this HPRT assay.

(Chang S, 2017c)

Report:	K-CA 5.8.1/~~13~~14 Naumann, S. (2017c). CGA304075 - Micronucleus test in human lymphocytes *in vitro*. Envigo CRS GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany. Report No. 1781700, 01 June 2017. Unpublished. Syngenta File No. CGA304075_10003.

GUIDELINES: OECD 487 (2016).

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

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

EXECUTIVE SUMMARY

The test substance CGA304075 was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in six independent experiments. In each experimental group two parallel cultures were analysed. Per culture 1000 binucleated cells were evaluated for cytogenetic damage, except for the positive control in Experiment IB without S9 mix, where only 500 binucleated cells were evaluated.

The highest applied concentration in this study (2041 µg/mL of the test substance) was chosen with regard to the purity (98%) of the test substance and with respect to the current OECD Guideline 487. Concentration selection of the cytogenetic experiment was performed considering the toxicity data and precipitation in accordance with OECD Guideline 487.

Precipitation was observed in Experiment IA in the absence and presence of S9 mix at ≥680 µg/mL at the end of treatment. In all other experiments no precipitation was observed.

In Experiment IA, no concentrations in the cytotoxic range were available for evaluation due to the steep increase in cytotoxicity. This experiment repeated with a more refined range of concentrations and designated as Experiment IB. In Experiment IB in the absence of S9 mix, the highest evaluated concentration (239 µg/mL) showed clear cytotoxicity, however, in the presence of S9 mix, again there were no evaluable concentrations present due to the steep increase in cytotoxicity.

Therefore, two further repeat experiments for the short exposure (4 hours) in the presence of S9 mix with a modified dose concentration range were performed (Experiments IIB and IIC, respectively). In the first repeat experiment (Experiment IIB) the concentration selection was further refined and a top concentration of 150 µg/mL was chosen, however, a shift in the cytotoxicity was observed and no concentrations with cytotoxicity were available for evaluation, hence the experiment was repeated. However, in Experiment IIC, moderate cytotoxicity was observed up to the highest applied concentration of 200 µg/mL in the presence of metabolic activation.

In the first experiment for the continuous exposure without S9 mix (Experiment IIA) a steep increase in cytotoxicity was observed and no concentrations with acceptable cytotoxicity were available for evaluation. The first repeat experiment (Experiment IIB) was declared invalid due to strong cytotoxicity which was observed at all concentrations. In Experiment IIC for the continuous exposure without S9 mix, the highest evaluated concentration of 160 µg/mL showed clear cytotoxicity.

In all experimental parts in the absence and presence of S9 mix, no relevant increases in the number of micronucleated cells were observed after treatment with the test item.

Appropriate mutagens were used as positive controls. They induced statistically significant increases in cells with micronuclei.

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

Therefore, CGA304075 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic or the highest applied concentrations.

MATERIALS AND METHODS

Materials:

Test Material:	CGA304075
Description:	Yellowish solid
Lot/Batch number:	MES 500/1
Molecular weight:	241.3 g/mol
Purity:	98% w/w (estimated error ± 2%) correction for purity was made.
Retest date:	30 November 2018

Control Materials:

Negative:	-
Solvent control (final concentration):	Deionised water (10 %)
Positive control:	Absence of S9 mix: MMC, 1.5 µg/mL (Experiment I), Absence of S9 mix: Demecolcin, 100.0 ng/ml (Experiment II) Presence of S9 mix: Cyclophosphamide 17.5 µg/mL

Mammalian metabolic system: S9 derived

X	Induced		Aroclor 1254	X	Rat	X	Liver
	Non-induced	X	Phenobarbitol		Mouse		Lung
			None		Hamster		Other
		X	Other β-naphthoflavone		Other		

X indicates those that apply

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/mL in the cultures. S9 mix contained $MgCl_2$ (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM) and NADP (4 mM) in sodium-ortho-phosphate-buffer (100 mM, pH 7.4).

Test cells: mammalian cells in culture

	V79 cells (Chinese hamster lung fibroblasts)
X	Human lymphocytes. Obtained on the days of culture initiation from healthy, non-smoking donors. All donors had a previously established low incidence of chromosomal aberrations in their peripheral blood lymphocytes.
	Chinese hamster ovary (CHO) cells

X indicates those that apply

Media: DMEM/Ham's F12 (1:1)				
Properly maintained?	X	Yes		No
Periodically checked for *Mycoplasma* contamination?		Yes		No
Periodically checked for karyotype stability?		Yes		No

X indicates those that apply

Test compound concentrations used:

Absence of S9 mix	Experiment IB Experiment IIC	35.1, 107, 207, 239 µg/mL 99.3, 120, 145, 160 µg/mL
Presence of S9 mix	Experiment IIC	136, 150, 182, 200 µg/mL

Study Design and Methods:

In-life dates: Start: 04 January 2017, End: 19 April 2017

TEST PERFORMANCE

Preliminary Cytotoxicity Assay: A preliminary cytotoxicity test was performed to determine the concentrations to be used in the main experiment. The pre-test was performed with 11 concentrations of the test item separated by no more than a factor of √10 and a solvent and positive control. All cell cultures were set up in duplicate. Exposure time was 4 h (with and without S9 mix). The preparation interval was 40 h after start of the exposure.

Cytogenetic Assay:

Cell exposure time:		**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix + S9 mix	Experiment 1	4h 4h	4h 4h	4h 4h
- S9 mix	Experiment 2	20h	20h	20h

Cytokinesis block:	Cytochalasin B (4 µg/mL)

Cell harvest time after termination of treatment:	**Test Material**	**Solvent Control**	**Positive Control**
- S9 mix (4 hour treatment)	36h	36h	36h
+ S9 mix (4 hour treatment)	36h	36h	36h
- S9 mix (20 hour treatment)	0h	0h	0h

Details of slide preparation

Pulse exposure

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation S9 mix (50 µL/mL culture medium) was added. After 4 h the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure (without S9 mix)

About 48 h after seeding, 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with complete medium (with

10 % FBS) containing the test item. After 20 hours the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in and washed with "saline G". The washing procedure was repeated once as described. After washing the cells were re-suspended in complete culture medium containing 10 % FBS (v/v). Cytochalasin B (4 µg/mL) was added and the cells were cultured for approximately 20 hours until preparation.

Preparation and analysis of cells: The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were washed and fixated. The slides were added to a microscope slide and stained with Giemsa. Evaluation of the slides was performed using NIKON microscopes with 40 x objectives. The micronuclei were counted in binucleated cells showing a clearly visible cytoplasm area. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture. Cytotoxicity is expressed as cytostasis, calculating the CBPI, and used therefore as a cut off criteria. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

Evaluation criteria: The percentages of micronuclei in binucleate cells were calculated for each treatment scored. The data have been interpreted as follows:

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly negative if, in all of the experimental conditions examined:

– None of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
– There is no concentration-related increase
– The results in all evaluated test item concentrations should be within the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval)

The test item is then considered unable to induce chromosome breaks and/or gain or loss in this test system.

Providing that all of the acceptability criteria are fulfilled, a test item is considered to be clearly positive if, in any of the experimental conditions examined:

– At least one of the test item concentrations exhibits a statistically significant increase compared with the concurrent solvent control
– The increase is concentration-related in at least one experimental condition
– The results are outside the range of the laboratory historical solvent control data (95% control limit realized as 95% confidence interval)

When all of the criteria are met, the test item is then considered able to induce chromosome breaks and/or gain or loss in this test system.

Statistical analysis: Statistical significance was confirmed by using the Chi-squared test ($\alpha < 0.05$) using a validated R Script for those values that indicate an increase in the number of cells with micronuclei compared to the concurrent solvent control.

RESULTS AND DISCUSSION

Preliminary cytotoxicity assay: A pre-experiment to evaluate the cytotoxicity of the test item was performed. The pre-experiment is reported as the main Experiment I since the criteria mentioned under 'Acceptability of the assay' were met.

Cytogenetic assay: The test substance CGA304075 was assessed for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix.

Five independent experiments were performed. In Experiment IA and IB, the exposure periods were 4 hours with and without S9 mix. In Experiment IIA, the exposure period was 20 hours without S9 mix. In Experiment IIB and IIC, the exposure periods were 4 hours with and 20 hours without S9 mix. The cells were prepared 40 hours after start of treatment with the test substance.

In each experimental group two parallel cultures were analysed. 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides making a total of 2000 binucleated cells per test substance concentration, except for the positive control in Experiment IB without S9 mix, where only 500 binucleated cells were evaluated due to strong clastogenic effects.

To assess cytotoxicity the CBPI (the proportion of second-division cells in the treated population relative to the untreated control) was determined in 500 cells per culture. Percentage of cytostasis (inhibition of cell growth) is also reported.

The highest treatment concentration in Experiment IA, 2041 µg/mL was chosen with regard to the purity (98%), the molecular weight of the test substance and with respect to the OECD Guideline 487 for the *in vitro* mammalian cell micronucleus test.

Precipitation was observed in Experiment IA in the absence and presence of S9 mix at ≥680 µg/mL at the end of treatment. In all other experiments no precipitation was observed.

No relevant influence on the osmolarity or pH was observed.

In Experiment IA, no concentrations in the cytotoxic range were available for evaluation due to the steep increase in cytotoxicity. This experiment was repeated with a more refined range of concentrations and designated as Experiment IB. In Experiment IB in the absence of S9 mix, the highest evaluated concentration (239 µg/mL) showed clear cytotoxicity, however, in the presence of S9 mix, again there were no evaluable concentrations present due to the steep increase in cytotoxicity.

Therefore, two further repeat experiments for the short exposure (4 hours) in the presence of S9 mix with a modified dose concentration range were performed (Experiments IIB and IIC, respectively). In the first repeat experiment (Experiment IIB) no cytotoxicity up to the highest applied concentration of 150 µg/mL was observed. However, in Experiment IIC, moderate cytotoxicity was observed up to the highest applied concentration of 200 µg/mL in the presence of metabolic activation.

In the first experiment for the continuous exposure without S9 mix (Experiment IIA) a steep increase in cytotoxicity was observed and no concentrations with acceptable cytotoxicity were available for evaluation. The first repeat experiment (Experiment IIB) was declared invalid due to strong cytotoxicity which was observed at all concentrations. In Experiment IIC for the continuous exposure without S9 mix, the highest evaluated concentration of 160 µg/mL showed clear cytotoxicity.

In neither the absence nor presence of S9 mix were relevant increases in the number of micronucleated cells observed after treatment with the test item in any of the experiments. The percentage of micronucleated cells at all of the evaluated concentrations was within the 95% confidence interval of the laboratory's historical solvent control data, none of the increases were

statistically significant and no trend was observed. Therefore, the outcome of the study was clearly negative.

Demecolcin (75 ng/mL), MMC (1.0 µg/mL) or CPA (17.5 µg/mL) were used as appropriate positive control chemicals and showed statistically significant increases in binucleated cells with micronuclei.

CONCLUSIONS: It can be stated that under the experimental conditions reported, the test substance did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes.

Therefore, CGA304075 is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to the highest required concentration.

(Naumann S, 2017c)

Table 5.8.1-52: Summary of Toxicity Studies of Metabolites

Metabolite	Study Type	Dose Levels	NO(A)EL / LD_{50}	Major effects at LOAEL	Reference
CGA249287	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Hartmann, 1992*
	90 Day Dietary Toxicity Rat	0, 300, 1000 and 4000 ppm (23.9, 79.5 and 305 mg/kg bw/day for males and 27.2, 90.5 and 343 mg/kg bw/day for females).	NOEL: 1000 ppm (79.5/90.5 mg/kg bw/day for males and females, respectively)	Reduced body weight development and food consumption.	Milburn, 2001*
CGA275535	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Sommer, 2000b*
NOA422054	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Sommer, 2000a*
CGA321915	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Winkler, 1996*
CGA232449	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Hartmann, 1994*
CGA263208	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Hartmann, 1994a
		2000 mg/kg bw	> 2000 mg/kg bw	-	Hartmann, 1991
		2000 mg/kg bw	> 2000 mg/kg bw	-	Hartmann, 1992a
	90 Day Dietary Toxicity Rat	0, 50, 300, 2000 and 8000 ppm (3.1, 17.8, 131, 536 mg/kg bw/day for males and 3.5, 22.1, 140, 616 mg/kg bw/day for females).	NOAEL: 300 ppm (17.8/22.1 mg/kg bw/day for males and females, respectively)	Reduced body weight development and food consumption.	Altmann, 2002
	Pre-natal Developmental Toxicity Rat	0, 20, 200, 400 and 600 mg/kg bw/day	Maternal NOAEL: 200 mg/kg bw/day Foetal NOAEL: 200 mg/kg bw/day	Maternal: Clinical signs, reduced body weight development and food consumption. Foetal: Reduced foetal weight and delayed ossification.	Khalil, 2002
CGA304075	Acute Oral LD_{50} Rat	2000 mg/kg bw	> 2000 mg/kg bw	-	Schoch, 1994

*Included in original EU review.

Toxicity Studies on CGA263208

Report:	K-CA 5.8.1/4416 Hartmann H.R. (1994a). CGA263208 tech. (plant metabolite of CGA219417): Acute oral toxicity in the rat. Novartis Crop Protection AG, 4332 Stein, Switzerland. Amendment no. 2, 2000; Amendment no. 1, 1996; Original report, 1994. Laboratory Report No. 943051, Amendment no. 2, 13 June 2000; Amendment no. 1, 08 August 1996, Original report 06 September 1994. Unpublished. Syngenta File No. CGA263208/00032.

GUIDELINES: OECD 401 (1987); 92/69/EEC, B.1.

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: The rat was selected as the test species as it is recognized by international guidelines as a standard test species.

EXECUTIVE SUMMARY

In an acute oral toxicity study, groups of fasted, young adult, Tif: RAI f (SPF) rats, five per sex, were given a single oral dose of CGA263208 tech. (plant metabolite of CGA219417) (purity 95.2%) in 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80 at doses of 1000 or 2000 mg/kg bw and observed for 14 days.

Two females were found dead following a dose of 2000 mg/kg bw (one 4 hours and the other 1 day after dosing). There were no other mortalities. Piloerection, hunched posture, exophthalmos and dyspnea were seen. Additionally, reduced locomotor activity was seen in all animals. Tremor and ataxia were present in one female at 2000 mg/kg. The surviving animals had recovered within 4 to 5 days. There was no effect on body weight. No deviations from normal morphology were found in any animal.

Conclusion: The acute oral LD50 value for CGA263208 tech. (plant metabolite of CGA219417) was greater than 2000 mg/kg bw in the rat. The LD50 was greater than 2000 mg/kg bw for males and approximately 2000 mg/kg bw for females.

MATERIALS AND METHODS

Materials:

Test Material:	CGA263208 tech. (plant metabolite of CGA219417)
Description:	Technical, solid
Lot/Batch number:	P201025
Purity:	95.2%
CAS#:	Not reported
Stability of test compound:	Reanalysis date June 1995 (stored at 0-5°C)

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

Test Animals:

Species	Rat
Strain	Tif: RAI f (SPF)
Age/weight at dosing	Young adult / 169-212 g
Source	Ciba-Geigy Limited, Animal Production, 4332 Stein, Switzerland
Housing	5 same sex animals/cage, in Macrolon Type 4 cages
Acclimatisation period	At least 5 days
Diet	NAFAG 890 Tox (Nafag, Gossau/SG, Switzerland) *ad libitum* (except for overnight fasting prior to dosing).
Water	Water *ad libitum*
Environmental conditions	Temperature: 22±2°C Humidity: 55±10% Air changes: Approximately 15/hour. Photoperiod: 12 hours light / 12 hours dark.

Study Design and Methods:

In-life dates: Start: 22 June 1994, End: 23 August 1994

Animal assignment and treatment: In an acute oral toxicity study, groups of fasted, young adult, Tif: RAI f (SPF) rats, five per sex, were given a single oral dose of CGA263208 tech. (plant metabolite of CGA219417) (purity 95.2%) at doses of 1000 or 2000 mg/kg bw and observed for 14 days.

The test substance was diluted in 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80 and dosed by oral gavage at a rate of 10 mL/kg body weight.

The animals were examined daily (am and pm on working days and am on weekends) for mortality and viability and were examined once daily for clinical signs for 14 days. Body weights were recorded immediately prior to dosing (day 0) and on days 7 and 14 and at death. All animals were necropsied and examined macroscopically.

Statistics: The oral LD_{50} values were estimated.

RESULTS AND DISCUSSION

Mortality: Two females were found dead following a dose of 2000 mg/kg bw (one 4 hours and the other 1 day after dosing). There were no other mortalities.

Table 5.8.1-6: Acute oral toxicity mortality data

Dose Level (mg/kg bw)	Day Number	Number of Deaths	
		Male	Female
1000	Total at day 14	0/5	0/5
2000	Day 0 (4 hours)	0	1
	Day 1	0	1
	Total at day 14	0/5	2/5

Clinical observations: Piloerection, hunched posture, exophthalmos and dyspnea were seen. Additionally, reduced locomotor activity was seen in all animals. Tremor and ataxia were present in one female at 2000 mg/kg. The surviving animals recovered within 4 to 5 days.

Body weight: The body weight of the animals increased from day 0 to day 14.

Necropsy: No deviations from normal morphology were found in any animal.

CONCLUSION: The acute oral LD_{50} value for CGA263208 tech. (plant metabolite of CGA219417) was greater than 2000 mg/kg bw in the rat. The LD_{50} was greater than 2000 mg/kg bw for males and approximately 2000 mg/kg bw for females.

(Hartmann H.R., 1994a)

Report:	K-CA 5.8.1/~~15~~17 Hartmann H.R. (1992a). CA1139A: Acute oral toxicity in the rat. RCC Ltd., Itingen, Toxicology Division, CH-4332 Stein, Switzerland. Report No. 923045, including Amendment 1, 12 February 2002. Unpublished. Syngenta File No. CA1139/0001.

GUIDELINES: OECD 401 (1987).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: The rat was selected as the test species as it is recognized by international guidelines as a standard test species for the determination of acute oral toxicity. The number of animals used was considered to be the minimum required to meet the scientific and regulatory objectives of the study.

EXECUTIVE SUMMARY

In an acute oral toxicity study, two groups of five males and five female, fasted, young adult, Tif: RAIf (SPF) rats were given a single oral dose of CA1139A (purity 76.2%) in 0.5% carboxymethylcellulose in 0.1% aqueous polysorbate 80 at doses of 1000 and 2000 mg/kg bw and observed for 14 days.

One female dosed with 1000 mg/kg and one male and two females dosed with 2000 mg/kg died spontaneously within two days of dosing. Piloerection, hunched posture, dyspnea and reduced locomotor activity were observed in all animals. Tremor, ataxia and respiratory sounds were present in the females dosed at 1000 mg/kg. Tonic spasms were observed in one female at 2000 mg/kg. All surviving animals recovered within 4 to 7 days. There was no effect on body weight. At necropsy, a spotted thymus was found in one female at 2000 mg/kg found dead two days after dosing. No deviations from normal morphology were found in the remaining animals.

Conclusion: The acute oral LD_{50} values for CGA263208 tech. was greater than 2000 mg/kg bw to male and female rats.

MATERIALS AND METHODS

Materials:

Test Material:	CA1139A
Description:	Powder
Lot/Batch number:	P.201025
Purity:	76.2 %
CAS#:	Not reported
Stability of test compound:	Reanalysis date: February 1993 (Stored at room temperature)

Vehicle and/or positive control: 0.5% carboxymethylcellulose in 0.1% aqueous polysorbate 80.

Test Animals:

Species	Rat
Strain	Tif: RAIf (SPF)
Age/weight at dosing	Young adult / 176-220 g
Source	Ciba-Geigy Limited, Animal Production, 4332 Stein, Switzerland
Housing	5 same sex animals/cage, in Macrolon Type 4 cages
Acclimatisation period	At least 5 days
Diet	NAFAG 890 Tox diet (Nafag, Gossau/SG, Switzerland) *ad libitum* (except for overnight fast prior to dosing).
Water	Water *ad libitum*
Environmental conditions	Temperature: 22±2°C Humidity: 55±10% Air changes: Approximately 15/hour. Photoperiod: 12 hours light / 12 hours dark.

Study Design and Methods:

In-life dates: Start: 09 June 1992, End: 03 August 1992.

Animal assignment and treatment: In an acute oral toxicity study, two groups of five males and five female, fasted, young adult, Tif: RAIf (SPF) rats were given a single oral dose of CA1139A (purity 76.2%) at doses of 1000 and 2000 mg/kg bw. The test substance was diluted in 0.5% carboxymethylcellulose in 0.1% aqueous polysorbate 80 and dosed by oral gavage at a rate of 10 mL/kg body weight.

The animals were examined daily (am and pm on working days and am on weekends) for mortality and viability and were examined once daily for clinical signs for 14 days. Body weights were recorded immediately prior to dosing (day 0) and on days 7 and 14 and at death. All animals were necropsied and examined macroscopically as soon as possible after death or at the end of the observation period, as appropriate.

Statistics: The oral LD_{50} was estimated.

RESULTS AND DISCUSSION

Mortality: One female dosed with 1000 mg/kg and one male and two females dosed with 2000 mg/kg died spontaneously within two days of dosing.

Clinical observations: Piloerection, hunched posture, dyspnea and reduced locomotor activity were observed in all animals. Tremor, ataxia and respiratory sounds were present in the females dosed at 1000 mg/kg. Tonic spasms were observed in one female at 2000 mg/kg. All surviving animals recovered within 4 to 7 days.

Bodyweight: The bodyweight of the animals was within the range commonly recorded for this age and strain.

Necropsy: A spotted thymus was found in one female at 2000 mg/kg found dead 2 days after dosing. No deviations from normal morphology were found in the remaining animals.

CONCLUSION: The acute oral LD_{50} values for CGA263208 tech. was greater than 2000 mg/kg bw to male and female rats.

(Hartmann H.R., 1992a)

Report:	K-CA 5.8.1/~~16~~18 Hartmann H. (1991). CGA263208 tech: Acute oral toxicity in the rat. Short-term Toxicology, Ciba-Geigy Limited, 4332 Stein, Switzerland. Report No. 911054. Unpublished. Syngenta File No. CA1059/0001.

GUIDELINES: OECD 401 (1987).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: The rat was selected as the test species as it is recognized by international guidelines as a standard test species.

EXECUTIVE SUMMARY

In an acute oral toxicity study, groups of fasted, young adult, Tif: RAI f (SPF) rats were given a single oral dose of CGA263208 tech. (purity 72.7%) in 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80 at doses of 1000 mg/kg bw (five males) and 2000 mg/kg bw (five males and five females) and observed for 14 days.

Two males were found dead following a dose of 2000 mg/kg bw (one 3hours and 1 day after dosing) and one female was found dead 1 day after dosing with 2000 mg/kg bw. None of the 1000 mg/kg bw males died. Piloerection, abnormal body positions, dyspnea were seen. Additionally trismus was seen in all animals, reduced locomotor activity was seen in both sexes at 2000 mg/kg bw and salivation was seen in females at 2000 mg/kg bw. All surviving animals recovered within 4-6 days. There was no effect on body weight. At autopsy, a spotted thymus was present in one of the male decedents at 2000 mg/kg bw. No deviations from normal morphology were found in the remaining animals.

Conclusion: The acute oral LD_{50} values for CGA263208 tech. was greater than 2000 mg/kg bw to male and female rats.

MATERIALS AND METHODS

Materials:

Test Material:	CGA263208 tech.
Description:	Technical, solid
Lot/Batch number:	P.010010
Purity:	72.7 %
CAS#:	Not reported
Stability of test compound:	Reanalysis date December 1992 (stored at room temperature)

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

Test Animals:

Species	Rat
Strain	Tif: RAI f (SPF)
Age/weight at dosing	6-8 weeks / 168-249 g
Source	Ciba-Geigy Limited, Animal Production, 4332 Stein, Switzerland
Housing	5 same sex animals/cage, in Macrolon Type 4 cages
Acclimatisation period	At least 5 days
Diet	NAFAG 890 Tox (Nafag, Gossau/SG, Switzerland) *ad libitum* (except for overnight fast prior to dosing).
Water	Water *ad libitum*
Environmental conditions	Temperature: 22±2°C Humidity: 55±10% Air changes: Approximately 15/hour. Photoperiod: 12 hours light / 12 hours dark.

Study Design and Methods:

In-life dates: Start: 15 April 1991, End: 14 May 1991

Animal assignment and treatment: In an acute oral toxicity study, groups of fasted, young adult, Tif: RAI f (SPF) rats were given a single oral dose of CGA263208 tech. (purity 72.7%) at doses of 1000 mg/kg bw (five males) and 2000 mg/kg bw (five males and five females) and observed for 14 days.

The test substance was diluted in 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80 and dosed by oral gavage at a rate of 10 mL/kg body weight.

The animals were examined daily (am and pm on working days and am on weekends) for mortality and viability and were examined once daily for clinical signs for 14 days. Body weights were recorded immediately prior to dosing (day 0) and on days 7 and 14 and at death. All animals were necropsied and examined macroscopically.

Statistics: The oral LD_{50} values were estimated.

RESULTS AND DISCUSSION

Mortality: Two males were found dead following a dose of 2000 mg/kg bw (one 3 hours and 1 day after dosing) and one female was found dead 1 day after dosing with 2000 mg/kg bw. None of the 1000 mg/kg bw males died.

Table 5.8.1-7: Acute oral toxicity mortality data

Dose Level (mg/kg bw)	Day Number	Number of Deaths	
		Male	Female
1000	Total at day 14	0/5	-
2000	Day 0 (3 hours)	1	0
	Day 1	1	1
	Total at day 14	2/5	1/5

Clinical observations: Piloerection, abnormal body positions and dyspnea were seen. Trismus was seen in all animals. In addition, reduced locomotor activity was seen in both sexes at 2000 mg/kg bw and salivation was seen in females at 2000 mg/kg bw. All surviving animals recovered within 4-6 days.

Body weight: The body weight of the animals increased from day 0 to day 14.

Necropsy: A spotted thymus was present in one of the male decedents at 2000 mg/kg bw. No deviations from normal morphology were found in the remaining animals.

CONCLUSION: The acute oral LD_{50} values for CGA263208 tech. was greater than 2000 mg/kg bw to male and female rats.

(Hartmann H, 1991)

Report:	K-CA 5.8.1/~~17~~19 Altmann B. (2002). CGA263208 tech: 90-Day oral toxicity study in rats. RCC Limited, Toxicology Division, 4332 Stein, Switzerland. Report No. 20013024, 08 February 2002. Unpublished. Syngenta File No. CGA263208/0005.

GUIDELINES: OECD 408 (1998); OPPTS 870.3100 (1998); JMAFF 12 Nousan No. 8147 (2000).

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: This toxicity study was conducted in order to determine the potential toxicity of CGA263208 tech. in albino rats (HanBrI:WIST (SPF)) following daily oral administration in their food for at least 90 days. CGA263208 tech. is the stable form (carbonate salt) of a cyprodinil (CGA219417) metabolite. Albino rats were selected as a standard rodent species. Historical control data for this strain are available at Syngenta Crop Protection AG, 4332 Stein, Switzerland. The oral route was chosen for administration as this represents a possible route of exposure in humans and other mammalian species.

EXECUTIVE SUMMARY

In a 90 day oral (dietary) toxicity study, CGA263208 tech., admixed to the diet, was administered at selected dose levels (50, 300, 2000 and 8000 ppm) to albino rats (HanBrI:WIST (SPF)), 10 animals per sex and dose group. CGA263208 tech. is the stable form (carbonate salt) of a cyprodinil (CGA219417) metabolite. In parallel, CGA219417 tech. was administered in the diet at 8000 ppm to 10 males and 10 females for comparison. A similarly constituted group of rats received untreated diet and served as controls.

Clinical signs, body weight, food consumption, water consumption and mortality were monitored throughout the study for all animals. Measurements for functional observational battery and motor activity were performed at treatment end in all animals. Ophthalmologic examinations were performed pretest in all animals and towards the end of the treatment period in control, high dose animals and the reference test item group only. Haematological, blood chemistry, and urine analyses were performed at the end of the treatment period on all animals. At sacrifice, animals were examined macroscopically and organ weights were recorded from surviving animals. Organs and tissues were collected and prepared for histopathological evaluation and examined microscopically.

The test items were homogeneously distributed in the diet and were stable at the target concentrations. Achieved concentration agreed with nominal. The mean daily intake of CGA263208 tech. was 3.06, 17.8, 131 and 677 mg/kg body weight for males and 3.52, 22.1, 147 and 638 mg/kg body weight for females at 50, 300, 2000 and 8000 ppm, respectively. The mean daily intake of CGA219417 tech. was 548 mg/kg body weight for males and 747 mg/kg body weight for females at 8000 ppm, respectively.

There were no treatment-related mortalities or effects on functional observational battery assessments, motor activity or ophthalmoscopy findings in any of the rats.

CGA263208 tech.: Treatment-related clinical signs were restricted to animals at 8000 ppm and included mainly transiently reduced muscle tone and mostly sporadic occurrence of piloerection. These alterations were more pronounced in females. A transient loss in mean body weight was noted in both sexes at 8000 ppm at week 2. Mean overall body weight gain was significantly and dose-related reduced in both sexes at 8000 and 2000 ppm. Mean food consumption was significantly reduced (dose-related) in both sexes at 2000 ppm at week 1. Afterwards, food consumption increased but food spillage was noted in single animals at 8000 ppm and in females at 2000 ppm. Calculation of food consumption by excluding affected animals, revealed in general lower food consumption values at 8000 ppm. Reduction (partly significantly) in food utilisation efficiency was seen in both sexes at 8000 ppm at week 1 and afterwards in males at 8000 and 2000 ppm. These changes reflect the observed effects on food consumption and body weight development in animals of this group. Mean water consumption was transiently reduced at 8000 ppm at week 1.

Males treated at 8000 ppm had slightly lower mean values for haemoglobin concentration and haematocrit. In addition, lower prothrombin activities were recorded for males treated at 2000 and 8000 ppm. Females (8000 ppm) had a slight leukocytosis associated with lymphocytosis and higher values for eosinophil, basophil, monocyte and large unstained cell counts.

Treatment at 8000 ppm resulted in increased plasma urea levels for both sexes. Females treated at ≥2000 ppm had also higher plasma creatinine levels. Lower mean values for protein and globulin, associated with higher values for albumin to globulin ratios were recorded for males ≥2000 ppm and for females at 8000 ppm. Females at 8000 ppm had also slightly lower albumin levels. In addition, males and females treated at 8000 ppm had higher activities of alkaline phosphatase, and gamma-glutamyl transpeptidase activity was increased in single animals of this group. Furthermore higher bilirubin values were recorded for males at 8000 ppm, and females of this group had higher potassium levels. Males and females treated at 8000 ppm had a slight proteinuria. In addition, females of this group excreted a more alkaline urine.

At macroscopic examination, the gonads, epididymides, prostate gland, seminal vesicle and testes of male group 5 (8000 ppm) were reduced in size. Absolute and relative spleen weights were reduced in both sexes at 8000 and 2000 ppm. Absolute organ weight of testes, epididymides, uterus and ovaries were reduced in animals at 8000 ppm. Microscopic examination showed treatment-related findings only in group 5 (8000 ppm). Severity of fatty atrophy of the bone marrow was increased and that of splenic hematopoietic activity decreased in males and females; in males, there was occurrence of precipitate and reduced spermatozoa in the epididymides, reduced secretion in the prostate gland and seminal vesicle, and reduced spermatogenic activity and increased incidence and severity of tubular atrophy in the testes; incidence and severity of hepatocellular glycogen deposition were decreased in males and females; cytoplasmic vacuolation of pancreatic acinar cells occurred in males.

CGA219417 tech.: There were no mortalities and no treatment-related clinical signs. Mean overall body weight gain was significantly reduced in both sexes at 8000 ppm. Mean food consumption was reduced in both sexes at 8000 ppm at week 1. Afterwards, values increased but food spillage was noted. Calculation of food consumption by excluding affected animals, revealed in general lower food consumption values in females. At 8000 ppm, efficiency of food utilization was reduced in both sexes (significantly in males) at the beginning of the study and reflects the observed effects on food consumption and body weight development in animals of this group. There was no treatment-related effect on water consumption.

Males treated at 8000 ppm had lower mean values for haemoglobin concentration and haematocrit, and a lower prothrombin activity. In females, haematological effects were confined to a leukocytosis associated with lymphocytosis. Males and females treated at 8000 ppm had lower globulin values, higher albumin to globulin ratios, and increased activities of alkaline phosphatase and gamma-glutamyl transpeptidase, and an increase of cholesterol. Males and females treated at 8000 ppm had a slight proteinuria and the mean urinary pH value was higher in females. In addition, glucosuria and ketonuria were recorded for individual male and female animals.

There were no treatment-related macroscopic findings. An increase in liver weight was noted in both sexes. In males, thymus weight was reduced (toxicological relevance considered doubtful) and relative thyroid weight was increased. Microscopic examination showed splenic haematopoietic activity had increased severity in males and decreased severity in females; fatty atrophy of bone marrow had increased severity in females and splenic haemosiderosis in males; incidence of renal tubular atrophy was increased in males, renal tubular dilatation occurred in one male and most females, and pigment deposition of renal tubular epithelia occurred in females; incidence of glycogen deposition was decreased in males and females and severity of this finding was decreased in females; severity of thyroid follicular hypertrophy was increased in males and females and incidence of this finding was increased in females.

Conclusion: Oral administration of CGA263208 tech. admixed to the diet for at least 90 days was tolerated in rats and most findings were restricted to the high dose group (8000 ppm). At this dose level clinical signs were noted and ≥2000 ppm resulted in depression in food consumption and body weight development. Laboratory investigations and histopathological examinations point to the hematopoietic and lymphoreticular system, reproductive system, liver and pancreas as potential target organs. In addition, changes on laboratory parameters indicate disturbance in kidney function. However, some of these alterations may be related to nutritional deficiency. Based on food consumption and body weight, the No-Observable-Adverse-Effect Level (NOAEL) of 300 ppm was defined for both males and females, corresponding to a daily test item intake of 17.8 mg/kg body weight/day for males and 22.1 mg/kg body weight/day for females.

Oral administration of the parallel tested parent test item CGA219417 tech. admixed to the diet for at least 90 days at 8000 ppm resulted in effects which were similar to the findings seen after treatment of the metabolite (CGA263208 tech.) at 8000 ppm. Effects on body weight development and food consumption were less pronounced. The haematopoietic and lymphoreticular system, kidney, liver and thyroid were identified as potential target organs.

MATERIALS AND METHODS

Materials:

Test Material:	CGA263208 tech.
Description:	Technical, white powder. Stable form (carbonate salt) of a cyprodinil (CGA219417) metabolite
Lot/Batch number:	01494D0
Purity:	94.4%
CAS#	Not reported
Stability of test compound:	Reanalysis date May 2002 (stored at room temperature)

Test Material:	CGA219417 tech.
Description:	Technical, beige granules
Lot/Batch number:	P.012011
Purity:	99.2%
CAS#	Not reported
Stability of test compound:	Reanalysis date April 2003 (stored at room temperature)

Vehicle and/or positive control: Pelleted, certified standard diet (Nafag No. 8900 FOR GLP).

Test Animals:

Species	Rat
Strain	HanBrl:WIST (SPF)
Age/weight at dosing	7-8 weeks / males 223-293, females 146-205g.
Source	RCC Ltd. Biotechnology & Animal Breeding Division, 4414 Füllinsdorf, Switzerland
Housing	Individually in macrolon type 3 cages.
Acclimatisation period	25 days
Diet	Pelleted, certified standard diet (Nafag No. 8900 FOR GLP) *ad libitum* (except for overnight fasting prior to blood and urine collection)
Water	Tap water *ad libitum* (except overnight during urine collection).
Environmental conditions	Temperature: 22±2°C Humidity: 55±10% Air changes: 16-20 changes/hour Photoperiod: 12 hours light / 12 hours dark

Study Design and Methods:

In-life dates: Start: 16 July 2001, End: 18 October 2001

Dose level selection: The doses were selected based on the results of previously conducted toxicity studies in rats (***Fankhauser, H, 199; Giessen, W, 1997***).

Animal assignment and treatment: Immediately after delivery, the animals were distributed into groups by means of computer-generated random numbers.

A total of 120 animals (10 males and 10 females per group) was used. The outline of the experimental groups and animal numbers is presented in the following table:

Table 5.8.1-8: Study design

Group	1	2	3	4	5	6
Treatment	Control	CGA263208				CGA219417
Dietary concentration (ppm)	0	50	300	2000	8000	8000
Number of males	10	10	10	10	10	10
Number of females	10	10	10	10	10	10

The test items were administered orally, admixed into the diet, continuously for 90 days. The animals of the control group (group 1) were fed with similarly prepared and pelleted food without the test items.

Test substance preparation and analysis: The test items were weighed (without adjustment for purity) on a calibrated balance. The pulverised diet was then homogeneously mixed with the appropriate concentrations of the test items and about 25% water was added before pelleting to ensure the necessary pellet quality. The pellets were subsequently air-dried. Fresh diets were prepared at about 3 weeks to monthly intervals and stored in stainless steel containers at room temperature in a separate area.

Prior to the start of the study, samples of the diet (pellets) containing the test items at concentrations covering the selected dose range were analysed for content, homogeneity and stability. Control analyses of the test items content in this study were undertaken for every preparation (weeks 1 to 2, 3 to 6, 7 to 10 and 11 to treatment end), and on test items homogeneity with diet used for treatment weeks 1 to 2. Stability investigations were performed at room temperature and 8 weeks with samples of the diet used for treatment weeks 1 to 2. Samples for homogeneity were collected from three different segments (beginning, middle and end) of the pelleting process. Stability was evaluated by analysing samples of

each dietary preparation after diet had been stored for 7 weeks at room temperature. Analysis was by high performance liquid chromatography (HPLC).

Results –

CGA263208 tech. Achieved concentration was satisfactory (92.0% to 109.4% of nominal content). The results showed a homogeneous distribution of the test item in the diet. The individual concentrations varied in the range from -3% to +3% of the mean concentrations. The overall means of nominal dietary concentrations calculated from analytical results of food batches used for treatment weeks 1-13 were in the range between 99.5% and 102.0%. Dietary preparations of CGA263208 tech. were stable in pelleted food for 7 weeks when stored at room temperature.

CGA219417 tech.: Achieved concentration was satisfactory (97.4% to 103.5% of nominal content). The results showed a homogeneous distribution of the test item in the diet. The individual concentrations varied in the range from ±0% of the mean concentrations. The overall mean of nominal dietary concentration (8000 ppm) calculated from analytical results of food batches used for treatment weeks 1-13 was 100.7%. Dietary preparations of CGA219417 tech. were stable in pelleted food for 7 weeks when stored at room temperature.

Mortality and clinical observations: All animals were examined twice daily for morbidity and mortality. General clinical observations were undertaken daily. Detailed clinical observations were undertaken pretest and once weekly thereafter.

Bodyweight: The bodyweight of each rat was recorded pretest and once weekly thereafter.

Food consumption and utilisation and test substance intake: Food consumption was recorded pretest and once weekly thereafter and calculated for periods of one week (expressed as grams food consumed/animal/day). Based on individual weekly food consumption and individual body weights, food utilisation efficiency was calculated (expressed as g bw gain/100 g food consumed). Test item intake (expressed as mg/kg body weight/day) was calculated.

Water consumption: Water consumption was recorded once per week.

Neurobehavioural Assessment:
Functional observational battery (FOB): All animals were evaluated in a FOB towards the end of the treatment period. Examinations were done in the morning, before the daily administration. To control for variations in test conditions and to make experimenters unaware of the animals' treatment, animals were randomised, the cage labels covered with the corresponding FOB number and the animals tested in FOB-number order.

Observations of animals was conducted in a structured way and included (1) observation of animals in the home cage, (2) observation and gait evaluation in a standard arena and (3) observation during handling and were followed by a neurological examination. Observations covered the functional domains of CNS activity, CNS overexcitation, sensorimotor, autonomic and physiological functions.

Observations in the home cage and open field included, but were not limited to, the following signs: recumbency, spasms, urination, click response, posture/gait, convulsions, Straub tail, palpebral closure, gait abnormalities, piloerection, stereotypies, eye prominence, paddling movements, unkempt fur, pupil size, distended abdomen, activity, faecal consistency, paralysis, respiratory abnormalities, fasciculations.

Signs assessed during handling included, but were not limited to, the following signs: ease of removal, paralysis, salivation, vocalization, ease of handling, chromodacryorrhea, lachrymation, emaciation, muscle tone, chromorhinorrhea, rhinorrhea, dehydration, tremor.

Neurological examinations included tests for:

sensorimotor functions (approach, touch, vision, audition, pain, vestibular)
autonomic functions (pupillary reflex, body temperature)
sensorimotor coordination (grip strength, landing foot splay)

Motor activity: Shortly after completion of the FOB, motor activity was measured. Treatment groups were counter balanced across test times and across devices. Motor activity was recorded over a total of 30 minutes using an automated open field device. This device has been shown to detect increases as well as decreases in locomotor activity. Data were analysed and summed over intervals of 3 minutes. The test boxes (40 cm x 40 cm x 40 cm) were made of transparent Plexiglas. 3cm above ground a sensor ring is mounted that contains 16 infrared beams on each side. This sensor ring measures the horizontal distance travelled by the rat. A second, identical sensor ring is mounted at an approximate height of 3/4 of the rats' body length and measures vertical activity.

Horizontal activity:	total distance (in cm)
Vertical activity:	number of rearings (counts)
Other parameters	time in central quadrant (in sec)

Ophthalmoscopy: The eyes from all rats were examined prior to the start of the study. The eyes from control (Group 1) and 8000 ppm CGA263208 tech. (Group 5) and 8000 ppm CGA219417 tech. (Group 6) rats were examined during week 13.

Haematology and coagulation: At the end of the treatment period, blood was collected from all animals after overnight starvation, from the sublingual vein, under light isoflurane anaesthesia. The following parameters were examined:

haemoglobin
haematocrit
red blood cell count
mean cell volume
mean cell haemoglobin
prothrombin time
haemoglobin concentration distribution width
mean cell haemoglobin concentration
red cell volume distribution width
leukocyte count
differential leukocyte (white cell) count
thrombocyte count

Clinical chemistry: At the end of the treatment period, blood was collected from all animals after overnight starvation, from the sublingual vein, under light isoflurane anaesthesia. The following parameters were examined:

urea
creatinine
glucose
albumin
total protein
total cholesterol
triglycerides
total bilirubin
globulin
chloride
alkaline phosphatase activity
aspartate aminotransferase activity
alanine aminotransferase activity
gamma-glutamyl transferase activity
calcium
inorganic phosphorus
sodium
potassium
A/G ratio
glutamate dehydrogenase activity

Urinalysis: At the end of the treatment period overnight urine samples were collected from all animals. Animals were placed in an individual metabolism cage without food or water. The following parameters were examined:

colour
volume
relative density
pH
leukocytes
erythrocytes
protein
urobilinogen
glucose
ketones
bilirubin

Termination and pathology:

Macroscopic examination: At the end of the treatment period all control and treated animals were exsanguinated in deep carbon dioxide anaesthesia and subjected to detailed necropsy.

Organ weights: From all animals surviving to scheduled termination, the following organs were removed, trimmed free of extraneous tissue and weighed:

adrenal glands*
brain
epididymides*
heart
kidneys*
liver
ovaries*
spleen
testes*
thymus
thyroid with parathyroids
uterus

* Paired organs were weighed together.

Tissue submission: The following tissues were examined *in situ*, removed and examined and fixed in an appropriate fixative:

gross lesions
adrenal gland
aorta
bone marrow (from femur and sternum)
brain
caecum
colon
duodenum
epididymis
eyes
femur (with bone marrow)
femurotibial joint
Harderian glands
heart
ileum
jejunum
kidney
lachrymal glands (exorbital)
liver
lung
lymph node - axillary
lymph node - mesenteric
mammary gland
optic nerves
ovary
oviduct
pancreas
parathyroids
Peyer's patches
pituitary
prostate
rectum
salivary gland (submandibular)
seminal vesicle
skeletal muscle
spinal cord (cervical, thoracic, lumbar)
skin/subcutis
spleen
stomach
testis
thymus
thyroid
tongue
trachea
urinary bladder
uterus

muzzle
nerve - sciatic
oesophagus
vagina
Zymbal's gland

Microscopic examination: All tissues preserved for examination (excluding those in bold type) were examined for all animals. Histological changes were described, whenever possible, according to their distribution, severity and morphologic character.

Statistics: Statistical analyses of in-life and organ weight data were carried out using the statistical routines contained in the NOVATOX System. Quantitative data such as body and organ weights were analysed either using parametric or non-parametric statistical tests following a pre-test for uniformity of the within group variances based upon the Bartlett's test of homogeneity of variances. In the case of a non-significant Bartlett's test ($p>0.05$) a one-way analysis of variance (ANOVA) was carried out. If the overall test of differences between the groups was significant ($p\leq0.05$), comparisons were made between the control group and each of the treatment groups using Dunnett's multiple comparison test. In cases where Bartlett's test was significant ($p\leq0.05$) the Kruskal-Wallis non-parametric test of the differences between the groups was carried out. If this test was significant ($p\leq0.05$), comparisons were made between the control group and each of the treatment groups using Dunn's multiple comparison test. Ordinal data such as urine components were analysed using the non-parametric Kruskal-Wallis test. If this test was significant ($p\leq0.05$), comparisons were made between the control group and each of the treatment groups using Dunn's multiple comparison test. All statistical tests were two-sided.

RESULTS AND DISCUSSION

Mortality: There were no treatment-related mortalities.

Clinical observations:

CGA263208 tech: Treatment-related clinical signs were restricted to animals of the highest dose group (8000 ppm). At 8000 ppm, reduced muscle tone was observed in 7 males and 8 females and piloerection was seen in 4 males and 8 females. The occurrence of reduced muscle tone was noted for the first time on day 37 and persisted in all animals to day 58 or 60. Occurrence of piloerection started in most animals on day 10 and disappeared in these animals at weeks 3 to 4. In three females, piloerection was seen afterwards again and in one male and female, each, this finding started at week 11. In addition, hunched posture was observed in one female at treatment end and discharge (genital region) was seen in four females during the second part of the study. Discharge (genital region) in one female (2000 ppm, day 79 to treatment end) was also observed. However, due to the restricted occurrence in one female and late time point, the toxicological relevance of this alteration is considered to be equivocal. Findings reported during the detailed clinical observations were consistent with those described above but, in addition, reduced muscle tone in group 5 (8000 ppm) was also seen in 3 males and one female at week 10 and in 5 males at week 13.

CGA219417 tech: There were no treatment-related clinical signs.

Body weight and body weight gain:

CGA263208 tech: A loss in mean body weight was noted in both sexes at the high dose level (8000 ppm) at week 2. Afterwards, body weight increased but body weight gain values were lower when compared to controls. Mean body weight at week 14 was significantly reduced by 33% in males and 22% in females of control values, respectively and mean overall body weight gain (weeks 1 to 14) was significantly reduced by 77% in males and 66% in females of control values, respectively. At 2000 ppm mean body weight change was reduced in males (occasionally significantly) during the whole treatment period and in females mainly at the beginning. Mean body weight at week 14 was reduced by 14% in males and 6% in

females of control values, respectively and mean overall body weight gain (weeks 1 to 14) was significantly reduced by 34% in males and 21% in females of control values, respectively. There were no treatment-related effects on body weight at the other dose levels.

CGA219417 tech.: Mean body weight change in both sexes was markedly reduced during the whole treatment period. Mean body weight at week 14 was significantly reduced by 14% in males and 12% in females of control values, respectively and mean overall body weight gain (weeks 1 to 14) was significantly reduced by 30% in males and 39% in females of control values, respectively.

Table 5.8.1-9: Intergroup comparison of bodyweight and body weight gains - selected times (g)

Week	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
1	253.4	255.5	251.7	256.1	254.3	247.2	177.3	174.3	180.0	177.7	170.4	174.1
2	285.5	287.6	283.7	273.4	235.8**	253.2**	191.3	189.7	195.9	186.1	153.3**	177.7
14	440.7	438.1	432.4	380.4**	296.7**	378.5**	248.1	247.4	254.3	233.8	194.3**	217.6**
1-14	187.3	182.6	180.6	124.3**	42.5**	131.3**	70.8	73.1	74.3	56.1*	23.9**	43.5**

** Statistically significant difference from control group mean at the 1% level

Food consumption and utilisation:

CGA263208 tech.: Mean food consumption was significantly and dose-related reduced in both sexes of groups 4 and 5 (2000 and 8000 ppm) at week 1. Compared to controls, food intakes were reduced by 20 and 54% in males and 24 and 65% in females of groups 4 and 5, respectively. Afterwards, food consumption values were comparable to those of controls or even higher (group 5 males). But the higher food intakes were correlated to food spillage in 5 high dose males. In addition, food spillage was also noted in females of groups 4 and 5 (2000 and 8000 ppm), but recorded in a lower number of animals and weeks. Due to food spillage, an exact evaluation of food consumption is difficult and calculation of food consumption, excluding the affected animals, revealed in general lower food consumption values in group 5 compared to controls. Food consumption of other treated groups was considered unaffected by treatment.

Efficiency of food utilisation was significantly reduced in both sexes of the high dose group (8000 ppm) at week 1 and occasionally significantly reduced in males in the further course of the study. In addition, food utilisation efficiency of group 4 males (2000 ppm) was significantly reduced at weeks 3 and 4. These changes reflect the observed effects on food consumption and body weight in animals of this group. Other treated groups were considered unaffected by treatment.

CGA219417 tech.: Mean food consumption was reduced (significantly in females) in both sexes at 8000 ppm at week 1 (12% in males and 31% in females when compared to controls). Afterwards, food consumption values were comparable to those of controls for males and partly higher for females. The higher food intakes were correlated by food spillage in 6 high dose females. Calculation of food consumption of females unaffected by food spillage revealed lower food consumption values compared to controls.

At 8000 ppm, efficiency of food utilization was reduced in both sexes (significantly in males) at the beginning of the study and reflects the observed food and body weight changes in animals of this group.

Table 5.8.1-10: Intergroup comparison of food consumption - selected times (g/rat/day)

Week	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
-1	22.54	23.70	22.16	24.05	23.65	23.00	16.77	16.66	17.25	17.73	16.06	16.98
1	22.20	23.12	21.26	17.79*	10.12**	19.56	16.12	15.38	15.86	12.23**	5.67**	11.09**
2	23.49	24.11	22.67	21.54	21.38	24.49	17.38	16.54	17.00	15.92	14.89	18.49
4	23.23	22.91	22.08	20.92	25.58	21.59	17.33	16.78	17.38	16.56	15.00	20.54
8	22.73	22.29	21.34	22.01	28.41	22.81	17.10	15.95	17.88	16.86	15.52	22.35
13	21.62	22.42	21.14	19.99	20.09*	20.13	15.67	15.27	17.16	15.79	14.52	15.82

* Statistically significant difference from control group mean at the 5% level
** Statistically significant difference from control group mean at the 1% level

Table 5.8.1-11: Intergroup comparison of efficiency of food utilisation (body weight gain/100 g food)

Week	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
-1	26	25	27	25	25	24	14	14	16	15	14	14
1	21	20	21	14	-30**	4**	12	14	14	10	-48**	5
2	17	15	18	12	13	13	8	9	7	9	12	6
3	19	16	16	11**	6**	14**	11	9	11	10	10	5
4	14	12	13	8**	3**	10*	8	8	8	6	6	7
13	2	4	3	3	-1	2	-1	1	-1	-6	-2	0

* Statistically significant difference from control group mean at the 5% level
** Statistically significant difference from control group mean at the 1% level

Table 5.8.1-12: Mean dose received (mg/kg bw/day)

	Group / Treatment / Dietary concentration (ppm)				
Sex/ details	2	3	4	5	6
	CGA263208				CGA219417
	50	300	2000	8000	8000
Males	3.06	17.8	131	677	548
Corrected $	-	-	-	536	527
Females	3.52	22.1	147	638	747
Corrected $	-	-	140	616	502

$ corrected for spillage - based on values for animals without food spillage only
- no spillage recorded for sex/group

Water consumption:

CGA263208 tech.: Mean water consumption was reduced in animals of group 5 (8000 ppm, significantly in females) in week 1 (21% in males and 33% in females of control values, respectively). Afterwards, water consumption increased to values comparable to controls. There were no treatment-related effects at other dose levels.

CGA219417 tech.: Water consumption was not affected by treatment.

Neurobehavioural Assessment:

Functional observational battery (FOB):

CGA263208 tech.: Functional measurements revealed no treatment-related neurophysiological findings.

Landing foot splay was reduced in high dose males (8000 ppm) and group comparisons showed a statistically significant decrease in forepaw and hind paw grip strength in group 5 males compared to controls, there was a similar trend in group 5 females and group 4 males. This effect was accompanied by lower body weight. Grip strength correlates with body weight and by calculation of the relative grip strength (grip strength / g body weight) the quotient was comparable or even higher to that of controls (forepaw males: control: 4.21, groups 4: 4.30 and group 5: 5.12; hind paw males: control: 3.35, group 4: 3.40 and group 5: 4.01; forepaw females: control: 5.58, group 5: 6.39; hind paw females: control: 3.88, group 5: 4.23). Therefore, this effect was considered to be secondary to body weight change and not a direct effect of the test item. Similarly to grip strength, the reduction in landing foot splay in group 5 males was considered to be incidental and not related to exposure to the test item. Other treated groups were not affected by treatment.

CGA219417 tech.: Functional measurements revealed no treatment-related neurophysiological findings.

Hind paw grip strength was significantly decreased in males. As discussed above, grip strength is correlated by body weight and by calculation of the relative grip strength (grip strength / g body weight) the quotient was comparable to that of controls (hind paw : control: 3.35, group 6: 3.31). Therefore, this effect was considered to be secondary to body weight change.

Motor activity:

There were no treatment-related effects on motor activity.

Ophthalmoscopy:

There were no treatment-related ophthalmoscopy findings.

Haematology:

CGA263208 tech.: Males treated at 8000 ppm had slightly lower mean values for haemoglobin concentration and haematocrit. In addition, lower prothrombin activities were recorded for males treated at 2000 and 8000 ppm. Females treated at 8000 ppm had a slight leukocytosis associated with lymphocytosis and higher values for eosinophil, basophil, monocyte and large unstained cell counts. Other treated groups were not affected by treatment.

CGA219417 tech.: Rats treated at 8000 ppm had similar effects as CGA263208 tech. on haematological parameters. In males, these effects included lower mean values for haemoglobin concentration and haematocrit, and a lower prothrombin activity. In females, these effects were confined to a leukocytosis associated with lymphocytosis.

Table 5.8.1-13: Intergroup comparison of selected haematology findings

Finding	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
Hb	9.44	9.24	9.26	9.12	8.83**	9.0**	8.82	9.11	8.86	8.59	8.84	8.97
Hct	0.4590	0.4500	0.4515	0.4490	0.4242**	0.4398*	0.4300	0.4400	0.4360	0.4170	0.4390	0.4360
PT	0.931	0.925	0.888	0.744**	0.764*	0.585**	1.073	0.968	1.171	1.006	0.948	1.015
WBC	7.864	6.866	5.707**	6.085*	7.521	6.026**	3.813	4.062	4.844	4.189	7.745**	5.182*
Eos	0.122	0.101	0.122	0.101	0.117	0.110	0.072	0.078	0.081	0.072	0.140*	0.079
Baso	0.082	0.062	0.048	0.047	0.076	0.049	0.030	0.045	0.044	0.028	0.069**	0.048
Lymph	6.216	5.324	4.315**	4.838*	5.964	4.515**	2.817	3.062	3.808	3.072	6.242**	4.092*
Mono	0.182	0.163	0.116*	0.103**	0.166	0.131	0.091	0.090	0.106	0.080	0.131*	0.115
Luc	0.065	0.057	0.038	0.040	0.070	0.047	0.027	0.031	0.034	0.027	0.071**	0.044

* Statistically significant difference from control group mean at the 5% level

** Statistically significant difference from control group mean at the 1% level

Hb = haemoglobin (mmol/L), Hct = haematocrit (relative), PT = prothrombin time (ratio of normal activity)

WBC = leukoctye count, Eos = eosinophil count, Baso = basophil count, Mono = monocyte count, Luc = large unstained cell count (all x 10^9/L)

Blood chemistry:

CGA263208 tech.: Males and females treated at 8000 ppm had increased plasma urea levels. Females treated at 2000 and 8000 ppm had also higher plasma creatinine levels. Lower mean values for protein and globulin, associated with higher values for albumin to globulin ratios were recorded for males at 2000 and 8000 ppm and for females at 8000 ppm. Females at 8000 ppm had also slightly lower albumin levels. In addition, males and females treated at 8000 ppm had higher activities of alkaline phosphatase, and gamma-glutamyl transpeptidase activity was increased in one male and 5 female rats of this group. Higher bilirubin values were recorded for males at 8000 ppm, and females of this group had higher potassium levels. Other treated groups were not affected by treatment.

CGA219417 tech.: Males and females treated at 8000 ppm had lower globulin values, higher albumin to globulin ratios, and increased activities of alkaline phosphatase and gamma-glutamyl transpeptidase and increased cholesterol.

Table 5.8.1-14: Intergroup comparison of selected blood chemistry findings

Finding	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
Urea	5.689	5.936	6.476	6.386	8.129**	6.058	7.252	7.130	7.124	8.015	9.975**	7.970
Creat	28.02	27.99	30.84	28.23	30.50	25.38	29.82	30.03	30.27	34.83**	34.05*	27.62
Prot	64.969	64.516	63.236	62.452*	56.782**	64.721	68.867	66.249	67.288	65.562	57.473**	67.847
Glob	24.658	23.328	22.771	20.961*	17.489**	20.656**	22.627	21.817	21.470	21.738	16.501**	19.951**
Alb	40.311	41.188	40.465	41.491	39.470	44.065**	46.240	44.432	45.818	43.824	40.972**	47.896
A/G	1.643	1.778	1.798	1.982**	2.261**	2.151**	2.047	2.039	2.138	2.033	2.513**	2.431*
ALP	63.04	63.54	63.56	66.25	92.30**	79.78**	22.93	28.46	31.02	29.58	80.55**	53.13**
GGT	0.00	0.00	0.00	0.00	0.26	2.41**	0.00	0.00	0.00	0.00	1.27**	1.41**
Bili	1.568	1.470	1.428	1.708	2.046*	1.690	1.732	1.373	1.534	1.679	1.931	2.218
K	4.231	4.116	4.530	4.035	4.949	4.391	3.543	3.734	3.452	3.303	4.120**	3.715

* Statistically significant difference from control group mean at the 5% level

** Statistically significant difference from control group mean at the 1% level

Urea = mmol/L, Creat = creatinine (µmol/L), Prot = total protein (g/L), Glob = globulin (g/L), Alb = albumin (g/L), A/G = albumin/globulin ratio, ALP = alkaline phosphatase activity (U/L), GGT = gamma-glutamyl transpeptidase activity (U/L), Bili = total bilirubin (µmol/L), K = potassium (mmol/L).

Urinalysis:

CGA263208 tech.: Males and females at8000 ppm had a slight proteinuria. In addition, females of this group excreted a more alkaline urine. Other treated groups were not affected by treatment.

CGA219417 tech.: Males and females at 8000 ppm had a slight proteinuria and the mean urinary pH-value was higher in females. Glucosuria and ketonuria were recorded for individual male and female animals.

Table 5.8.1-15: Intergroup comparison of selected urinalysis findings

Finding	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
Protein (g/L)	0.650	0.700	0.700	0.825	0.975*	0.975*	0.500	0.300	0.350	0.500	1.325*	0.750
pH	6.60	6.30	6.45	6.35	6.95	6.60	5.20	5.45	5.55	5.80	6.70**	6.25**
Glucose (mmol/L)	0.0	0.0	0.0	0.0	0.0	0.9*	0.0	0.0	0.0	0.0	0.0	0.3
Ketones (mmol/L)	0.80	1.30	1.40	1.10	1.00	5.60**	0.90	0.65	0.75	0.90	0.65	1.85

* Statistically significant difference from control group mean at the 5% level

** Statistically significant difference from control group mean at the 1% level

Sacrifice and pathology:

Macroscopic findings:

CGA263208 tech.: At macroscopic examination, the gonads, epididymides, prostate gland, seminal vesicle and testes of male group 5 (8000 ppm) were reduced in size. These findings were associated with microscopic observations such as reduced secretion, reduced spermatozoa, reduced spermatogenic activity and testicular tubular atrophy. There were no other findings considered to be treatment-related

CGA219417 tech.: There were no treatment-related macroscopic findings.

Table 5.8.1-16: Intergroup comparison of selected macroscopic findings in males

Finding	Group / Treatment / Dietary concentration (ppm)					
	1	2	3	4	5	6
	Control	CGA263208				CGA219417
	0	50	300	2000	8000	8000
Epididymides reduced in size	0	0	0	0	3	0
Prostate reduced in size	0	0	0	1	6	0
Testes reduced in size	0	0	0	0	3	0
Seminal vesicles reduced in size	0	1	0	1	6	1

Organ weights:

CGA263208 tech.: A significant reduction for absolute spleen weight was seen in males and females of groups 4 and 5 (2000 and 8000 ppm) and for relative spleen weight in males of group 5 and in females of groups 4 and 5 (2000 to 8000 ppm). Absolute organ weights of the reproductive system (testes, epididymides, uterus and ovaries) were reduced in animals of group 5. There were no associated microscopic findings in the ovary or uterus. Other changes in organ weights were considered to be secondary to reduced carcass weight and not compound-related, because of absence of a histopathological correlation and/or the relative organ weights were within the normal range.

CGA219417 tech.: A treatment-related increase in liver weight was noted in both sexes. Mean absolute liver weight was slightly increased in males and mean relative liver weight was significantly increased in both sexes (30% in males and 14% in females). Mean absolute and relative thymus weight was reduced in males (absolute: 29% and relative: 18%), but in view of the reduced body weight development in this group, the absence of histopathological correlation and any similar effects in females, a treatment-related reduction of thymus weight is considered doubtful. Relative thyroid weight was increased in males but absolute thyroid weight was in the normal range. Other changes in organ weights were considered to be secondary to reduced carcass weight and not compound-related, because of absence of a histopathological correlation and/or the relative organ weights were within the normal range.

Table 5.8.1-17: Intergroup comparison of selected organ weights [absolute (g) and relative to carcass weight]

Organ	Group / Treatment / Dietary concentration (ppm)											
	1	2	3	4	5	6	1	2	3	4	5	6
	Control	CGA263208				CGA219417	Control	CGA263208				CGA219417
	males						females					
	0	50	300	2000	8000	8000	0	50	300	2000	8000	8000
Carcass	410.3	416.6	406.8	358.1**	282.5**	355.4**	234.4	234.6	241.5	221.8	182.7**	204.5**
Spleen	0.7861	0.7622	0.7044	0.6203*	0.4389**	0.7312	0.6327	0.6017	0.5680	0.4825**	0.3920**	0.5146**
(rel)	1.91	1.83	1.74	1.74	1.55*	2.07	2.70	2.56	2.36*	2.18**	2.14**	2.51
Testes	4.0396	3.9105	3.8087	3.7013	2.9506**	3.7680	-	-	-	-	-	-
Epidid	1.3977	1.4152	1.4071	1.2352	0.9266**	1.3510	-	-	-	-	-	-
Uterus	-	-	-	-	-	-	0.8417	0.8616	0.9166	0.7452	0.6040	0.8525
Ovaries	-	-	-	-	-	-	0.1475	0.1609	0.1666	0.1570	0.1041**	0.1356
Liver	13.873	14.815	14.426	13.178	10.039**	15.602	8.744	8.647	9.146	8.303	6.272**	8.722
(rel)	33.8	35.5	35.4	36.7*	35.5	43.9**	37.3	36.9	37.9	37.5	34.4	42.7**
Thymus	0.2744	0.2762	0.3055	0.2537	0.2179	0.1943*	0.2295	0.2397	0.2594	0.2234	0.1905	0.2141
(rel)	0.670	0.666	0.748	0.722	0.764	0.551	0.976	1.03	1.08	1.01	1.04	1.04
Thyroid	0.0246	0.0253	0.0224	0.0225	0.0249	0.0267	0.0226	0.0219	0.0240	0.0222	0.0196	0.0229
(rel)	0.0598	0.0606	0.0550	0.0637	0.0878**	0.0753	0.0966	0.0940	0.0991	0.101	0.108	0.112

* Statistically significant difference from control group mean at the 5% level
** Statistically significant difference from control group mean at the 1% level
Epid = epididymides, Thyroid = thyroids and parathyroids

Microscopic findings:

CGA263208 tech.: Fatty atrophy of the bone marrow occurred with a slightly increased severity in male and female group 5 (8000 ppm). Splenic haematopoietic activity had a decreased severity in male and female group 5. In group 5, the epididymides of 3/10 males contained precipitate, and those of 5/10 males exhibited reduced spermatozoa. The prostate gland of 7/10 males and the seminal vesicle of 6/10 males had reduced secretion. The testes of 5/10 males showed tubular atrophy and those of 3/10 males reduced spermatogenesis. Testicular tubular atrophy occurring occasionally in some control and treated animals of other experimental groups was considered as incidental finding, owing to its low incidence and severity and even distribution among the groups. The reproductive findings in males of group 5 were associated with pronounced body weight loss and may be non-specific findings due to the poor general condition of animals. In male and female group 5 the incidence and severity of hepatocellular glycogen deposition was decreased. In male group 5, 3/10 individuals had vacuolation of pancreatic acinar cells. Both glycogen deposition in the hepatocytes and vacuolation of pancreatic acinar cells are physiological changes which probably reflected the decrease in body weight of the affected groups. There were no other findings that were considered to be treatment-related.

CGA219417 tech.: Fatty atrophy of the bone marrow had a slightly increased severity in females. Splenic hematopoietic activity was increased in males and decreased in females. Splenic haemosiderosis had a slightly increased severity in males. The incidence of renal tubular atrophy was slightly increased in males. One male and 8/10 females had minimal renal tubular dilatation. Slight to moderate deposition of pigment in renal tubular epithelia was observed in 5/10 females. In males the incidence of hepatocellular glycogen deposition was decreased and in females both the incidence and severity were decreased. In

females the grading was decreased as well. Males exhibited an increased grading and females a slightly increased incidence and grading of hypertrophy of the thyroid follicular cells. There were no other findings that were considered to be treatment-related.

Table 5.8.1-18: Incidence (and mean severity) of selected microscopic findings in males

Finding		Group / Treatment / Dietary concentration (ppm)					
		1	2	3	4	5	6
		Control	CGA263208				CGA219417
		0	50	300	2000	8000	8000
Bone marrow	fatty atrophy	10 (2.0)	9 (1.9)	10 (2.3)	10 (2.3)	10 (3.8)	10 (2.2)
Spleen	extramedullary haematopoiesis	10 (2.2)	10 (2.5)	10 (2.1)	10 (2.5)	10 (1.8)	10 (3.4)
	haemosiderosis	10 (2.3)	10 (2.2)	10 (2.2)	10 (2.2)	10 (2.4)	10 (2.8)
Epididymides	precipitate					3 (1.3)	
	reduced spermatozoa					5 (2.2)	
Prostate	reduced secretion					7 (1.7)	
Seminal vesicle	reduced secretion					6 (2.5)	
Testes	tubular atrophy	1 (1.0)	2 (1.0)	3 (1.0)	1 (1.0)	5 (1.2)	2 (1.0)
	reduced spermatogenesis					3 (2.0)	
Kidneys	tubular atrophy	3 (1.3)	2 (1.0)	2 (1.0)	1 (1.0)	4 (1.0)	5 (1.2)
	tubular dilation						1 (1.0)
Liver	glycogen deposition	6 (1.2)	7 (1.4)	7 (1.7)	8 (1.3)		2 (1.5)
Pancreas	cytoplasmic vacuolation					3 (1.7)	
Thyroid	follicular hypertrophy	9 (1.2)	9 (1.4)	10 (1.4)	10 (1.7)	9 (1.4)	10 (2.7)

Table 5.8.1-19: Incidence (and mean severity) of selected microscopic findings in females

Finding		Group / Treatment / Dietary concentration (ppm)					
		1	2	3	4	5	6
		Control	CGA263208				CGA219417
		0	50	300	2000	8000	8000
Bone marrow	fatty atrophy	8 (2.0)	10 (2.5)	8 (2.1)	8 (2.3)	10 (3.6)	9 (3.1)
Spleen	extramedullary haematopoiesis	10 (3.6)	10 (3.4)	10 (3.6)	10 (3.4)	10 (2.6)	10 (2.8)
Kidneys	pigment deposition						5 (2.2)
	tubular dilation						8 (1.0)
Liver	glycogen deposition	5 (1.8)	6 (1.5)	8 (1.4)	8 (1.5)	2 (1.0)	3 (1.0)
Thyroid	follicular hypertrophy	2 (1.0)		1 (1.0)	2 (1.0)	3 (1.3)	5 (1.4)

CONCLUSION: Oral administration of CGA263208 tech. admixed to the diet for at least 90 days was tolerated in rats and most findings were restricted to the high dose group (8000 ppm). At this dose level clinical signs were noted and ≥2000 ppm resulted in depression in food consumption and body weight development. Laboratory investigations and histopathological examinations point to the hematopoietic and lymphoreticular system, reproductive system, liver and pancreas as potential target organs. In addition, changes on laboratory parameters indicate disturbance in kidney function. However, some of these alterations may be related to nutritional deficiency. Based on food consumption and body weight,

the No-Observable-Adverse-Effect Level (NOAEL) of 300 ppm was defined for both males and females, corresponding to a daily test item intake of 17.8 mg/kg body weight/day for males and 22.1 mg/kg body weight/day for females.

Oral administration of the parallel tested parent test item CGA219417 tech. admixed to the diet for at least 90 days at 8000 ppm resulted in effects which were similar to the findings seen after treatment of the metabolite (CGA263208 tech.) at 8000 ppm. Effects on body weight development and food consumption were less pronounced. The haematopoietic and lymphoreticular system, kidney, liver and thyroid were identified as potential target organs.

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Fankhauser, H. (1991), CGA219417: 3-Month Oral Toxicity Study in Rats. Test Number 891321, 19.06.1991. Unpublished study, Toxicology, Ciba Geigy, Stein, Switzerland.

Giessen, W. (1997), CGA219417: 90-Day Subchronic Neurotoxicity Study in Rats. Test Number 963031, 15.05.1997. Unpublished study, Toxicology, Novartis AG, Stein, Switzerland.

(Altmann B, 2002)

Report:	K-CA 5.8.1/~~18~~20 Khalil S. (2002). CGA263208 tech.: Prenatal developmental toxicity study in the rat. RCC Limited, Toxicology Division, 4332 Stein, Switzerland. Report No. 20013025, 09 February 2002. Unpublished. Syngenta File No. CGA263208/0006.

GUIDELINES: OECD 414 (2001); OPPTS 870.3700 (1998); JMAFF 12 Nousan No. 8147 (2000).

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

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

EXECUTIVE SUMMARY

In a prenatal developmental toxicity study, CGA263208 tech. (purity 94.4%) was tested for its embryonic, foetotoxic, and teratogenic potential in rats. The test material was administered by gavage in an aqueous solution of carboxymethylcellulose (0.5% w/w) supplemented with 0.1 % (w/w) Tween 80 at daily doses of 0, 20, 200, 400 and 600 mg/kg body weight to 24 mated Wistar rats per group from day 6 to 20 of pregnancy inclusive, using a dose volume of 10 mL/kg body weight. Dams were killed on day 21 p.c., just prior to the expected delivery and foetuses were removed by Caesarean section for examination.

Two high dose group animals were found dead on day 20 of gestation. Necropsy of both animals revealed fluid contents in the small and large intestine and/or solid contents in the stomach/reddish small, large intestine and thymus/haemorrhagic vagina. Piloerection, salivation and vaginal bloody/clear discharge were seen in most of the 400 and 600 mg/kg bw/day treated animals. At 400 and 600 mg/kg bw/day, food consumption and body weight gain were reduced and significantly different from the control group throughout the dosing period. Overall weight gain during gestation (days 0 to 21 p.c.) at both dose levels was significantly lower than that of the controls. At 200 mg/kg bw/day, food consumption was slightly and significantly reduced during gestation days 6 to 13; during the rest of the treatment period, differences from the control value were minimal. Since body weight change for females at 200 mg/kg bw/day was similar or superior to that of the control group and because overall weight gain (days 0 to 21 p.c.) was similar to that of the control group, the observed reduction of food consumption during the first week of treatment was considered not to be biologically relevant.

Gravid uterus weights and carcass weights were reduced at 400 and 600 mg/kg). Net weight change from day 6 p.c. was reduced at400 mg/kg bw/day and there were weight losses at 600 mg/kg bw/day. With the exception of solid content in the stomach (filled with bedding material) seen in groups treated with 400 and 600 mg/kg bw/day, necropsy examination revealed no significant findings in surviving animals.

For all groups, there was no evidence of an adverse effect on any of the reproductive parameters (pre-implantation loss, implantation sites, post-implantation loss, live litter size and sex ratios). At 400 and 600 mg/kg bw/day, mean foetal body weights were significantly lower than those of the controls.

There were no foetal external or visceral observations that were considered to be related to treatment. At skeletal examination, there was a statistically significant increase in incomplete ossification of metacarpal-5 at 600 mg/kg bw/day. A high incidence of the following skeletal variants was observed in the high dose group - incomplete ossification or unossified sternebra -6, calcaneus, metatarsal-1, cervical vertebral centres, proximal and distal phalanges of anterior and posterior digits. At 400 mg/kg bw/day, increased incidence of the following skeletal variants was observed – unossified metatarsal -1, incomplete ossification and *I* or unossified proximal and distal phalanges of anterior and posterior digits. These skeletal anomalies and variations are consistent with a slight delay in foetal development (reduced ossification) and correlated with the reduction in mean foetal body weight in the 400 and 600 mg/kg bw/day groups.

Conclusion: Maternal toxicity (two deaths at 600 mg/kg bw/day, reduced food consumption and body weight gain at 400 and 600 mg/kg bw/day) were seen. At both dose levels, minor developmental delays (reduced ossification) and reductions in foetal body weights were recorded.

On the basis of these results, the NOAEL for both maternal and developmental toxicity was 200 mg/kg bw/day. There was no indication of teratogenic potential.

MATERIALS AND METHODS

Materials:

Test Material:	CGA263208 tech.
Description:	Technical, solid
Lot/Batch number:	014941DO
Purity:	94.4 %
CAS#:	Not reported
Stability of test compound:	Reanalysis date May 2002 (stored at room temperature)

Vehicle and/or positive control: The test substance was administered in 0.5% (w/w) aqueous solution of sodium carboxymethylcellulose supplemented with 0.1 % (w/w) aqueous polysorbate 80/Tween 80.

Test Animals:

Species	Rat
Strain	Albino rats, Hanlbm:WIST (SPF)
Age/weight at dosing	At least 9 weeks / 168-249 g
Source	RCC Ltd., Biotechnology & Animal Breeding Division, Wolferstrasse 4, 4414 Füllinsdorf, Switzerland
Housing	Individually in Macrolon Type 3 cages
Acclimatisation period	Approximately 1-2 weeks prior to mating
Diet	Pelleted certified standard diet (Nafag No. 890 FOR GLP) *ad libitum*
Water	Tap water *ad libitum*
Environmental conditions	Temperature: 22±3°C Humidity: 55±20% Air changes: Approximately 16-20/hour. Photoperiod: 12 hours light / 12 hours dark.

Study Design and Methods:

In-life dates: Start: 01 July 2001, End: 16 July 2001

Mating procedure: Nulliparous females were mated overnight with males of the same stock and proven fertility, at an initial ratio of three females to one male, in mating cages**.** The following morning, successful mating was assessed by the presence of a vaginal plug or of spermatozoa in a vaginal smear. This day was designated as day 0 of pregnancy= day 0 post coitum (p.c.). Presumed pregnant females were removed from the mating cages and the procedure repeated for remaining females until sufficient positively mated dams were produced.

Dose selection rationale: CGA263208 tech. is the stable form (carbonate salt) of a cyprodinil (CGA219417 tech.) metabolite. The dose levels were selected based on the results of a developmental toxicity study with CGA219417 tech. in rats.

The selected dose levels were 0, 20, 200, 600 and 1000 mg/kg bw/day. Because of high maternal toxicity due to 1000 mg/kg bw/day (8 females found dead after 1-3 days of treatment), treatment was discontinued and an additional treated group treated at 400 mg/kg bw/day was included.

Animal assignment: Mated females were allocated to experimental and control groups using a method of randomisation by weight stratification.

Table 5.8.1-20: Animal numbers and treatment groups

Dose level (mg/kg bw/day)				
Control	**Low**	**Low-mid**	**High-mid**	**High**
0	**20**	**200**	**400**	**600**
24	24	24	24	24

Dose preparation and analysis: The test material was administered as a suspension in the specified vehicle. Suspensions at the appropriate concentrations were freshly prepared every day immediately prior to dosing of the animals (within approximately 2 hours). Test material-vehicle mixtures were prepared with a high-speed homogeniser. Homogeneity of the mixtures during administration was maintained with a magnetic stirrer.

Verification analyses of the actual test material concentrations, stability and homogeneity in the prepared suspensions were performed on three dates during the administration. All samples were analysed by HPLC.

Concentration analysis results: The mean concentrations of CGA263208 tech. in suspensions were 99.0%, 98.1 %, 100.9% and 102.1 % of the nominal concentrations (2, 20, 40 and 60mg/mL respectively).

Homogeneity results: The homogeneity of CGA263208 tech. in suspension was considered to be in an acceptable range.

Stability results: CGA263208 tech. was found to be stable in suspension for 2 hours at room temperature.

Treatment: CGA263208 tech. was administered once daily on days 6 to 20 of presumed gestation at a volume of 10 mL/kg body weight (adjusted daily to individual body weights) by oral gavage.

Observations:

Maternal observations: Animals were examined twice daily for mortality and daily for signs of toxicity. Body weights were recorded daily. Food consumption was recorded on days 3, 6, 9, 11, 13, 16, 19 and 21 p.c.

Maternal necropsy: The animals were sacrificed on day 21, just prior to expected delivery, by carbon dioxide inhalation, and foetuses were removed by hysterectomy. The following were recorded:

- Macroscopic pathological examination of the main organs of the thoracic and abdominal cavities, in particular the genitals.
- Number of corpora lutea in each ovary.
- Weight of the uterus including contents.
- Uterine contents in dams at scheduled necropsy (number and location of live and dead foetuses; number and location of early and late (embryonic/foetal) losses; uteri that appeared non-gravid were further examined (e.g. by ammonium sulphide staining) to confirm the non-pregnant status.
- Uterine contents in dams sacrificed or dying before scheduled termination (number and location of implantation sites; uteri that appeared non-gravid were further examined to confirm the non-pregnant status.

Foetal observations: The foetuses were numbered, sexed (on the basis of anogenital distance), externally examined and weighed. They were then killed and processed for visceral or skeletal examination.

Foetuses were assigned to either visceral or skeletal evaluation at an approximate 1: 1 ratio within each litter, independent of sex (starting with skeletal). In the case of gross external anomaly or malformation, foetuses were allocated to the alternate technique depending on the type and incidence of finding.

Statistical analyses: Quantitative data such as body and organ weights were analysed either using parametric or non-parametric statistical tests following a pre-test for uniformity of the within group variances based upon the Bartlett's test of homogeneity of variances. In the case of a non-significant Bartlett's test ($p>0.05$) a one-way analysis of variance (ANOVA) was carried out. If the overall test of differences between the groups was significant ($p\leq0.05$), comparisons were made between the control group and each of the treatment groups using Dunnett's multiple comparison test. In cases where Bartlett's test was significant ($p\leq0.05$) the Kruskal-Wallis non-parametric test of the differences between the groups was carried out. If this test was significant ($p\leq0.05$), comparisons were made between the control group and each of the treatment groups using Dunn's multiple comparison test.

Ordinal data such as the percentage of foetal malformations in a litter were analysed using the non-parametric Kruskal-Wallis test. If this test was significant ($p\leq0.05$), comparisons were made between the control group and each of the treatment groups using Dunn's multiple comparison test.

Binomial data such as the presence or absence of foetal malformations in a litter were analysed using a chi-square test of association. A significant chi-square value ($p \leq 0.05$) was followed by comparisons made between the control group and each of the treatment groups in turn using Fisher's exact test with Bonferroni correction for multiple testing. All statistical tests were two-sided.

RESULTS AND DISCUSSION

Maternal toxicity:

Mortality and clinical signs: Two high dose group animals (600 mg/kg bw/day) were found dead on day 20 of gestation. Prior to death, both animals exhibited piloerection, salivation, vaginal bloody / clear discharge and / or reduced activity / respiratory sounds. Necropsy of both animals revealed fluid contents in the small and large intestine and / or solid contents in the stomach / reddish small, large intestine and thymus / haemorrhagic vagina. There were no premature mortalities in the other groups.

Piloerection, salivation and vaginal bloody / clear discharge were seen in most of 400 and 600 mg/kg bw/day animals.

Body weight: At 400 and 600 mg/kg bw/day, body weight gain was significantly reduced from day 6 of gestation. The resulting lower body weights were significantly different from controls from gestation day 10 and 9 for the females at 400 and 600 mg/kg bw/day, respectively. At both dose levels, overall weight gain during gestation (days 0 to 21 p.c.) was significantly lower than that of the controls. At 20 and 200 mg/kg bw/day, body weights and body weight change were similar to that of the controls throughout the study.

Table 5.8.1-21: Intergroup comparison of body weight gain (g) – selected time points

Days	Dose level (mg/kg bw/day)				
	0 (control)	20	200	400	600
6-9	9.3	9.4	7.1	3.0**	-0.3**
11-13	8.8	8.1	10.0	5.0**	2.8**
19-21	25.7	22.9	23.6	18.1**	9.3**
6-21	105.4	95.1	99.5	78.9**	46.0**
0-21	128.2	117.4	124.4	102.1**	66.6**
Net change	30.2	27.4	29.6	13.1**	-7.9**

* Statistically significant difference from control group mean, $p<0.05$
** Statistically significant difference from control group mean, $p<0.01$
Net change = carcass weight minus day 6 body weight

Food consumption: At 400 and 600 mg/kg bw/day, food consumption was reduced and significantly different from the control group throughout the dosing period (days 6 to 21 p.c.). At 200 mg/kg bw/day, food consumption was slightly and significantly reduced during gestation days 6 to 13; during the rest of the treatment period, differences from the control value were minimal. Since body weight change and overall weight gain (days 0 to 21 p.c.) for females at 200 mg/kg bw/day was similar or superior to that of the control group, the observed reduction of food consumption during the first week of treatment was considered not to be biologically relevant. At 20 mg/kg bw/day, food consumption was similar to controls throughout the study.

Table 5.8.1-22: Intergroup comparison of food consumption (g/animal/day) – selected time points

Days	Dose level (mg/kg bw/day)				
	0 (control)	20	200	400	600
6-9	21.9	21.1	19.3**	15.8**	13.3**
11-13	25.1	23.6	22.4**	19.0**	15.0**
16-19	26.8	25.3	24.6	21.6**	16.2**
19-21	26.0	24.3	23.3	19.6**	13.2**

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

Sacrifice and pathology:

Maternal gross pathology: With the exception of the solid content in the stomach (filled with bedding material) seen in groups treated with 400 and 600 mg/kg bw/day (12/24 and 6/24 females respectively compared with 0/24 in controls), necropsy examination revealed no significant findings in surviving animals.

Caesarean section data: Post-implantation loss, live litter size and sex ratios were similar in all groups. At 400 and 600 mg/kg bw/day, mean foetal body weights were significantly lower than controls.

Table 5.8.1-23: Caesarean section observations for all pregnant females

Observation	Dose level (mg/kg bw/day)				
	0 (control)	20	200	400	600
Number of females mated	24	24	24	24	24
Number non pregnant	1	2	4	1	4
Number pregnant	23	22	20	23	20
Number deaths	0	0	0	0	2
Number with viable litters day 21	23	22	20	23	18
Mean number of corpora lutea	12.3	11.5	12.1	12.0	12.0
Mean number of implantation sites	11.4	10.5	10.9	11.2	11.3
% Pre-implantation loss	6.8	10.6	9.7	5.9	5.6
Mean number live foetuses	11.0	9.9	10.5	10.7	10.3
Mean number early resorptions	0.3	0.6	0.4	0.5	0.9
Mean number late resorptions	0	0	0	0	0.1
% post implantation loss	0.4	0.6	0.4	0.5	1.0
Total number viable foetuses - males	126	100	104	114	86
Total number viable foetuses - females	128	118	106	132	99
% Males	49.4	45.0	48.4	45.5	46.5
Mean foetal body weight (g)	4.9	4.9	4.8	4.4**	3.7**
Mean male foetal body weight (g)	5.0	5.0	4.9	4.5**	3.8**
Mean female foetal body weight (g)	4.8	4.8	4.7	4.3**	3.6**

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

Developmental Toxicity:

External examinations: Two foetuses had findings at external examination – one 600 mg/kg bw/day foetus had micrognathia and a second at the same dose had cleft palate, however, due to their isolated occurrence, these malformations were considered not to be treatment-related.

Visceral examinations: There were no observations that were considered to be related to treatment.

Skeletal examinations: There was no significant difference between the groups with respect to the overall incidence of skeletal anomalies. Anomalies were observed at low incidences in controls as well as treated groups. A statistically significant increase in incomplete ossification of metacarpal-5 seen at 600 mg/kg bw/day correlated with the reduction in mean foetal body weight.

Skeletal variations occurred in all groups, including controls (foetal incidence between 98.2 and 100%). A high incidence of the following skeletal variants was observed at 600 mg/kg bw/day: incomplete ossification or unossified sternebra -6, calcaneus, metatarsal-1, cervical vertebral centres, proximal and distal phalanges of anterior and posterior digits. At 400 mg/kg bw/day, increased incidences of the following skeletal variants were observed – unossified metatarsal -1, incomplete ossification and/or unossified proximal and distal phalanges of anterior and posterior digits.

Table 5.8.1-24: Selected foetal skeletal anomalies and variations (% foetuses affected per litter)

Observation	Dose level (mg/kg bw/day)				
	0 (control)	20	200	400	600
Unossified metacarpal 5	0.00	1.82	1.00	3.19	3.33
Incomplete ossification metacarpal 5	0.00	0.91	0.00	1.59	8.52**
Unossified sternebra 6	0.00	0.00	1.00	1.45	5.37**
Incomplete ossification sternebra 6	1.59	0.00	1.67	3.19	10.19*
Unossified calcaneus	72.98	84.44	80.88	91.34	97.96**
Unossified metatarsal 1	8.53	11.49	22.72	34.18*	64.15**
Incomplete ossification cervical vertebral centers	6.31	5.39	10.29	7.33	13.86
Unossified cervical vertebral centers	24.01	19.00	22.58	43.44	70.74**
Anterior digit 2 unossified proximal phalanx	29.93	25.54	46.36	60.59*	65.48**
Posterior digit 2 unossified proximal phalanx	56.05	56.49	69.54	86.33**	91.30**
Posterior digit 3 unossified proximal phalanx	32.84	27.42	45.67	71.02**	81.43**
Posterior digit 4 unossified proximal phalanx	28.30	24.74	43.74	68.91**	82.04**
Posterior digit 5 unossified proximal phalanx	80.37	93.57	90.54	95.80**	96.11*

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

These skeletal anomalies and variations are consistent with a slight delay in foetal development (ossification) and correlated with the reduction in mean foetal body weight in the 400 and 600 mg/kg bw/day dose groups.

CONCLUSIONS: Maternal toxicity (two deaths at 600 mg/kg bw/day, reduced food consumption and body weight gain at 400 and 600 mg/kg bw/day) were seen. At both dose levels, minor developmental delays (reduced ossification) and reductions in foetal body weights were recorded.

On the basis of these results, the no observable adverse effect level (NOAEL) for both maternal and developmental toxicity was 200 mg/kg bw/day. There was no indication of teratogenic potential.

(Khalil S, 2002)

Toxicity Studies on CGA304075

Report:	K-CA 5.8.1/~~19~~21 Schoch M. (1994). CGA304075 tech. (plant metabolite of CGA219417): Acute Oral Toxicity in the Rat. Ciba-Geigy Limited, Basle, Switzerland. Report No. 943053, 21 July 1994. Unpublished. Syngenta File No. CGA304075/0001.

GUIDELINES: OECD 401 (1987); 92/69/EEC, B.1.

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

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

JUSTIFICATION FOR TEST SYSTEM SELECTION: The rat was selected as the test species as it is recognized by international guidelines as a standard test species.

EXECUTIVE SUMMARY

In an acute oral toxicity study, groups of fasted, young adult, Tif: RAI f (SPF) rats, five per sex, were given a single oral dose of CGA304075 tech. (plant metabolite of CGA219417) (purity 99%) in 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80 at 2000 mg/kg bw and observed for 14 days.

Piloerection, hunched posture and dyspnea were seen, being common symptoms in acute tests. Additionally, reduced locomotor activity was observed in all animals. The animals recovered within 3 days. At necropsy, no deviations from normal morphology were found in all animals.

Conclusion: The acute oral LD_{50} value for CGA304075 tech. (plant metabolite of CGA219417) was greater than 2000 mg/kg bw for male and female rats.

MATERIALS AND METHODS

Materials:

Test Material:	CGA304075 tech. (plant metabolite of CGA219417)
Description:	Technical, solid
Lot/Batch number:	MU-4421/6
Purity:	99%
CAS#:	Not reported
Stability of test compound:	Reanalysis date 10 July 1994 (stored at 0-5°C)

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

Test Animals:

Species	Rat
Strain	Tif: RAI f (SPF)
Age/weight at dosing	Young adult / 181-214 g
Source	Ciba-Geigy Limited, Animal Production, 4332 Stein, Switzerland
Housing	5 same sex animals/cage, in Macrolon Type 4 cages
Acclimatisation period	At least 5 days
Diet	NAFAG 890 Tox (Nafag, Gossau/SG, Switzerland) *ad libitum* (except for overnight fasting prior to dosing).
Water	Water *ad libitum*
Environmental conditions	Temperature: 22±2°C Humidity: 55±10% Air changes: Approximately 15/hour. Photoperiod: 12 hours light / 12 hours dark.

Study Design and Methods:

In-life dates: Start: 22 June 1994, End: 11 July 1994

Animal assignment and treatment: In an acute oral toxicity study, groups of fasted, young adult, Tif: RAI f (SPF) rats, five per sex, were given a single oral dose of CGA304075 tech. (plant metabolite of CGA219417) (purity 99%) at 2000 mg/kg bw and observed for 14 days.

The test substance was diluted in 0.5% (w/v) carboxymethylcellulose in 0.1% (w/v) aqueous polysorbate 80 and dosed by oral gavage at a rate of 10 mL/kg body weight.

The animals were examined daily (am and pm on working days and am on weekends) for mortality and viability and were examined once daily for clinical signs for 14 days. Body weights were recorded immediately prior to dosing (day 0) and on days 7 and 14 and at death. All animals were necropsied and examined macroscopically.

Statistics: The oral LD_{50} values were estimated.

RESULTS AND DISCUSSION

Mortality: There were no mortalities observed in this study.

Table 5.8.1-25: Acute oral toxicity mortality data

Dose Level (mg/kg bw)	Day Number	Number of Deaths	
		Male	Female
2000	Total at day 14	0/5	0/5

Clinical observations: Piloerection, hunched posture, and dyspnea were seen. Additionally, reduced locomotor activity was seen in all animals. The animals recovered within 3 days.

Body weight: The body weight of the animals increased from day 0 to day 14.

Necropsy: No deviations from normal morphology were found in any animal.

CONCLUSION: The acute oral LD_{50} value for CGA304075 tech. (plant metabolite of CGA219417) was greater than 2000 mg/kg bw in male and female rats.

(Schoch M, 1994)

CA 5.8.2 Supplementary studies on the active substance

Studies on the immunotoxicological potential

According to Commission regulation (EU) No 283/2013 supplementary studies on the immunotoxicological potential are required for an active substance when they are necessary to further clarify observed effects on the immune system.

Cyprodinil does not fulfil these criteria and specific studies on immunotoxicity would not be required, however, an immunotoxicity study (Crittenden, 2010, Syngenta File No. CGA219417_11448) has been conducted with cyprodinil (US EPA OPPTS 870.7800 Immunotoxicity (1998)) as part of a response to a regulatory data call by the US EPA. This study was not submitted initially as immunotoxicity studies are not an EU data requirement, however, this study is now included after a request from the RMS and has subsequently been summarised in K-CA 5.8.2/02 below.

In addition, a review of the currently available toxicity studies on cyprodinil has been undertaken and endpoints considered relevant for the identification of potential immunotoxicity have been evaluated.

Report:	K-CA 5.8.2/01 Bhandal H. (2015). Cyprodinil - Position Statement Concerning Immunotoxicity Potential. Syngenta Ltd. Jealott's Hill International Research Centre, Bracknell, Berks RG42 6EY. Report No. TK0223683, 27 July 2015. Unpublished. Syngenta File No. CGA219417_11652.

EXECUTIVE SUMMARY

A detailed review of parameters related to immune function has been conducted on the existing toxicity database for cyprodinil. Repeated-dose studies in rats, mice and dogs were reviewed for any treatment-related changes in a variety of indicators of potential immunotoxicity including leukocyte counts, lymphocyte counts, globulin concentration, macroscopic findings (lymph nodes, thymus, and spleen), organ weights (spleen and thymus), and microscopic findings (bone marrow, lymph nodes, spleen, and thymus).

A thorough review of the toxicology database for cyprodinil has shown no evidence of adverse effects on the immune system in rats, mice or dogs. In addition, cyprodinil does not belong to a class of chemicals (e.g., the organotins, heavy metals, or halogenated aromatic hydrocarbons) that would be expected to be immunotoxic.

(Bhandal H., 2015)

Report:	K-CA 5.8.2/02 Crittenden P. (2010). Cyprodinil - A 28-Day Dietary Immunotoxicity Study in CD-1 Female Mice. WIL Research Laboratories, LLC, 1407 George Road, Ashland, Ohio 44805-8946, USA. Report No. WIL-639075, 18 November 2010. Unpublished. Syngenta File No. CGA219417_11448.

GUIDELINES: OPPTS 870.7800 (1998).

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

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

EXECUTIVE SUMMARY

The test substance, cyprodinil, was offered *ad libitum* in the diet for a minimum of 28 consecutive days to 3 groups (Groups 2-4) of Crl:CD1 (ICR) female mice at dietary concentrations of 500, 2000, and 5000 ppm, respectively. Mice in Group 5 were administered the positive control substance, cyclophosphamide (CPS), via intraperitoneal injection (50 mg/kg/day) for 4 consecutive days (study days 24 through 27). The vehicle control and positive control groups (Groups 1 and 5, respectively) were offered the basal diet on a comparable regimen as the cyprodinil-treated groups. Additionally, all mice were immunized with an intravenous injection of sheep red blood cells (sRBC) on study day 24, approximately 96 hours prior to the scheduled necropsy. Each group consisted of 10 females. All animals were euthanized on study day 28.

All animals were observed twice daily for mortality and moribundity. Clinical examinations were performed once daily for all animals. Detailed physical examinations were performed once weekly and on the day of the scheduled necropsy. Individual body weights were recorded twice weekly and food consumption was recorded approximately weekly. Complete necropsies were conducted on all animals. The liver, spleen, and thymus were collected from all animals and weighed at the scheduled necropsy. Spleens were placed in EBSS/HEPES buffer and shipped to ImmunoTox®, Inc. After arrival at ImmunoTox®, Inc., spleen cell suspensions were prepared, spleen cell counts were performed, and the number of specific IgM antibody-forming cells directed towards the sRBC were determined.

Mean test substance consumptions in the 0, 500, 2000, and 5000 ppm groups were 0, 103.8, 468.3, and 1245.3 mg/kg/day, respectively, over the entire 4-week study.

All animals survived to the scheduled necropsy. There were no test substance-related clinical observations. There were no test substance-related effects on body weights, food consumption, or macroscopic findings. There were no cyprodinil-related effects on absolute, adjusted, or relative spleen or thymus weights. There were no effects attributed to cyprodinil on the specific activity or total activity of splenic IgM antibody-forming cells to the T cell-dependent antigen sRBC.

Higher mean absolute, relative (to body weight), and adjusted liver weights noted in the 5000 ppm cyprodinil-treated group were consistent with previous studies assessing cyprodinil at similar exposure levels.

Lower mean absolute and adjusted spleen and thymus weights were noted for the CPS (positive control) group when compared to the vehicle control group. Additionally, CPS administration produced statistically significantly lower spleen cell numbers (58% lower), specific activity (99% lower), and total spleen activity (99% lower) of IgM antibody-forming cells when compared to the vehicle control group. These effects were consistent with the known immunosuppressant effects of CPS and validated the appropriateness of the AFC assay.

Based on the results of this study, treatment of female Crl:CD-1(ICR) mice with cyprodinil on a continuous basis in the diet for a minimum of 28 consecutive days resulted in no suppression of the humoral component of the immune system. The no observed effect level (NOEL) for suppression of the immune response in female CD-1 mice offered cyprodinil on a continuous basis in the diet for a minimum of 28 days at 0, 500, 2000, and 5000 ppm was 5000 ppm (equivalent to 1245.3 mg/kg/day). The only effect attributed to cyprodinil treatment was higher mean absolute, relative (to body weight), and adjusted liver weights for the 5000 ppm group.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	Light beige powder
Lot/Batch number:	P.012011
Purity:	99.2% (w/w)
CAS#	Not reported
Stability of test compound:	The test substance was stored at room temperature (<30ºC), and was considered stable under this condition.

Vehicle and/or positive control: The test substance was administered via PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002 (meal). Control and positive control animals also received PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002 (meal).

Test Animals:

Species:	Mouse
Strain:	Crl:CD-1(ICR)
Age/weight at dosing:	38 days old; 21.9 g to 26.5g
Source:	Charles River Laboratories Inc., Kingston, New York, USA
Housing:	Upon arrival, all animals were housed 2 to 3 per cage by sex for approximately 3 days. Thereafter, all animals were housed individually in clean, stainless steel, wire mesh cages suspended above cage board.
Acclimatisation period:	13 days
Diet:	PMI Nutrition International, LLC, Certified Rodent LabDiet® 5002 (meal) *ad libitum*
Water:	Tap water ad libitum
Environmental conditions:	Temperature: 19-25 °C Humidity: 30-70% Air changes: 10 per hour Photoperiod: 0600-1800 hours

Study Design and Methods:

In-life dates: Start: 13 April 2010, End: 24 May 2010

Animal assignment: On 22 April 2010 (4 days prior to the initiation of control and test diet administration), all available mice were weighed and examined in detail for physical abnormalities. Based on the review of all appropriate pre-test data by the Study Director, which were collected using WTDMS™, animals judged suitable for assignment to the study were selected for use in a computerized randomization procedure. A printout containing the animal numbers, corresponding body weights, and individual group assignments was generated based on body weight stratification in a block design. The animals were then arranged into groups according to the printout. Individual body weights at randomization were within ± 20% of the mean. Animals not assigned to study were euthanized by carbon dioxide inhalation and discarded.

Table 5.8.2-1: Study design

Test group	Dietary Cyprodinil Dose Level (ppm)	Cyclophosphamide dose Level (mg/kg/day IP)	sRBC (mL/mouse IV)	# females
Vehicle control	0	0	0.2	10
Low Cyprodinil	500	0	0.2	10
Mid Cyprodinil	2500	0	0.2	10
High Cyprodinil	5000	0	0.2	10
CPS	0	50	0.2	10

Diet preparation and analysis: The control and test diets were prepared weekly and stored at room temperature.

Analyses to determine the homogeneity of the test substance in the diet at 450 and 5500 ppm was conducted prior to the dosing phase of the study using test substance diets prepared solely for homogeneity testing. Following preparation of the test diet formulations, samples (approximately 100 g each) were collected from the top, middle, and bottom strata of each formulation for homogeneity analysis.

Duplicate samples for stability determinations were collected from the middle strata of the test diets used during study week 0 and one set of samples was stored at room temperature for 11 days.

Duplicate samples were collected from the top, middle, and bottom of the prepared test substance dietary formulations administered during study weeks 0 and 3 (*i.e.*, n=6 samples/formulation, including the control group), except for the basal diet sample collected for study week 0, which was only collected from the middle strata. In addition, the study week 0 dietary formulations had an additional sample collected from the middle stratum to be used as the initial (time 0) concentration for stability assessments. One set of samples prepared for the first (study week 0) and the fourth (study week 3) weeks of dosing were transferred to the WIL Analytical Chemistry Department for analysis. Analyses of dietary formulations during the dosing period were conducted prior to dosing. The remaining samples were stored frozen at approximately -20ºC as back-up samples to be discarded after acceptance of the analytical results or issuance of the final report. All analyses were conducted by the WIL Analytical Chemistry Department using a validated high performance liquid chromatography method using ultraviolet absorbance detection.

Table 5.8.2-2: Results of homogeneity analysis

Homogeneity Assessment of the 20 April 2010 Formulations		
	400 ppm	5500 ppm
Mean Concentration (mg/mL)	420	5420
RSD (%)	4.5	2.8
Mean % of Target	93.3	98.5

Table 5.8.2-3: Results of stability analysis

	Mean Concentration, ppm (% of Target)		
	Group 2 (500 ppm)	Group 3 (2000 ppm)	Group 4 (5000 ppm)
11-Days Room Temperature Storage	452 (92.1%)	1804 (92.6%)	4533 (91.6%)

Table 5.8.2-4: Results of concentration analysis

	Mean Concentration, ppm (% of Target)		
Date of Preparation	**Group 2 (500 ppm)**	**Group 3 (2000 ppm)**	**Group 4 (5000 ppm)**
22 April 2010	491 (98.1)	1948 (97.4)	4950 (99.0)
13 May 2010	NA [a]	1958 (97.9)	5084 (102)
17 May 2010	496 (99.3)		

[a] = Formulation not used for dose administration.

Cyclophosphamide preparation: The positive control substance, CPS, was prepared in a PBS solution at a concentration of 5 mg/mL. The solution was prepared once, divided into 4 daily aliquots, and stored frozen at approximately ≤20ºC. On each day of dosing (study days 24-27) a vial was quickly thawed, stored on ice, and administered within 3 hours on each day of dosing.

Statistics: Analyses were conducted using two-tailed tests (except as noted otherwise) for minimum significance levels of 1% and 5%, comparing each cyprodinil-treated group to the vehicle control group. Each mean was presented with the standard deviation (S.D.), standard error (S.E.), and the number of animals (N) used to calculate the mean. Due to the different rounding conventions inherent in the types of software used, the means and standard deviations on the summary and individual tables may differ by ±1 in the last significant figure.

Body weight, body weight change, and food consumption were subjected to a parametric one-way ANOVA (Snedecor and Cochran, 1980) to determine intergroup differences. Following the ANOVA, Dunnett's test (Dunnett, 1964) was used to compare the CPS- and cyprodinil-treated groups to the vehicle control group.

Absolute organ weights were analysed using ANOVA followed by Dunnett's test (Dunnett, 1964). The adjusted organ weights (*i.e.*, organ weights adjusted based on terminal body weight) were analysed using ANCOVA followed by Dunnett's test (Dunnett, 1964). Summary values of organ to body weight ratios for spleen, thymus, and liver were tabulated but not analysed statistically.

The terminal body weight, thymus, and spleen weight (absolute and relative to terminal body weight), and AFC assay data obtained in this study was analyzed by ImmunoTox®, Inc. In the tables, all data were presented as means with standard deviations. Due to possible differences in rounding procedures, the mean and standard deviation values for the organ weight data generated by ImmunoTox®, Inc., may differ slightly from those generated by WIL. The AFC data were expressed as both specific activity, IgM antibody forming cells per million spleen cells (AFC/10^6 spleen cells), and as IgM Total Spleen Activity (AFC/spleen). Data were first tested for homogeneity of variances using the Bartlett's Chi Square Test (Bartlett, 1937). Homogenous data were evaluated using parametric one-way ANOVA (Kruskal and Wallis, 1952). Following the ANOVA, a Dunnett's test (Dunnett, 1964) was performed regardless of whether the ANOVA indicated a statistically significant difference ($p \leq 0.05$). Non-homogenous data were evaluated using a nonparametric analysis of variance (Wilson, 1956). When significant differences occurred, the treatment groups were compared to the vehicle control group using the Gehan-Wilcoxon Test (Gross and Clark, 1975) as appropriate. The Jonckheere's Test (Hollander and Wolfe, 1973) was used to test for dose-related trends across the vehicle control and treatment groups. The positive control data were evaluated using the Student's t-Test (Sokal and Rohlf, 1981) and compared to the vehicle control group. The criteria for accepting the results of the positive control group was a statistically significant ($p \leq 0.05$) decrease in the response compared to that of the appropriate vehicle control group.

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

Observations: All animals were examined/observed once daily. Detailed physical examinations were conducted on all animals approximately weekly, beginning approximately 1 week prior to randomization, at the time of randomization, and on the day of the scheduled necropsy.

Body weight: The body weight of each animal was recorded twice weekly throughout the study, beginning approximately 1 week prior to randomization, at the time of randomization, on study day 0, and prior to the scheduled necropsy.

Food consumption and test substance intake: Food consumption for each animal was recorded approximately weekly, beginning approximately 1 week prior to randomization and ending just prior to the scheduled necropsy. Food intake was calculated as g/animal/day for each interval.

The mean amounts of cyprodinil consumed (mg/kg/day) by each treatment group were calculated from the mean food consumed (g/kg of body weight/day using the average of the first and last body weight during the week) and the appropriate target concentration of cyprodinil in the food (mg/kg).

Clinical pathology: Blood samples were collected following euthanasia by carbon dioxide inhalation from the inferior vena cava of all animals on the day of the scheduled necropsy. The blood samples (approximately 0.1 mL) were collected into a tube containing potassium EDTA as an anticoagulant for the preparation of blood smears for possible future evaluation.

IgM Antibody Analysis: Blood samples were collected following euthanasia by carbon dioxide inhalation from the inferior vena cava of all animals at the scheduled necropsy. The blood samples (approximately 0.75 mL, if possible) were collected into borosilicate glass tubes and processed to obtain serum samples. The serum was transferred to cryovials and stored frozen at approximately -70ºC at WIL for possible future IgM antibody analysis.

Investigations *post mortem:*

Macroscopic examination: All animals were examined *postmortem*. This involved an external observation and an internal examination of all organs and structures.

Organ weights: The following organs were removed, trimmed free of extraneous tissue, and weighed from all animals at the scheduled necropsy:

liver
spleen
thymus

Spleen "wet" weights were recorded at the scheduled necropsy, and were provided to ImmunoTox®, Inc.

Tissue submission: The following tissues were examined *in situ*, removed and examined, and fixed in an appropriate fixative (except as noted):

bone marrow smear [a]
femur
liver
lymph node
mesenteric
peyer's patches (GALT)
spleen [b]
thymus

[a] = Bone marrow slides were not placed in formalin
[b] = Placed in EBSS/HEPES buffer; prepared for immunotoxicological evaluation

Spleen Processing for Immunotoxicological Evaluation: Spleens were collected from all animals at the scheduled necropsy immediately following blood collection. Individual spleens were placed into individual, tared tubes maintained on ice containing EBSS with 15 mM HEPES and supplemented with gentamicin as a bacteriostat. Each tube was then weighed to provide a "wet" weight for each spleen. Spleen samples from Group 1 to 4 animals were randomized and coded for analysis. This was done to ensure that the analyst was unaware from which treatment group the spleen sample had been collected. Spleen samples from Group 5 were labelled as positive control samples for analysis. The spleen samples were placed on crushed ice and shipped to ImmunoTox®, Inc. via overnight courier for AFC analysis.

Upon receipt at ImmunoTox®, Inc., the spleens were accessioned in accordance with the ImmunoTox®, Inc., SOP for receipt of biological samples. Spleen weight data previously recorded by WIL was provided to ImmunoTox®, Inc. along with thymus weight data for subsequent organ weight analysis.

The spleen samples were processed into single-cell suspensions prepared in accordance with the ImmunoTox®, Inc. SOP for mouse spleens. The cell suspensions were centrifuged and resuspended in EBSS with HEPES. Spleen cell counts were performed using a Model Z1 Coulter Counter®. Viability of splenocytes was determined using propidium iodide and the Coulter® EPICS® XL-MCL Flow Cytometer. The AFC assay served to determine the number of specific IgM antibody-forming cells directed towards sRBC and was a modification of the Jerne plaque assay (**Jerne *et al.*, 1963**).

Microscopic examination: Tissues were stored as appropriate for future histopathological examination.

RESULTS AND DISCUSSION

Mortality: All animals survived to the scheduled necropsy.

Clinical observations: There were no test substance-related clinical observations.

Body weight and weight gain: Body weights were unaffected by cyprodinil administration. There were no statistically significant differences in mean body weight when the control and cyprodinil-treated groups were compared.

Statistically significantly lower mean body weight gain was noted from study day 24 to 28 in the 2000 ppm group; however, in the absence of a dose-related trend and correlating differences in mean and cumulative body weights this was not considered to be related to cyprodinil administration.

Food consumption and compound intake: Food consumption was unaffected by cyprodinil administration. There were no statistically significant differences when the control and cyprodinil-treated groups were compared, except for higher food consumption for the 5000 ppm group on study day 21 to 28; however, this value was generally similar to pretest values and other intervals during the dosing period, and was not in a direction or of a magnitude considered to be toxicologically relevant. Therefore, this difference was not considered related to cyprodinil administration.

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

Table 5.8.2-5: Mean Dose Received (mg/kg of body weight/day)

Cyprodinil (ppm)	500	2000	5000
Females	103.8	468.3	1245.3

Sacrifice and pathology:
Organ weights: weights as compared to the vehicle control group. Higher mean absolute and relative liver weights (12.5% and 15.3%, respectively) and a statistically significantly higher mean adjusted liver weight was noted for the 5000 ppm group as compared to the vehicle control group.

As expected, statistically significant decreases were observed in the positive control group for spleen and thymus weights. The positive control group had 45.4% and 42.9% lower absolute and relative spleen weights and 67.8% and 66.8% lower absolute and relative thymus weights, respectively, compared to the vehicle control group. These effects were consistent with the known immunosuppressant effects of CPS and validate the appropriateness of the assay.

Macroscopic findings: There were no test substance-related macroscopic findings at the scheduled necropsy.

Antibody Forming Cell (AFC) Assay: There were no cyprodinil-related effects on spleen cell numbers. However, higher spleen cell numbers were observed in the 500 and 2000 ppm groups (20% and 24% higher, respectively), but the values were not statistically significantly different from the vehicle control group. Therefore, in the absence of higher spleen cellularity in the 5000 ppm group and the lack of a dose-response, the slightly higher spleen cell numbers observed for the 500 and 2000 ppm group was not considered treatment-related. As expected, statistically significantly lower (58%) spleen cell numbers were observed in the positive control group when compared to the vehicle control group.

In the functional evaluation of the IgM antibody-forming cell response, treatment with cyprodinil did not suppress the humoral immune response when evaluated as either specific activity (AC/10^6 spleen cells) or total spleen activity (AFC/spleen). Specific activity was higher (38%) in the 5000 ppm group; however, the value was not statistically significantly different from the vehicle control group. In addition, there was higher total spleen activity in all test substance-treated groups; however, values were not statistically significantly different from the vehicle control group. As anticipated, statistically significantly lower specific activity (100%) and total spleen activity (100%) was observed in the positive control group (CPS) animals when compared to the vehicle control group animals.

Natural Killer Cell Activity Assay: There were no indications in this study that cyprodinil was immunotoxic and therefore the need to conduct additional immunotoxicity assessments, specifically a natural killer cell activity assay, was not identified at this time.

CONCLUSION: Based on the results of this study, treatment of female Crl:CD-1(ICR) mice with cyprodinil on a continuous basis in the diet for a minimum of 28 consecutive days resulted in no suppression of the humoral component of the immune system.

The No-Observed-Effect Level (NOEL) for suppression of the immune response in female CD-1 mice offered cyprodinil on a continuous basis in the diet for a minimum of 28 days at 0, 500, 2000, and 5000 ppm was 5000 ppm (equivalent to 1245.3 mg/kg/day). The only effect attributed to cyprodinil-treatment was higher mean absolute, relative (to body weight), and adjusted liver weights for the 5000 ppm group.

(Crittenden P, 2010)

The publications listed below have been taken from the open literature. 8 papers (K-CA 5.8.2/03, K-CA 5.8.2/04, K-CA 5.8.2/05, K-CA 5.8.2/06, K-CA 5.8.2/07, K-CA 5.8.2/08, K-CA 5.8.2/09 and K-CA 5.8.2/10) were found as part of the comprehensive literature search (**M-CA Section 9**).

Report:	K-CA 5.8.2/03. Shah I, Houck K, Judson R, Kavlock R, Martin M, Reif D, Wambaugh J, and Dix D (2011). Using nuclear receptor activity to stratify hepatocarcinogens. Published paper. *PLoS ONE*. Vol **6**, Issue 2, e14584. Syngenta File No. NA_13828.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The method objective is to stratify chemicals based on their putative mode of action for human toxicity using data ranging from *in vitro* molecular assays to *in vivo* rodent outcomes from ToxCast and other available resources. Nuclear receptors (NR) are a superfamily of ligand-activated transcription factors that control a range of cellular processes. Persistent stimulation of some NR is a non-genotoxic mechanism of rodent liver cancer with unclear relevance to humans. A systematic analysis of new *in vitro* human NR activity data on 309 environmental chemicals in relationship to their liver cancer-related chronic outcomes in rodents is conducted.

EXECUTIVE SUMMARY

Note that only the results for cyprodinil are reported within this summary. General statements are included in the publication such that cyprodinil specific information is not reported.

The effects of 309 environmental chemicals on human constitutive androstane receptors (CAR/NR1I3), pregnane X receptor (PXR/NR1I2), aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptors (PPAR/NR1C), liver X receptors (LXR/NR1H), retinoic X receptors (RXR/NR2B) and steroid receptors (SR/NR3) were determined using *in vitro* data. Hepatic histopathology, observed in rodents after two years of chronic treatment for 171 of the 309 chemicals, was summarized by a cancer lesion progression grade. Chemicals that caused proliferative liver lesions in both rat and mouse were generally more active for the human receptors, relative to the compounds that only affected one rodent species, and these changes were significant for PPAR ($p<0.001$), PXR ($p<0.01$) and CAR ($p<0.05$). Though most chemicals exhibited receptor promiscuity, multivariate analysis clustered them into relatively few NR activity combinations. The human NR activity pattern of chemicals weakly associated with the severity of rodent liver cancer lesion progression ($p<0.05$).

The rodent carcinogens had higher *in vitro* potency for human NR relative to non-carcinogens. Structurally diverse chemicals with similar NR promiscuity patterns were weakly associated with the severity of rodent liver cancer progression. While these results do not prove the role of NR activation in human liver cancer, they do have implications for nuclear receptor chemical biology and provide insights into putative toxicity pathways. More importantly, these findings suggest the utility of *in vitro* assays for stratifying environmental contaminants based on a combination of human bioactivity and rodent toxicity.

Cyprodinil was assigned to nuclear receptor group A. NRG A contains 41 chemicals that tend to activate AhR, PXR, CAR, PPAR and in some cases also SR or LXR. Cyprodinil was assigned to lesion progression group VIII i.e. the group with the lowest promiscuity and potency.

MATERIALS AND METHODS

Materials:

Test Material: Cyprodinil
No further information provided

Study Design and Methods:

In-life dates: Not applicable.

Human *in vitro* NR assay data for hundreds of environmental chemicals is being generated as a part of the ToxCast project (***Dix et al 2007***). Most of the Phase I ToxCast chemicals have undergone long-term testing experiments in rodents and their chronic hepatic effects have been curated and made publicly available in the Toxicology Reference Database (ToxRefDB) (***Martin et al 2009***). Although small sets of chemicals have been evaluated using selected NR in the past, ToxCast is the largest public data set on chemicals, encompassing concentration-dependent NR activity and chronic outcomes including liver cancer. Hence, these data provide a unique opportunity to investigate relationships between *in vitro* NR activation and rodent hepatic effects. The method objective is to stratify chemicals based on their putative mode of action for human toxicity using data ranging from in vitro molecular assays to *in vivo* rodent outcomes from ToxCast (***Judson et al 2010***) and other available resources. In this analysis, an unsupervised multivariate analysis of NR activities and rodent liver lesions to investigate a potential mode of action for non-genotoxic hepatocarcinogenesis has been used.

Nuclear Receptor Activity: Human NR activity for 309 environmental chemicals was obtained from *in vitro* high-throughput screening (HTS) experiments. Duplicates and triplicates for eight chemicals were included for quality control purposes. HTS data were collected for 10 out of the 48 human NR, selected based on availability of assays and potential relevance to toxicology. A total of 54 HTS assays were used to interrogate different facets of receptor activation.

Combinatorial Nuclear Receptor Activity: The chemicals were clustered by similarity of aggregate NR activity into 7 putative groups and ordered by promiscuity and potency.

Comparing NR Activity with Cancer Lesion Progression: *In vivo* rat and mouse long-term histopathology outcomes were organized by severity of lesions progressing to cancer.

Lesion Progression and Nuclear Receptor Activities: The chemicals were graded according to their hepatic effects. The severity and concordance of hepatic lesions was clustered by similarity and allocated to nuclear receptor activity groups.

RESULTS AND DISCUSSION

The effects of 309 environmental chemicals on human constitutive androstane receptors (CAR/NR1I3), pregnane X receptor (PXR/NR1I2), aryl hydrocarbon receptor (AhR), peroxisome proliferator-activated receptors (PPAR/NR1C), liver X receptors (LXR/NR1H), retinoic X receptors (RXR/NR2B) and steroid receptors (SR/NR3) were determined using *in vitro* data. Hepatic histopathology, observed in rodents after two years of chronic treatment for 171 of the 309 chemicals, was summarized by a cancer lesion progression grade. Chemicals that caused proliferative liver lesions in both rat and mouse were generally more active for the human receptors, relative to the compounds that only affected one rodent species, and these changes were significant for PPAR ($p<0.001$), PXR ($p<0.01$) and CAR ($p<0.05$). Though most chemicals exhibited receptor promiscuity, multivariate analysis clustered them into relatively few NR activity combinations. The human NR activity pattern of chemicals weakly associated with the severity of rodent liver cancer lesion progression ($p<0.05$).

Cyprodinil was assigned to nuclear receptor group A. NRG A contains 41 chemicals that tend to activate AhR, PXR, CAR, PPAR and in some cases also SR or LXR. Cyprodinil was assigned to lesion progression group VIII i.e. the group with the lowest promiscuity and potency.

CONCLUSION: Cyprodinil was assigned to nuclear receptor group A. NRG A contains 41 chemicals that tend to activate AhR, PXR, CAR, PPAR and in some cases also SR or LXR. Cyprodinil was assigned to lesion progression group VIII i.e. the group with the lowest promiscuity and potency.

REFERENCES:

Dix DJ, Houck KA, Martin MT, Richard AM, Setzer RW, et al. (2007) The ToxCast program for prioritizing toxicity testing of environmental chemicals. Toxicological sciences: an official journal of the Society of Toxicology 95: 5–12.

Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, et al. (2010) In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environmental health perspectives 118: 485.

Martin MT, Judson RS, Reif DM, Kavlock RJ, Dix DJ (2009) Profiling chemicals based on chronic toxicity results from the U.S. EPA ToxRef database. Environmental Health Perspectives 117: 392–399.

(Shah I, *et al*., 2011)

Report:	K-CA 5.8.2/04. Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, Reif DM, Rotroff DM, Shah I, Richard AM, and Dix DJ. (2010). *In vitro* screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Published paper. *Environ Health Perspect 118*:485-492. Syngenta File No. NA_13831.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: This project aims to evaluate the use of *in vitro* assays for understanding the types of molecular and pathway perturbations caused by environmental chemicals and to build initial prioritization models of *in vivo* toxicity.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

Chemical toxicity testing is being transformed by advances in biology and computer modelling, concerns over animal use, and the thousands of environmental chemicals lacking toxicity data. The U.S. Environmental Protection Agency's ToxCast program aims to address these concerns by screening and prioritizing chemicals for potential human toxicity using *in vitro* assays and *in silico* approaches.

309 chemicals which were mostly pesticide active chemicals were tested in 467 assays across nine technologies, including high-throughput cell-free assays and cell-based assays, in multiple human primary cells and cell lines plus rat primary hepatocytes. Both individual and composite scores for effects on genes and pathways were analysed.

Cyprodinil is <u>not</u> included as one of the 21 chemicals investigated as causing rat liver tumours. This

approach of examining results from *in vitro* assays across a battery of tests promises to provide meaningful data on the thousands of untested environmental chemicals and to guide targeted testing of environmental contaminants.

MATERIALS AND METHODS

Materials:

Test Material: Cyprodinil
Description: In ToxCast™ Database

Vehicle: not specified.

Study Design and Methods:

In-life dates: Not reported.

Data sources: The data used to develop the prioritization profiles for the 309 unique chemicals are housed in U.S. EPA's ToxMiner database, an internal repository for assay data from ToxCast.

Screening assays: Nine separate assay technologies, with assays run in concentration–response format and in some cases with multiple time points were included. Assays encompass both direct, primary interactions between chemicals and molecular targets and downstream cellular events such as gene expression. These are summarised below:

Assay set	Assays	Cell type(s)
Cell-free HTS	239	Cell free
Cell-based HTS	13	HEK293, HeLa, HepG2, FAO
High-content cell imaging	19	HepG2 and primary rat hepatocytes
Quantitative Nuclease protection	16	Primary human hepatocytes
Multiplex transcription reporter	81	HepG2
Biologically multiplexed activity profiling (BioMAP	87	HUVEC, HDFn, HBEC, ASMC, KC, PBMC
Phase I and II XME cytotoxicity	4	Hep3B
HTS genotoxicity	1	TK6
Real-time cell electronic sensing	7	A549

RESULTS AND DISCUSSION

Cyprodinil is not included as one of the 21 chemicals investigated as causing rat liver tumours. Cyprodinil is an anilino-pyrimidine fungicide and will not be included in the data presented for chemical classes with at least 10 chemicals are included.

Cyprodinil is not specifically mentioned in the results section of this paper.

CONCLUSION: Cyprodinil is not included as one of the 21 chemicals investigated as causing rat liver tumours. This approach of examining results from *in vitro* assays across a battery of tests promises to provide meaningful data on the thousands of untested environmental chemicals and to guide targeted testing of environmental contaminants.

(Judson RS, *et al.,* 2010)

Report:	K-CA 5.8.2/05. Wetmore B, Wambaugh J, Ferguson S, Sochaski M, Rotroff D, Freeman K, Clewell H, Dix D, Andersen M, Houck K, Allen B, Judson R, Singh R, Kavlock R, Richard A and Thomas R (2012). Integration of dosimetry, exposure, and high-throughput screening data in chemical toxicity assessment. Published paper. Toxicological Sciences 125(1), 157-174. Syngenta File No. NA_13821.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: To address concerns regarding the large number of relatively untested chemicals and to improve chemical risk management, the U.S. Environmental Protection Agency (EPA) has implemented the ToxCast research program to evaluate hundreds to thousands of chemicals in a broad panel of in vitro high-throughput screening (HTS) assays at a fraction of the cost and time of in vivo animal studies. However, relying on nominal assay concentrations may misrepresent potential in vivo effects of these chemicals due to differences in bioavailability, clearance, and exposure. Hepatic metabolic clearance and plasma protein binding were experimentally measured for 239 ToxCast Phase I chemicals (including cyprodinil). Experimental data were used in a population-based in vitro–to–in vivo extrapolation model to estimate the daily human oral dose, called the oral equivalent dose, necessary to produce steady-state in vivo blood concentrations equivalent to in vitro AC_{50} or lowest effective concentration values across more than 500 in vitro assays. The estimated steady-state oral equivalent doses associated with the in vitro assays were compared with chronic aggregate human oral exposure estimates to assess whether in vitro bioactivity would be expected at the dose-equivalent level of human exposure.

EXECUTIVE SUMMARY

Note that where possible, only the results for cyprodinil are reported within this summary. General statements are included in the publication such that cyprodinil specific information is not always reported.

Hepatic metabolic clearance and plasma protein binding were experimentally measured for 239 ToxCast Phase I chemicals (including cyprodinil). The experimental data were used in a population-based in vitro–to–in vivo extrapolation model to estimate the daily human oral dose, called the oral equivalent dose, necessary to produce steady-state in vivo blood concentrations equivalent to in vitro AC_{50} or lowest effective concentration values across more than 500 in vitro assays. The estimated steady-state oral equivalent doses associated with the in vitro assays were compared with chronic aggregate human oral exposure estimates to assess whether in vitro bioactivity would be expected at the dose-equivalent level of human exposure.

A total of 18 (9.9%) chemicals for which human oral exposure estimates were available had oral equivalent doses at levels equal to or less than the highest estimated U.S. population exposures. Ranking the chemicals by nominal assay concentrations would have resulted in different chemicals being prioritized. The in vitro assay endpoints with oral equivalent doses lower than the human exposure estimates included cell growth kinetics, cytokine and cytochrome P450 expression, and cytochrome P450 inhibition.

The incorporation of dosimetry and exposure provide necessary context for interpretation of in vitro toxicity screening data and are important considerations in determining chemical testing priorities.

MATERIALS AND METHODS

Materials:

Test Material: Cyprodinil

Test substance was obtained in neat form or as 20mM stock solutions in DMSO supplied in 96-well polypropylene plates. Solutions were prepared from the neat chemicals to generate the analytical calibration curves. All stock solutions were stored at <-70°C. (Further information provided in Supplementary Table 1).

Vehicle: DMSO.

Study Design and Methods:

In-life dates: Not reported.

Plasma protein binding assay: Plasma protein binding was determined for each chemical using the rapid equilibrium dialysis (RED) method with slight modification (***Rotroff et al., 2010; Waters et al., 2008***). Human plasma with anti-coagulant (K_2EDTA) that tested negative for HBSAg, HIV 1/2 Ab, HIV-1, RNA, HCV Ab, HCV RNA, and STS was used. Chemical stock solutions (prepared in DMSO) were added to plasma chambers to achieve a final concentration of 10µM. After incubation, aliquots were removed and equal volumes of PBS or plasma were added to aliquots from the plasma or PBS chambers, respectively, for matrix matching. The RED assays were performed in triplicate.

Metabolic clearance assay: The rate of hepatic metabolism of the parent compound was determined based upon the method described by (***Rotroff et al., 2010***). Two concentrations (1 and 10 µM) were incubated over a 4-h period with cryopreserved primary human hepatocytes. Both concentrations fall in the middle of the range of concentrations tested in the ToxCast assays. The hepatocytes were characterized for metabolism (CYP1A2, CYP2C9, CYP2D6, CYP3A4/5, CYP2C19, ethoxycoumarin glucuronidation, and ethoxycoumarin sulfation) and viability (Trypan Blue exclusion), and the values were within acceptable ranges compared with historical quality control limits. Chemical stock solutions (prepared at 0.2 and 2mM in DMSO) were added to prewarmed (37°C) incubation medium in polypropylene tubes to achieve working stock chemical concentrations of 2 and 20µM. These solutions were then transferred to 96-well polypropylene plates (0.05 mL per well) and incubated at 37°C and 5% CO_2 for 10–30 min prior to addition of the cells. The final cell density was 0.5 x 10^6 viable cells/mL and the final chemical concentrations were 1 and 10µM. A media-only (no cell) negative control and a negative matrix control (boiled hepatocytes) were included for each chemical. Each sample was run in triplicate.

Bidirectional permeability (Caco-2) assay: To assess the impact of bioavailability on the IVIVE of the ToxCast chemical library, a subset of the chemicals were tested in the bidirectional permeability (Caco-2) assay. Caco-2 cells (passage numbers 65–66; 21–28 days old) were grown to confluence in Hank's balanced salt solution (HBSS) on polycarbonate Transwell inserts. Chemical stock solutions prepared at 10mM in DMSO were administered in duplicate to the apical (for apical to basolateral assessment, A→B) or basolateral (for basolateral to apical assessment, B→A) side at pH 7.4 ± 0.2 to achieve a final concentration of 5µM. Media was collected from the receiver and donor wells 120 min after chemical addition. Percent recovery and the apparent permeability (P_{app}) in both directions (apical to basolateral [P_{app} A-B] and basolateral to apical [P_{app} B-A]) were determined.

Red blood cell partitioning assay: To assess the impact of red blood cell partitioning on the IVIVE of the ToxCast chemical library, a subset of the chemicals were tested in this assay (not clear if cyprodinil was in the subset). Reference plasma was isolated from an aliquot of fresh whole human blood. Chemical stock solutions prepared at 50mM in DMSO were administered to both whole blood and reference plasma to achieve a final concentration of 5µM and incubated. Whole blood was centrifuged and the plasma

stored on ice prior to addition of acetonitrile. Samples were shaken at RT for 5 min and then centrifuged prior to measurement of the compound in the supernatant. Samples were run in duplicate. The $K_{RBC/PL}$ was determined. The quality of the assay was assessed by concurrent analysis of reference compound verapamil.

Chemical analysis by high performance liquid chromatography with mass spectrometric detection: samples from the metabolic stability and plasma protein binding assays.

Chemical analysis by selective ion-monitoring gas chromatography with mass spectrometric detection: samples from the metabolic stability and plasma protein binding assays.

Chemical analysis by HPLC with UV/Vis detection: samples from the metabolic stability and plasma protein binding assays.

Plasma protein binding data analysis: To calculate percent of unbound chemical (F_{ub}), the mean concentration of the test compound in the PBS chamber was divided by the mean concentration in the matched plasma and multiplied by 100. A minimum measurable F_{ub} was set to 0.005. If the concentration of the chemical in the free fraction was below this value or below the analytical limits of detection, a default F_{ub} of 0.005 was assumed.

Metabolic clearance data analysis: Metabolic clearance data were plotted separately in semilog format (log concentration vs. time) with three replicates at each time point. The disappearance of the chemical over time was analyzed using linear regression. Clearance was normalized to cell number. Considering three replicates at each of the six time points, a standard F-test (degrees of freedom = 1,16; α=0.10) was used to determine whether the slope of the line was significantly different from 0. Chemicals that had no statistically significant change ($p > 0.10$) were assigned a metabolic clearance of 0.

In vitro bioactivity data: The initial phase of the ToxCast program measured activity of 309 compounds against a set of approximately 500 in vitro assays. Nine separate technologies were used, including receptor-binding and enzyme activity assays, cell-based protein and RNA expression assays, real-time growth measured by electronic impedance, and fluorescent cellular imaging. Each chemical-assay combination was run in dose response and an AC_{50} or LEC value was estimated depending on the range of the dose response data. The in vitro bioactivity was assumed to be solely the result of the parent compound. Although two assays used primary hepatocyte cultures with some metabolic capacity, most of the assays lacked known metabolic activity. A detailed description of the assays and associated data are provided in earlier publications.

Estimation of oral equivalents using IVIVE: A simple pharmacokinetic equation (***Wilkinson and Shand, 1975***) was used to estimate expected steady-state blood concentrations. The equation was based on zero-order uptake of a daily dose from the gut (assuming 100% oral bioavailability) with both renal and hepatic clearance. For the metabolic clearance, either the 1 or 10µM value was used depending on which value was closer to the AC_{50} or LEC concentration.

Simulations were performed using a dose of 1 mg/kg/day and the Simcyp software (Simcyp Limited, Sheffield, U.K.). Monte Carlo analysis was performed within the Simcyp software to simulate variability across a population of 100 healthy individuals of both sexes from 20 to 50 years of age. A coefficient of variation of 30% was used for intrinsic and renal clearance. The median, upper, and lower fifth percentiles for the concentration at steady state (Css) were obtained as output.

Reverse dosimetry was used to relate blood or tissue concentrations to an exposure concentration. The median, upper, and lower fifth percentiles for the C_{ss} were used as conversion factors to generate oral equivalent doses. An oral equivalent value was generated for each chemical and each AC_{50} or LEC value across the 500 in vitro assays.

Estimation of human oral exposure. For most of the chemicals, exposure estimates were obtained from available EPA Office of Pesticide Programs documents and Federal Register notices.

RESULTS AND DISCUSSION

Plasma protein binding assay: No specific result for cyprodinil reported. Most of the chemicals were highly bound to plasma, with 59% of the chemicals having a fraction unbound 0–5%. Only 7.1% of the chemicals tested had fraction unbound values greater than 80%, whereas no unbound chemical was detected for 77 of the chemicals (32%).

Hepatic metabolic clearance assay: No specific result for cyprodinil reported. The metabolic clearance measurements ranged from 0 to 250.30 and 0 to 234.42 µL/min/10^6 cells at the 1 and 10µM concentrations, respectively. Of these chemicals, 82 (34%) showed either no metabolism or saturation at the 10µM concentration and 37 (15%) showed either no metabolism or saturation at both the 1 and 10µM concentrations. Conversely, 202 of the 239 (85%) chemicals displayed clearance in at least one of the two concentrations. Most (78%) of the chemicals had no metabolism or had clearance values < 0 µL/min/10^6 cells.

IVIVE Modeling: No specific result for cyprodinil reported. Further information provided in Supplementary Table 8.

Estimated Human Oral Exposures: Chronic aggregate human oral exposure estimates were obtained for 182 of the 239 chemicals (76%). A comparison of the exposure estimates with the oral equivalent doses revealed that 18 chemicals (9.9%), including cyprodinil, possessed human exposure estimates for the most highly exposed subpopulation that overlapped with the range of nonoutlier oral equivalent doses.

To more broadly characterize the differences between the oral equivalent dose ranges and the human exposure estimates, activity-to-exposure ratios (AERs) were calculated for each chemical. The AER values were log-normally distributed. The median, upper, and lower quartiles of the log-normal AER distribution for the general U.S. population were 123.03, 1122.02, and 11.48, respectively. The median, upper, and lower quartiles of the lognormal AER distribution for the most highly exposed subpopulation were 44.69, 478.63, and 6.03, respectively. Of the 182 Phase I chemicals for which exposure estimates were available, 9.9% (18 chemicals) had an AER ≤1. The value for cyprodinil was 0.9138.

Table 5.8.2-6: In Vitro Assay Endpoints with Oral Equivalent Doses Lower than the Estimated Human Oral Exposure

Assay endpoint	AC_{50} or LEC (lM)	Oral equivalent dose (mg/kg/day)	Human exposure (mg/kg/day)	AER (Or Eq/Hum exp)
Change in CYP1A2 expression, 48 h	10.474	0.020499	0.0257	0.7976
Change in CYP2B6 expression, 24 h	11.428	0.022366	0.0257	0.8703
Change in HMGCS2 expression, 24 h	8.8588	0.017338	0.0257	0.67463
Change in SULT2A1 expression, 24 h	9.6951	0.018974	0.0257	0.7383
Change in UGT1A1 expression, 24 h	9.973	0.019518	0.0257	0.7595
Competitive binding of muscarinic receptor M2	12	0.023485	0.0257	0.9138
Competitive binding of muscarinic receptor M5	9.3	0.018201	0.0257	0.7082

Table 5.8.2-7: In Vivo Effects, In Vitro Assay Hits, and the AER Range with Overlapping Oral Equivalents and Human Exposure Estimates

Use pattern	In vivo effects	Assay endpoint hits	AER value/range
Fungicide (almonds, grapes, pome, and stone fruits)	Liver and kidney effects	Changes in expression of multiple cytochrome P-450s and UGT1A1; competitive binding of muscarinic receptors	0.67–0.91

CONCLUSION: The incorporation of dosimetry and exposure provide necessary context for interpretation of *in vitro* toxicity screening data and are important considerations in determining chemical testing priorities.

REFERENCES:

Rotroff, D. M., Wetmore, B. A., Dix, D. J., Ferguson, S. S., Clewell, H. J., Houck, K. A., Lecluyse, E. L., Andersen, M. E., Judson, R. S., Smith, C. M., et al. (2010). Incorporating human dosimetry and exposure into high throughput in vitro toxicity screening. Toxicol. Sci. 117, 348–358.

Waters, N. J., Jones, R., Williams, G., and Sohal, B. (2008). Validation of a rapid equilibrium dialysis approach for the measurement of plasma protein binding. J. Pharm. Sci. 97, 4586–4595.

Wilkinson, R. G., and Shand, D. G. (1975). A physiological approach to hepatic drug clearance. Clin. Pharmacol. Ther. 18, 377–389.

(Wetmore BA, *et al*., 2012)

Report:	K-CA 5.8.2/06. Chorfa A, Lazizzera C, Bétemps D, Morignat E, Dussurgey S, Andrieu T and Baron T, 2014. A variety of pesticides trigger in vitro α-synuclein accumulation, a key event in Parkinson's disease. Published paper. Arch Toxicol. DOI 10.1007/s00204-014-1388-2. Syngenta File No. NA_13827.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: In Parkinson's disease (PD), a neurodegenerative disease characterized by the aggregation of the alpha-synuclein (αS) protein, numerous epidemiological and experimental studies have suggested a possible role of exposure to some pesticides. Epidemiological studies have largely failed to identify pesticides specifically involved in PD, so it is of critical importance to set up *in vitro* toxicity studies of pesticides. Therefore, changes of αS levels following pesticide exposures of human cell lines *in vitro*, using either ELISA detection of endogenous αS or flow cytometry after overexpression using recombinant adenoviruses were measured.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

Lewy bodies and Lewy neurites represent deposits of aggregated αS in the brain of patients suffering with some neurodegenerative disorders including PD and dementia with Lewy bodies (***Spillantini et al. 1997***). Even slight overexpression or slower degradation of the protein could constitute a risk factor for PD

(***Ibanez et al, 2004***). Braak and colleagues had postulated the hypothesis that a putative, but still unidentified, environmental pathogen entering by olfactory and/or digestive route could induce αS misfolding and aggregation in specific cell types and reach the brain via a consecutive series of projection neurons (***Braak et al. 2006; Hawkes et al. 2009***).

Changes of αS levels following exposure of human cell lines (SH-SY5Y) to cyprodinil at its IC50 (104 µM) *in vitro*, using either ELISA detection of endogenous αS or flow cytometry after over expression using recombinant adenoviruses, were measured.

Three pesticides which have frequently been associated with PD, produced a dose dependent increase in cellular αS levels and released αS into the culture medium. From examination of an additional series of 20 pesticides from different families and chemical structures, it was found that majority of 12 studied fungicides also produced αS accumulation with cyprodinil having more pronounced effects than the three pesticides tested.

A variety of pesticides, including cyprodinil, can disrupt αS homeostasis *in vitro*. Data in this paper illustrate an experimental strategy that could help in the identification of chemicals that could be specifically involved in Parkinson's disease etiology.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	Further information contained within online version of article only

Vehicle: Dimethylsulfoxide ≤5% (Euromedex)

Cell culture and αS overexpression:
SH-SY5Y dopaminergic neuroblastoma and SK-MEL-2 malignant melanoma human cell lines were obtained from the "American Type Culture Collection" (Rockville, MD, USA).

FM516-SV, a human SV-40 immortalized normal melanocyte cell line, was provided by M. Herlyn, from "The Wistar Institute of Anatomy and Biology" (Philadelphia, PA, USA).

Cells were maintained in DMEM culture medium and transduced by recombinant adenoviruses encoding human αS as previously described (***Chorfa et al. 2013***).

Study Design and Methods:

In-life dates: Not reported.

Pesticide exposure: At approximately 80 % cell confluence (day 3), cells were transduced by adenoviruses and exposed (or not) to cyprodinil at its IC50 (104 µM). After a change at day 5 of the medium, supplemented (or not) with the pesticide, the cells were sampled at day 6 for analyses by flow cytometry.

In some experiments, after 3 days of cell exposure to pesticide (days 3 to 6), the culture medium was replaced with pesticide-free DMEM and the cells further maintained in culture for 4 days (until day 10). Cells were seeded in individual plates, each corresponding to one time of cell culture (days 4, 5, 6, 9 and 10). Both cells and culture medium were harvested at each of these time points. Cell lysates and culture media were finally analyzed at the same time by ELISA.

Cell death and viability assays: The MTT assay was performed as described in (***Chorfa et al. 2013***), and the IC50 value determined.

αS quantification by ELISA: Plates were coated with 100 ng/mLof capture clone 42 monoclonal antibody (MAb) (overnight at +4 °C), and cell lysates (samples) or recombinant αS (standard) were revealed with 8 ng/mL of reporter (C-20)-R polyclonal antibody (PAb) (1 h at room temperature (RT)). The plates were then incubated with anti-rabbit IgG HRP conjugate at 0.4 µg/mL (1 h at RT). Between each steps, the plates were washed 5 times in PBS 1X—0.05 % Tween-20. Finally, 3,3′,5,5′-tetramethylbenzidine solution was added to each well (15 min). The reaction was stopped with 1 N HCl, and the absorbance was measured at 450 nm. Nine independent experiments in duplicate were conducted.

αS quantification by flow cytometry: After cell permeabilization and fixation, the cell pellets were incubated in 96-well round-bottom plates with clone 42 MAb in permwash solution for 30 min at 4°C and then incubated with goat anti-mouse IgG R-phycoerythrin conjugate at 4°C for 30 min. The specific fluorescence intensities were measured with a MACSQuant analyser and the data analysed.

Statistics: For cytotoxicity assays and dose–response effects, the responses to the pesticide concentrations were modelled with 3-parameter log-logistic models. An estimate of IC50 and its related confidence intervals were derived from the model. Statistical analyses were performed using R software. The package drc was used for the normalized analysis of the dose–response curves. Analysis of variance was used to examine the different variables of interest and differences between αS levels for ELISA assays and flow cytometry. The comparisons of the mean values were performed for each condition and compared with the level of significance at $p < 0.05$ using Bonferroni's method. Statistical analysis was performed using R software.

RESULTS AND DISCUSSION

ELISA measures of the endogenous αS of untransduced SH-SY5Y cells: αS levels were very significantly increased ($p < 0.001$) by 104 µM cyprodinil.

Flow cytometry measures of cellular αS levels after pesticide exposure (72 h) of SH-SY5Y cells transduced with WT or A53T αS adenoviruses: Cyprodinil significantly increased WT and A53TαS levels ($p < 0.001$). These were still significantly increased ($p < 0.001$) when cells were over expressing the mutated A53T αS, compared with WT αS, consistent with the notion that this mutated protein, associated with a genetic form of PD, is more prone to aggregation. This increase in A53T αS levels was especially high (≈×2.5 that observed with the WT αS) with cyprodinil on endogenous or WT adenoviral αS.

CONCLUSION: A variety of pesticides, including cyprodinil, can disrupt αS homeostasis *in vitro*. Data in this paper illustrate an experimental strategy that could help in the identification of chemicals that could be specifically involved in Parkinson's disease etiology.

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Report:	K-CA 5.8.2/07. Coleman M, O'Neil J, Woehrling E, Ndunge O, Hill E, Menache A and Reiss C (2012). A preliminary investigation into the impact of a pesticide combination on human neuronal and glial cell lines in vitro. Published paper. PLoS ONE 7(8): e42768. Syngenta File No. NA_13832.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: There has been little investigation into the possible synergistic biological effects on human neuronal and glial cellular systems considering the use of many combinations of biocides, their known persistence through foodstuff processing and their potential neurological human impact. In this study, two cell lines have been used, gliomal U251 and neuroblastomal SH-SY5Y cells, which model the basic cell types of the human central nervous system, the astrocytes and neurones. The effects of a combination of three commonly used biocides (pyrimethanil, ciprodinil and fludioxonil) on cell viability, mitochondrial health and generation of oxidative stress, alone and in combination have been investigated.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil and cyprodinil combinations are reported within this summary.

Many pesticides are used increasingly in combinations during crop protection and their stability ensures the presence of such combinations in foodstuffs. The effects of three fungicides, pyrimethanil, cyprodinil and fludioxonil, were investigated together and separately on U251 and SH-SY5Y cells, which can be representative of human CNS glial and neuronal cells respectively.

Over 48h, all three agents showed significant reductions in cellular ATP, at concentrations that were more than tenfold lower than those which significantly impaired cellular viability. The effects on energy metabolism were reflected in their marked toxic effects on mitochondrial membrane potential.

In addition, evidence of oxidative stress was seen in terms of a fall in cellular thiols coupled with increases in the expression of enzymes associated with reactive species formation, such as GSH peroxidase and superoxide dismutase. The glial cell line showed significant responsiveness to the toxin challenge in terms of changes in antioxidant gene expression, although the neuronal SH-SY5Y line exhibited greater vulnerability to toxicity, which was reflected in significant increases in caspase-3 expression, which is indicative of the initiation of apoptosis.

Cyprodinil was the most toxic agent individually, although oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents.

Cyprodinil was the most toxic agent individually, although oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents.

The impact of some pesticides, both individually and in combinations, merits further study in terms of their impact on human cellular health.

MATERIALS AND METHODS

Materials:

Test Materials:	Cyprodinil (no details reported) Pyrimethanil (no details reported) Fludioxonil (no details reported)

Vehicle: Dimethylsulfoxide

Study Design and Methods:

In-life dates: Not reported.

Study design: The pesticides were dissolved completely in stock DMSO solutions and serially diluted in media prior to addition to the cells. Cyprodinil was studied singly in the various assays and in combination with the other pesticides across a concentration range of 0–1 mM. in 100 mL phenol red free DMEM PenStrep solution, in triplicate wells. Cells were incubated with increasingly cytotoxic concentrations of pesticides with the aim of the calculation of IC_{50} values for the various assays. In the combination studies, all three agents were added so that the same net weight of pesticide was present in the incubations as those containing cyprodinil alone. The vehicle controls contained DMSO alone.

Note that all the following experiments were conducted on three separate occasions in quadruplicate.

Cell Viability Assay in the presence of Pesticides: Effects on SHSY5Y and U251 cell viability were evaluated through the CellTiter Blue™ (resazurin assay; Promega), singly and in combinations. Untreated negative controls were run together with the treated cells. Reciprocal absorbance values were calculated and the percent viability was expressed as the value in the presence of toxicant as a percentage of that in the medium only control (set as 100%).

JC-1 Mitochondrial membrane potential assay: Following a 48 hour exposure to the test chemicals, the cell culture medium in each well was completely replaced with phenol red free DMEM containing 5 µM JC-1and incubated. The medium was removed, cells washed three times with PBS and 100 mL PBS added to each well. The red substrate fluorescence intensity was read at 544 nm/590 nm (excitation/emission) and the results were expressed as a percentage of the fluorescence value from the medium only control well (set as 100%).

Total glutathione assay: Following a 48 hour exposure to increasingly cytotoxic concentrations of test chemical, the cell culture medium in each well was completely replaced with 5% (w/v) sulfosalicylic acid (SSA) solution and the plates subjected to three freeze-thawing steps. A 5 U/mL GSR enzyme solution was prepared. Aliquots from each SSA treated well were removed , an NADPH/DTNB working solution added ,followed by incubation at 37°C for 15 min. GSR enzyme solution was added, the plate incubated and the A_{405} measured. The total glutathione content of samples was determined from standard curves generated with known amounts of GSH using the same procedure and expressed as a percentage of the medium only control well (set as 100%).

ATP Assay: Following a 48 hour exposure increasingly cytotoxic concentrations of test chemical, ATP levels were determined using the ATP luminescence assay kit from Invitrogen. The ATP content of samples was determined from standard curves generated with known amounts of ATP using the same procedure and expressed as a percentage of the medium only control well (set as 100%).

Real-Time PCR: Three enzymes that are strongly associated with oxidative stress, (caspase-3, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx), were studied using qRT-PCR.

Total RNA was extracted , reverse transcribed and Real-Time PCR: cDNAs were amplified in a PCR reaction using optimised sequence specific primers for caspase-3, superoxide dismutase (SOD) and glutathione peroxidase (GSHPx) , 3 enzymes strongly associated with oxidative stress.

GAPDH was selected as a stably expressed gene for normalisation of test gene expression after GeNorm analysis. The comparative CT method was used to calculate the relative quantification of gene expression.

Statistics: Fold changes in gene expression using the comparative CT method and statistical analysis were determined using the Relative Expression Software Tool (REST 2009, www.qiagen.com).

RESULTS AND DISCUSSION

CellTiter Blue™ results: Both cell lines showed similar levels of sensitivity with regard to the combination of pesticides, as well as to cyprodinil alone. For both cell lines, the combination was the most toxic. With the JC-1 assay, in both cell lines, cyprodinil and the combination were the most toxic ($P<0.001$). Cyprodinil showed an extremely potent reduction in ATP levels, which was approximately ten-fold greater than their effects in the CellTiter Blue™ viability assay ($P<0.001$). ATP reductions for cyprrodinil were significantly different with regard to cell line ($P<0.05$) than those of pyrimethanil in the SHSY5Y cells. The pattern of toxicity of the pesticide combination, with respect to the mitochondrial effects (JC-1 and ATP assays) differed markedly between the neuronal and glial cell lines. Whilst the viability, ATP and JC-1 data assumed an approximately linear increase in toxicity, the U251 cells appeared to demonstrate a more sigmoidal appearance in terms of the ATP and JC-1 data, with the glial lines exhibiting apparently greater resistance to mitochondrial membrane attrition compared with the SH-SY5Y cells ($P<0.001$).

Studies on GSH cellular content indicated that the presence of cyprodinil caused significant reductions in both cell lines at low agent concentrations. However, the pesticide combination showed significant reductions in GSH at low and high agent concentrations in both cell lines ($P<0.001$). Cyprodinil at 62.5 mM appeared to show a net increase in GSH levels with respect to control ($P<0.01$). Overall, with the U251 cells even at the highest concentrations tested, GSH levels did not actually fall to 50% so an IC_{50} could not be calculated. In contrast, in terms of thiol depletion, the SHSY5Y cells were more vulnerable than the glial cell line. IC_{50}'s for thiol depletion for the values for combination (476.7+/234.6 mM) as well as cyprodinil alone (535.8+/254.8 mM) were not significantly different from each other, but they were significantly lower than the values for pyrimethanil and fludioxonil ($P<0.001$).

qPCR gene changes: There was a marked difference between the two cell lines investigated, with the U251 cells demonstrating the most significant responses to the toxicants.

U251 cells: Cyprodinil at both concentrations resulted in a marked decrease in the expression of GSHPx ($P<0.05$). In contrast, SOD expression was elevated to varying degrees by both concentrations, with a 19.93-fold increase with the higher concentration of cyprodinil ($P<0.05$).

Caspase-3 expression was enhanced by 11.53-fold in the presence of 500 mm cyprodinil ($P<0.05$).

The high dose of the compounds in combination resulted in a large increase in the expression of GSHPx, SOD and caspase-3 by 28.54, 18.46 and 13.21-fold, respectively.

SH-SY5Y cells: A 19.79 fold increase was recorded in SOD expression in the neuronal line in the presence of the combination (P<0.05). In the neuronal cells, a significant 5.3-fold increase in caspase-3 expression was also recorded (P<0.05). GSHPx expression was also found to be elevated in the presence of the higher concentration of the combination (5.12-fold, not statistically significant).

Table 5.8.2-8: Respective IC_{50} values (mM) after exposure of U251 and SH-SY5Y cell lines to cyprodinil and cyprodinil, fludioxonil and pyrimethanil in combination using three viability assays (CellTiter Blue™, JC-1 and ATP).

Assay	Pesticide	U251 (µM IC_{50})	SH-SY5Y (µM IC_{50})
CellTiter Blue	cyprodinil	332.3 b**, c*** &	304.4 b***, c***
	combination	257.2	269.9 b***
JC-1	cyprodinil	203.7	40.5
	combination	273.6	63.9
ATP	cyprodinil	28	33 @
	combination	30.9	39.3

* P<0.05, **P<0.001, ***P<0.001.
b significant difference between respective CellTiter Blue™ and JC-1 IC_{50} values.
c significant difference between respective CellTiter Blue™ and ATP IC_{50} values.
@ P<0.05.
& P<0.001

Pesticide effects on energy metabolism: The toxicity of cyprodinil towards the cell lines appeared to be directly linked with their potent impact on ATP production; this was in turn connected with their detrimental effects on the mitochondrial membrane potential, as indicated by the JC-1 assay. ATP production has a critical effect on cell viability and whether cell death is either necrotic or apoptotic and it is strongly dependent on the level of cellular ATP depletion (***McConkey, 1998***). Cyprodinil is sufficiently lipophilic to penetrate the human blood brain barrier.

In the U251 cell line, cyprodinil directly impaired the expression of a major antioxidant defence enzyme (GSHPx) at low and high concentrations, whilst negatively affecting mitochondrial performance, an effect that should in itself lead to an increase in GSHPx expression. This impact on GSHPx occurred at cyprodinil concentrations when thiol levels were increased and this may be linked with the absence of thiol demand that resulted from the fall in GSHPx expression. Although the pesticides caused some cellular response to increases in reactive species to occur, such as the increase in SOD expression, in both cell lines, the suppression of GSHPx expression in this report suggests that in the U251 line, cell defence could potentially be partially undermined by the pesticides. This effect was not apparent in the neuronal cells.

Pesticide effects on Caspase-3 and detoxification response: Cyprodinil caused caspase-3 activation and the combination of pesticides with the U251 line showed very significant increases in the three key protective enzyme systems, GSHPx, SOD and caspase-3. Whilst this suggests that the combination of these agents was responsible for sufficient oxidative stress generation to elicit appropriate cellular defence gene responses in the U-251 cells, such a response to the combination was not evident in the SH-SY5Y line. At higher pesticide concentrations, it was apparent that thiol levels were also eroded in both cell lines and it is likely that this was a consequence of the combination of oxidative stress, attenuation of the ATP supply and mitochondrial toxicity. Although high consumption of thiols usually triggers a 'thermostatic' increase in thiol synthesis, this requires both reducing power and ATP to recycle GSH through GSSG reductase and *de novo* synthesis. The impact of the pesticides on energy metabolism and antioxidant protection may have amplified the toxic effects of the pesticides alone and in combination.

Context of findings in terms of modes of action and application: The toxicity of cyprodinil and the combination of fungicides seen in this study may be linked with commonality in basic cellular function

between mammalian and other target species and this may have significantly eroded these agents' fungal specificity. It is known that concerning mitochondrial structure and function, there are many similarities between mammalian and fungal systems (***Ragan, 1987***). In addition, a recent report has shown cyprodinil to inhibit human erythrocytic SOD, an effect that may also promote oxidative stress and cellular attrition (***Karadag and Bilgin, 2010***).

CONCLUSION: Cyprodinil was the most toxic agent individually, although oxidative stress-related enzyme gene expression increases appeared to demonstrate some degree of synergy in the presence of the combination of agents.

The impact of some pesticides, both individually and in combinations, merits further study in terms of their impact on human cellular health.

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Report:	K-CA 5.8.2/08. Fang C-C, Chen F-Y, Chen C-R, Liu C-C, Wong L-C, Liu Y-W and Su J-G J, 2013. Cyprodinil as an activator of aryl hydrocarbon receptor. Published paper. Toxicology 304:32-40. Syngenta File No. CGA219417_11675.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The aim of this study was to determine whether cyprodinil activates the aryl hydrocarbon receptor (AhR) and induces CYP1A1 expression in ovarian granulosa and liver cell lines, although its structure distinctly differs from that of the prototype of AHR ligands, such as benzo[a]pyrene (BaP) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD).

Cyprodinil is a pyrimidinamine fungicide and acts by inhibiting the biosynthesis of methionine and other thionic amino acids of fungi.

Upon interaction with xenobiotics, such as TCDD and BaP, the ligand-bound aryl hydrocarbon receptor (AhR) is translocated to the nucleus to form a heterodimer with the AhR nuclear translocator (ARNT), followed by recognition of the aryl hydrocarbon response element (AhRE) (also called the dioxin response element (DRE) and xenobiotic response element (XRE) motifs) on target genes. Cytochrome P450 CYP1A1 is the best-known AhR-sensitive target and the expression level of CYP1A1 is used as an indicator for activation of the AhR. BaP and TCDD are toxic environmental pollutants.

The AhR protein is abundantly expressed in mouse oocytes and granulosa cells of follicles at all stages of development and is reported to be is important in the physiology of ovarian follicles.

It has been reported that the Extracellular signal-regulated kinase (ERK) signal cascade is involved in regulating ovarian steroidogenesis in vitro by gonadotropin.

EXECUTIVE SUMMARY

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It has been reported that the Extracellular signal-regulated kinase (ERK) signal cascade is involved in regulating ovarian steroidogenesis in vitro by gonadotropin. The aim of this study was to determine whether cyprodinil activates AhR and induces CYP1A1 expression in ovarian granulosa and liver cell lines.

Cyprodinil induced nuclear translocation of the AhR, and induced the transcriptional activity of aryl hydrocarbon response element (AhRE).

Cyprodinil induced the expression of cytochrome P450 (CYP) 1A1, a well-known AhR-targeted gene, in ovarian granulosa cells, HO23, and hepatoma cells, Hepa-1c1c7. Its induction did not appear in AhR signal-deficient cells, and was blocked by the AhR antagonist, CH-223191.

Cyprodinil decreased AhR expression in HO23 cells, resulting in CYP1A1 expression decreasing after it peaked at 9 h of treatment in HO23 cells.

Dexamethasone is a synthetic agonist of glucocorticoids. Cyprodinil enhanced dexamethasone-induced gene expression, and conversely, its induction of CYP1A1 expression was decreased by dexamethasone in HO23 cells, indicating its induction of crosstalk between the AhR and glucocorticoid receptor and its role as a potential endocrine disrupter.

In addition to BaP, TCDD, and an AhR agonist, β-NF, cyprodinil also phosphorylated extracellular signal-regulated kinase (ERK) in HO23 and Hepa-1c1c7 cells, indicating its deregulation of ERK activity.

Conclusion: Cyprodinil, although its structure is distinctly different from that of the prototype of AHR ligands, such as BaP and TCDD, is an AhR activator and induces expression of CYP1A1, an AhR targeted gene.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil (4-cyclopropyl-6-methyl-N-phenylpyrimidin-2-amine)
Description:	Not reported
Supplied by:	Sigma (St. Louis, MO, USA).
Lot/Batch number:	Not reported
Purity:	Not reported
CAS#	Not reported
Stability of test compound:	Not reported

Vehicle: dimethyl sulfoxide (DMSO)

Media: Minimum essential medium alpha (MEMα); Dulbecco's modified Eagle medium/nutrient mixture F12 (DMEM/F12).

Cell culture: HO23 (human ovarian granulosa cell line) cells. were maintained in DMEM/F12, supplemented with 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.

Mouse hepatoma cell lines, Hepa-1c1c7, c4 (B13NBii1), and c12 (B15ECiii2) were maintained in MEMα supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. All cultured cells were kept at 37°C in a 95% air/5% CO_2 environment.

Plasmid construction and reporter activity assay: HO23 cells were subcultured at 2.8×10^5 cells/well in a 24-well plate overnight, and then the luciferase reporter plasmid and RSV-lacZ plasmids were transiently transfected into cells using the LipofectamineTM 2000 Transfection Reagent (Invitrogen, Carlsbad, CA) for 6 h, followed by treatment with the test compounds. Cell lysates were harvested at the appropriate time points after treatment with the test compounds and were assayed for both luciferase and β-galactosidase activities using Britelite (PerkinElmer) and the Galacto-StarTM System (Tropix, Bedford, MA), according to the manufacturer's instructions.

Western blotting: HO23 cells were seeded at 5.6×10^6 cells/6-cm dish overnight. Hepa-1c1c7, c4 and c12 cells were seeded at 4×10^5 cells/6-cm dish overnight. Cells were then cultured with test compounds for 1-11h, then cell lysates were prepared and Western blots performed.

Immunocellular fluorescence staining: To detect CYP1A1 expression, Hepa-1c1c7 cells were plated at 2×10^5 cells/well in 6-well plates with microscope cover glasses in the well overnight and then were cultured with test compounds for appropriate time periods, followed by washing with phosphate-buffered saline (PBS) and being fixed with ethanol. Expression of the CYP1A1 protein was probed using an antibody against CYP1A1, as revealed by fluorescence of a rabbit polyclonal secondary Ab to Goat IgG-H&L (DyLight® 488).. Fluorescence emitted by cells was viewed using a fluorescence microscope equipped with U-MWB2 optical filters at excitation/emission wavelengths of 460–490/520 nm.

To monitor nuclear localization of the AhR, Hepa-1c1c7 cells were plated at 2×10^5 cells/well in 6-well plates with microscope cover glasses in the well overnight, then cultured with test compounds for appropriate time periods, followed by washing with PBS and fixing with 4% formaldehyde in PBS. The formaldehyde was removed by washing with PBS, and then 0.2% TritonX-100 in PBS was added to break open cells. After removing TritonX-100 by PBS, 3% bovine serum albumin (BSA) in PBS was added for blocking and was then removed. A primary Ab against the AhR was added and incubated overnight at 4°C. The target protein was probed by an Ab against the AhR, followed by a rabbit polyclonal secondary Ab to goat IgG-H&L. Finally the bisbenzimide dye, Hoechst 33342 (5 µg/mL), was added to stain chromosomes and reveal the nuclear location, and Fluoromount-G was added to reduce fluorochrome quenching during analysis of slides by fluorescence microscopy. Fluorescence emitted by

DyLight® 488 was viewed using a fluorescence microscope equipped with U-MWB2 optical filters at excitation/emission wavelengths of 460–490/520 nm. Fluorescence emitted by Hoechst 33342 was viewed using a fluorescence microscope, equipped with U-MWU optical filters at excitation/emission wavelengths of 330–385/420 nm.

Statistical analysis: All data were from at least three independent experiments under identical conditions. Analysis was by Student's t-test (means considered significantly different at $p < 0.05$).

RESULTS AND DISCUSSION

Transactivation activity of the AhR: Following treatment of HO23 cells with 10mM cyprodinil and the p1646P1Luc3 reporter for 1-11 hours, promoter activity was increased. Cyprodinil at 5, 10, and 20 µM induced transactivation activity of the AhR in HO23 cells in a dose-dependent manner with both the p1646P1Luc3 reporter and the pAhRDtkLuc3 reporter.

Table 5.8.2-9: Effect of cyprodinil on transactivation activity of the CYP1A1 promoter and aryl hydrocarbon response element (AhRE) with reporter plasmid transfected into HO23 cells

Cyprodinil (µM)	Time (h)	Reporter plasmid	Increase in activity
10	1	p1646P1Luc3	2.7-fold*
	3		3.6-fold*
	5		4.5-fold*
	9		6.6-fold*
	11		5.3-fold*
5	9	p1646P1Luc3	3.5-fold*
10			4.9-fold*
20			6.8-fold*
5	9	pAhRDtkLuc3	4.1-fold***
10			6.2-fold***
20			8.0-fold***
10	9	p1646P1Luc3	3.0-fold
10 [pre-treated with CH-223191]	9		1.1-fold#
10	9	pAhRDtkLuc3	5.1-fold
10 [pre-treated with CH-223191]	9		1.4- fold#

Significant difference from control *$p < 0.05$, ***$p < 0.001$.
Significant difference from cells not pre-treated with CH-223191 $p < 0.05$.

Expression of the CYP1A1 protein: Treatment with 10 µM cyprodinil greatly increased the CYP1A1 protein with time course- and dose-dependence in HO23 and Hepa-1c1c7 cells. The induction of CYP1A1 protein expression was detectable after 3 h of treatment with 10 µM cyprodinil in both HO23 and Hepa-1c1c7 and reached a maximum level after 9 h, and decreased thereafter in HO23 cells. It reached a plateau level from t 9-15 h in Hepa-1c1c7 cells. Cyprodinil also dose-dependently increased CYP1A1 protein expression, and 1 µM of cyprodinil caused distinct induction in both cell lines.

Following treatment of hepa-1c1c7 cells with 10 µM cyprodinil for 3, 6 and 9 h, its CYP1A1 expression was revealed by immunofluorescence images. BaP-induced CYP1A1 expression was used as a positive control.

AhR dependent CYP1A1 expression: Following treatment of Hepa-1c1c7, c4 (Arnt-deficient), and c12 (AhR deficient) cells with cyprodinil, CYP1A1 expression was induced in Hepa-1c1c7 cells, but not in c4 or c12 cells. When cells were treated with cyprodinil plus CH-223191, CYP1A1 expression was suppressed in HO23 and Hepa-1c1c7 cells.

The AhR was localized in the nucleus (monitored by an immunofluorescence image) when Hepa-1c1c7 cells were treated with cyprodinil for 1 and 2 h. BaP-induced AhR nuclear localization was used as a positive control.

Expression pattern of the AhR: Following treatment of HO23 and Hepa-1c1c7 cells with cyprodinil for 12 and 18 h cyprodinil time course-dependently decreased AhR expression in HO23 cells, but not in Hepa-1c1c7 cells. BaP (used as positive control) abolished AhR expression in both cell lines.

Dexamethasone cell-specific suppression of cyprodinil-induced CYP1A1 expression: HO23 and Hepa-1c1c7 cells were treated with dexamethasone (a synthetic agonist of glucocorticoids) at 10 and 100 nM plus cyprodinil (10 μM) for 9 h. Dexamethasone itself did not induce CYP1A1 expression, and it showed a dose-dependent inhibition of cyprodinil-induced CYP1A1 protein expression in HO23 cells, but not in Hepa-1c1c7 cells.

Dexamethasone-stimulated GRE-mediated transcriptional activity: GRE and the MMTV promoter and reporter plasmids, GREtkLUC and MMTVLUC, were used to detect their transcriptional activities in response to activation of the GR. Dexamethasone highly stimulated GRE-mediated transcription by up to 17- and 12-fold in HO23 cells with GREtkLUC and MMTVLUC, respectively In contrast, cyprodinil itself only caused respective 1.2- and 1.3-fold increases in GRE- and MMTV-mediated transcription. However, HO23 cells being co-treated with cyprodinil and dexamethasone (10 nM) caused further 1.5- and 1.6-fold increases in dexamethasone-induced GRE-mediated transcriptional activity of GREtkLUC and MMTVLUC, respectively.

Phosphorylation of ERK1/2: Phosphorylation of ERK1/2 was detectable at 5 min of treatment and peaked at 15 min of treatment with cyprodinil in HO23 cells. Cyprodinil-inducing phosphorylation of ERK1/2 was dose-dependent (0.5–20 μM). In addition to cyprodinil, the other AHR ligands, BaP, TCDD, and β-NF, also induced phosphorylation of ERK1/2 in HO23 cells. Cyprodinil also dose-dependently induced phosphorylation of ERK in Hepa-1c1c7 cells, as revealed by Western blots and immunofluorescent images. In addition, cyprodinil also dose-dependently induced phosphorylation of ERK in c4 cells, which are deficient in the AhR signal.

CONCLUSIONS: Cyprodinil, although its structure is distinctly different from that of the prototype of AhR ligands, such as BaP and TCDD, is an AhR activator and induces expression of CYP1A1, an AhR targeted gene.

(Fang C-C., *et al*., 2013)

Report:	K-CA 5.8.2/09. Rotroff DM, Wetmore BA, Dix DJ, Ferguson SS, Clewell HJ, Houck KA, Lecluyse EL, Andersen ME, Judson RS, Smith CM, Sochaski MA, Kavlock RJ, Boellmann F, Martin MT, Reif DM, Wambaugh JF, and Thomas RS. (2010). Incorporating Human Dosimetry and Exposure into High-Throughput In Vitro Toxicity Screening. Published paper. Toxicological Sciences 117(2), 348–358. Syngenta File No. NA_13825.

KLIMSCH RELIABILITY SCORE: Not Applicable

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Many chemicals in commerce today have undergone limited or no safety testing. To reduce the number of untested chemicals and prioritize limited testing resources, several governmental programs are using high-throughput *in vitro* screens for assessing chemical effects across multiple cellular pathways.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

In this study, metabolic clearance and plasma protein binding were experimentally measured for 35 ToxCast phase I chemicals, including cyprodinil. The experimental data were used to parameterize a population-based *in vitro*-to-*in vivo* extrapolation model for estimating the human oral equivalent dose necessary to produce a steady-state *in vivo* concentration equivalent to *in vitro* AC_{50} (concentration at 50% of maximum activity) and LEC (lowest effective concentration) values from the ToxCast data. For 23 of the 35 chemicals (not including cyprodinil), the range of oral equivalent doses for up to 398 ToxCast assays was compared with chronic aggregate human oral exposure estimates in order to assess whether significant *in vitro* bioactivity occurred within the range of maximum expected human oral exposure. Cyprodinil did not have an overlapping oral equivalent dose and estimated human oral exposure.

Conclusion: Cyprodinil did not have an overlapping oral equivalent dose and estimated human oral exposure as no chronic aggregate human oral exposure estimate was included in this investigation. The authors suggest that integrating both dosimetry and human exposure information with the high-throughput toxicity screening efforts provides a better basis for making informed decisions on chemical testing priorities and regulatory attention. The authors consider these tools are necessary to move beyond hazard rankings to estimates of possible *in vivo* responses based on *in vitro* screens.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	Not reported
Batch	3301X
Purity	99.9%
Source	Sigma-Aldrich

Vehicle: DMSO

Stock solutions (20 mM) were prepared in DMSO and stored in amber vials at -80°C. The stock solutions were diluted to the 0.2 and 2mM working concentrations at the time the assays were conducted.

Study Design and Methods:

In-life dates: Not reported.

Plasma protein binding assay. Plasma protein binding was estimated using the rapid equilibrium dialysis (RED) method (***Waters et al., 2008***).

Metabolic clearance assay. The rate of hepatic metabolism of the parent chemical was measured at two concentrations (1 and 10μM) over a 2-h period using cryopreserved primary human hepatocytes.

Analytical chemistry analysis by LC/MS: Samples from the metabolic clearance assay were thawed at room temperature, vortexed briefly, and centrifuged at 6000 x g for 1 min. Samples were analysed by Liquid Chromatography/Mass Spectrometry (LC/MS).

Analytical chemistry analysis by gas chromography-mass spectrometry: Samples were thawed at room temperature, vortexed briefly and then centrifuged at 10,000 x g for 10 min. Solid phase extraction was conducted and analysed using electron impact mode and selective ion monitoring.

Plasma protein binding data analysis: To calculate percent of unbound chemical, the test compound concentration in the chamber without plasma protein was divided by the concentration in the chamber containing plasma and multiplied by 100. The default value was estimated based on two standard deviations over the minimum amount of binding detected in a previous study (***Waters, et al., 2008***).

Metabolic clearance data analysis: Metabolic clearance for the 1 and 10µM starting concentrations were plotted separately in semilog format (log concentration vs. time) with two replicates at each time point.

In vitro* bioactivity data:** The ToxCast program measured activity against a set of ~400 *in vitro* assays using nine separate technologies, including cell-based and cell-free binding assays, protein and RNA expression, cell imaging, and real-time electronic impedance measurements. Each chemical-assay combination was run in concentration response format and AC_{50} or LEC values were estimated. *The in vitro* bioactivity was assumed to be solely the result of the parent compound. Many assays were metabolically inactive, although the CellzDirect, Attagene, and Cellumen assays possessed some metabolic capacity. A detailed description of the assays and associated data are provided in an earlier publication (Judson, et al., 2010***).

RESULTS AND DISCUSSION

Human exposure estimates were not included for cyprodinil. Cyprodinil was not one of the chemicals where the highest estimated human oral exposure values fall within the range of predicted oral equivalents. The results for cyprodinil are shown below:

Concentration	Half life	Clearance
1 µM	36.6998	37.7738
10 µM	1652.5564	0.8389

CONCLUSION: Cyprodinil did not have an overlapping oral equivalent dose and estimated human oral exposure as no chronic aggregate human oral exposure estimate was included in this investigation. The authors suggest that integrating both dosimetry and human exposure information with the high-throughput toxicity screening efforts provides a better basis for making informed decisions on chemical testing priorities and regulatory attention. The authors consider these tools are necessary to move beyond hazard rankings to estimates of possible *in vivo* responses based on *in vitro* screens.

REFERENCES:

Judson, R., Richard, A., Dix, D. J., Houck, K., Martin, M., Kavlock, R., Dellarco, V., Henry, T., Holderman, T., Sayre, P., *et al*. (2009). The toxicity data landscape for environmental chemicals. Environ. Health Perspect. 117, 685–695.

Waters, N. J., Jones, R., Williams, G., and Sohal, B. (2008). Validation ofa rapid equilibrium dialysis approach for the measurement of plasma protein binding. J. Pharm. Sci. 97, 4586–4595.66

(Rotroff D., *et al,* 2010)

Report:	K-CA 5.8.2/10. Rotroff DM, Beam AL, Dix DJ , Farmer A, Freeman KM, Houck KA , Judson RS, LeCluyse EL, Martin MT, Reif DM and Ferguson SS. (2010a). Xenobiotic-Metabolizing Enzyme and Transporter Gene Expression in Primary Cultures of Human Hepatocytes Modulated by Toxcast Chemicals. Published paper. Journal of Toxicology and Environmental Health, Part B: Critical Reviews, 13:2-4, 329-346, DOI: 10.1080/10937404.2010.483949. Syngenta File No. NA_13823.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The U.S. Environmental Protection Agency (EPA) ToxCast research project is evaluating a substantial collection of *in vitro* screening assays for the ability to profile the bioactivity of environmental compounds and generate data predictive of *in vivo* toxicity.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

Primary human hepatocyte cultures are useful *in vitro* model systems of human liver because when cultured under appropriate conditions the hepatocytes retain liver-like functionality such as metabolism, transport, and cell signalling. This model system was used to characterize the concentration- and time-response of the 320 ToxCast chemicals for changes in expression of genes regulated by nuclear receptors. Fourteen gene targets were monitored in quantitative nuclease protection assays: six representative cytochromes P-450, four hepatic transporters, three Phase II conjugating enzymes, and one endogenous metabolism gene involved in cholesterol synthesis. These gene targets are sentinels of five major signalling pathways: AhR, CAR, PXR, FXR, and PPARa. Besides gene expression, the relative potency and efficacy for these chemicals to modulate cellular health and enzymatic activity were assessed. Results demonstrated that the culture system was an effective model of chemical induced responses by prototypical inducers such as phenobarbital and rifampicin. Gene expression results identified various ToxCast chemicals that were potent or efficacious inducers of one or more of the 14 genes, and by inference the 5 nuclear receptor signalling pathways. Significant relative risk associations with rodent *in vivo* chronic toxicity effects are reported for the five major receptor pathways. These gene expression data are being incorporated into the larger ToxCast predictive modelling effort.

Conclusion: In conclusion, the bioactivity of the 309 unique chemicals currently in the ToxCast library was characterized in cultures of primary human hepatocytes over concentration and time-course measurements. Many of these chemicals induced the expression of these human genes, providing distinct bioactivity profiles that may be useful in classifying and ranking these chemicals based on their potential to impact xenobiotic metabolism pathways regulated by nuclear receptors and often associated with toxicity. In addition, direct associations were observed between the activation of key human receptor pathways and specific and relevant rodent *in vivo* toxicity endpoints. These findings indicate the potential of metabolically competent, *in vitro* hepatocyte culture systems for generating data useful in predictive toxicity modelling, and identify putative human toxicity pathways for specific disease endpoints.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	Not reported
Purity	> 97%
Source	not reported

Vehicle: DMSO, water, culture medium

The stock solutions were diluted daily in culture medium such that the final DMSO concentration was 0.2% to achieve the final dosing concentrations of 0.004, 0.04, 0.4, 4 or 40 µM.

Study Design and Methods:

In-life dates: Not reported.

Hepatocyte cultures: Primary cultures of human hepatocytes were prepared from human liver tissue derived from two separate male donors. From the data it appears that cyprodinil was tested with hepatocytes from 1 donor only (hu 776). Tissue specimens used for these studies were derived from the normal margins of resected liver tissue that was resected due to the presence of metastatic colon tumors.

Hepatocytes were isolated by a modification of the two-step collagenase perfusion method described previously (***LeCluyse et al., 2005***). Final cell viability, prior to plating, was determined by the trypan blue exclusion test and was 91% (donor ref 776). Following isolation, hepatocytes were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum, insulin (4 µg/ml), and DEX (1 µM) and added to 96-well plates coated with a simple collagen, type I, substratum. Hepatocytes were allowed to attach for 4–6 h at 37ºC in a humidified culture chamber with 95% relative humidity/5% air/CO2. After attachment, culture vessels were swirled and medium containing debris and unattached cells was aspirated. Fresh ice-cold serum-free DMEM/Ham's F12 containing 50 nM DEX, 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenium (ITS+), and 0.25 mg/ml ECM was added to the culture vessels and immediately returned to the culture chamber. Medium was changed on a daily basis thereafter. Cultures of hepatocytes were maintained for 24–48 h prior to initiating experiments.

Treatment of Hepatocytes with Chemicals: This was done daily for two consecutive days with fresh dosing solutions. Time points were 6, 24 and 48 hours.

Cell Morphology Assessment: Cell morphology and integrity were evaluated using phase-contrast microscopy as an indicator of hepatocyte cell health (***Tyson & Green, 1987***). Nuclease Protection Assays (qNPA). At the conclusion of each treatment period, hepatocyte cultures (96-well) were washed with 1 volume of HBSS, lysed by addition of 25 µl ArrayPlate lysis buffer, and 70 µl/well of Denaturation Oil, denatured by incubation at 95ºC for 10 min, and frozen at approximately –70ºC until analysis by nuclease protection assay (qNPA) (***Roberts et al., 2007***).

qNPA analysis: Cell lysates were thawed, qNPA probes were added, and samples were incubated at 95ºC for 10 min to begin the detection process by denaturing the target RNA, dissociating the duplexes and secondary structure hybridization. At the conclusion of the hybridization period, S1 nuclease reagent was added to each sample to digest all nonprotected nucleotides at 50ºC for 60–90 min. At the conclusion of the S1 nuclease digestions, all reactions were stopped by transfer of all the samples to fresh plates containing stop solution and incubated at 95ºC for 15 min to deactivate the enzyme, dissociate the mRNA/ DNA probe heterodimers, and hydrolyze the resulting single stranded mRNA, leaving a stoichiometric amount of single-stranded DNA nuclease protection probe, unmodified in sequence, as the only intact oligonucleotide left in the sample. Half of the nucleotides comprising each nuclease protection

probe are utilized for capture hybridization to the array. At the completion of the array capture of probes, plates were washed, detection linkers were hybridized to the other half of each nuclease protection probe, plates were washed again, and detection enzymes were applied. The final step in the process was the imaging of the plates with the OMIX Imaging System The quantity of protected nuclease protection probe, and hence target mRNA in each well, was proportional to the luminescence intensity of the labeled detection oligonucleotides that bind each of the 16 spots within each well of a 96- well plate. Luminescence data were generated using the OMIX Imaging System software to generate endogenous control normalized data. These data were exported for bioinformatic analyses.

Marker genes: For cyprodinil the following genes were investigated:

6 hours: CYP1A2, UGT1A1, SULT2A1, CYP3A4, CYP1A1, HMGCS2, CYP2B6, GSTA2, CYP2C19, CYP2C9, SLCO1B1, ABCG2, ABCB11, ABCB1

24 hours CYP2C19, CYP1A2, UGT1A1, SULT2A1, CYP3A4, CYP1A1, HMGCS2, ABCG2, CYP2B6GSTA2, CYP2C9, SLCO1B1, ABCB11, ABCB1

48 hours: CYP1A2, UGT1A1, SULT2A1, CYP2C9, CYP3A4, SLCO1B1, CYP1A1, CYP2B6 GSTA2, CYP2C19, HMGCS2, ABCG2, ABCB11, ABCB1

Data Management and Analysis: Data were annotated with matching chemical and dosage information and compiled in a database. Foldover- control values for each respective time point were calculated for each treatment group.

Concentration-Response Curves Representative: Not done for cyprodinil.

RESULTS AND DISCUSSION

Cyprodinil is not cited as one of the most active chemicals. The dose response and pathway analysis has not been reported for cyprodinil.

Based on supplementary data cyprodinil gave AC_{50} values for the following genes only:

6 hours: CYP1A2, CYP1A1, CYP2B6

24 hours: CYP1A2, UGT1A1, SULT2A1, CYP3A4, CYP1A1, HMGCS2, CYP2B6

48 hours: CYP1A2, UGT1A1, CYP2B6

NOTE: There were significant differences in the response between donors after exposure to the positive control chemical 3-MC for both CYP1A1 and CYP1A2. For both genes, donor Hu 776 (used for cyprodinil) was observed to have a much greater response to 3-MC treatment.

Association of *In Vitro* (Human) Gene Expression to *In Vivo* (Rodent) Toxicity: NOTE cyprodinil is not specifically mentioned. The authors provide the following associations:

CYP1A1 and CYP1A2 induction are reflective of the AhR receptor pathway.

HMGCS2 is a PPARα sentinel gene. For the 21 current ToxCast chemicals, with evidence of rat liver tumors captured in the ToxRefDB, 4 were identified as “hits” (true positives) for HMGCS2, while 17 did not meet the ’hit’ criteria (false negatives).

CYP2B6 is the sentinel gene for the CAR nuclear receptor pathway. CYP2B6 induction and is associated with rat liver hypertrophy, rat thyroid tumors, and rat liver apoptosis/necrosis. When examining the 29

chemicals in ToxRefDB that produced thyroid tumors in rats, 23 were considered to be CYP2B6 "hits" in this assay (true positives) and 6 did not meet the set criteria (false negatives) Sixty-three (63) chemicals were observed to produce rat liver hypertrophy. Of those 63, 49 were found to associate with "hits" for CYP2B6 at the 48-h time point, this includes cyprodinil. Of the 21 chemicals that produced rat liver apoptosis/necrosis, 17 were regarded as CYP2B6 "hits"

Induction of, SULT2A1 (another PXR regulated gene), was associated with rat thyroid hyperplasia.

CONCLUSIONS: In conclusion, the bioactivity of the 309 unique chemicals currently in the ToxCast library was characterized in cultures of primary human hepatocytes over concentration and time-course measurements. Many of these chemicals induced the expression of these human genes, providing distinct bioactivity profiles that may be useful in classifying and ranking these chemicals based on their potential to impact xenobiotic metabolism pathways regulated by nuclear receptors and often associated with toxicity. In addition, direct associations were observed between the activation of key human receptor pathways and specific and relevant rodent *in vivo* toxicity endpoints. These findings indicate the potential of metabolically competent, *in vitro* hepatocyte culture systems for generating data useful in predictive toxicity modelling, and identify putative human toxicity pathways for specific disease endpoints.

REFERENCES:

LeCluyse E, Bullock P, Madan A, Carroll K and Parkinson A. (1999). Influence of extracellular matrix overlay and medium formulation on the induction of cytochrome P-450 2B enzymes in primary cultures of rat hepatocytes. Drug Metab Dispos 27:909–915.

Roberts RA, Sabalos CM, LeBlanc ML, Martel RR, Frutiger YM, Unger JM, Botros IW, Rounseville MP, Seligmann BE, Miller TP, Grogan TM and Rimsza LM. (2007). Quantitative nuclease protection assay in paraffin-embedded tissue replicates prognostic microarray gene expression in diffuse large-B-cell lymphoma. Lab Invest 87:979–997.

Tyson C and Green C (1987). Cytotoxicity measures: Choices and methods In The isolated hepatocyte: Use in toxicology and xenobiotic biotransformation New York: Academic Press 119–158.

(Rotroff D., *et al,* 2010a)

CA 5.8.3 Endocrine disrupting properties

According to Commission regulation (EU) No 283/2013 specific studies are required for an active substance which induces specific indications of potential for endocrine disruption to understand mode of action and the adversity of the effects. Cyprodinil does not fulfil these criteria and specific endocrine disruption studies would not be required.

A review of the all relevant data for potential endocrine disruption in mammalian species has been undertaken to fulfil this new data requirement.

Report:	K-CA 5.8.3/01 Charlton A.J. (2015). Cyprodinil - Review for Potential for Endocrine Disruption in Mammalian Species. Syngenta Ltd. Jealott's Hill International Research Centre, Bracknell, Berks RG42 6EY. Report No. TK0223684, 02 July 2015. Unpublished. Syngenta File No. CGA219417_11641.

EXECUTIVE SUMMARY

This report reviews and summarises all of the relevant available data, including open scientific literature, on cyprodinil for potential for endocrine disruption using a weight of the evidence approach proposed by

the European Chemical Industry Council (CEFIC) Endocrine Modulators Steering Group (EMSG), structured according to the OECD Conceptual Framework for Testing and Assessment of Endocrine Disruptors.

Cyprodinil has been extensively tested, with the relevant data from the regulatory and open scientific literature studies covering a wide range of study types both *in vitro* and *in vivo*. These data fall into levels 1, 2, 4 and 5 of the OECD Conceptual Framework.

Following, evaluation of each of the relevant studies individually and a subsequent weight of evidence evaluation, it can be concluded that cyprodinil is not an endocrine disruptor as defined by WHO/IPCS (2002):

"An endocrine disruptor is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations."

(Charlton A.J., 2015)

The publications listed below have been taken from the open literature. 10 papers (K-CA 5.8.3/02, K-CA 5.8.3/03, K-CA 5.8.3/04, K-CA 5.8.3/05, K-CA 5.8.3/06, K-CA 5.8.3/07, K-CA 5.8.3/08, K-CA 5.8.3/09 & K-CA 5.8.3/10) were found as part of the comprehensive literature search (**M-CA Section 9**).

Report:	K-CA 5.8.3/02. Reif, D., *et al*. (2010). Endocrine profiling and prioritization of environmental chemicals using ToxCast™ data. Published paper. *Environ. Health Perspec*. **118(12)**: 1714-1720. Syngenta File No. NA_13716.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The aim of this study was to develop a flexible method to facilitate the rational prioritization of chemicals for further evaluation and to demonstrate its application as a candidate decision-support tool for the endocrine disruptor screening programme (EDSP).

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

The prioritization of chemicals for toxicity testing is a primary goal of the U.S. Environmental Protection Agency (EPA) ToxCast™ program. Phase I of ToxCast used a battery of 467 *in vitro*, high-throughput screening assays to assess 309 environmental chemicals. One important mode of action leading to toxicity is endocrine disruption, and the U.S. EPA's Endocrine Disruptor Screening Program (EDSP) has been charged with screening pesticide chemicals and environmental contaminants for their potential to affect the endocrine systems of humans and wildlife. The authors focussed on oestrogen, androgen, and thyroid pathways, defined putative

endocrine profiles and derived a relative rank or score for the entire ToxCast library of 309 unique chemicals. Effects on other nuclear receptors and xenobiotic metabolizing enzymes were also considered, as were pertinent chemical descriptors and pathways relevant to endocrine-mediated signalling.

Combining multiple data sources into an overall, weight-of-evidence Toxicological Priority Index (ToxPi) score for prioritizing further chemical testing resulted in more robust conclusions than any single data source taken alone.

Cyprodinil is not highlighted as an EDSP chemical. The ToxPi profiles developed here provide graphical insight into the relative contributions of multiple data domains considered in this chemical profiling and prioritization. It is amenable to incorporating extant prioritization schemes and relevant data from diverse sources, thereby facilitating meta-analysis across resources from the U.S. EPA and elsewhere. Because ToxPi scores are intended for relative ranking, particular implementations of this framework can be continually updated with new chemicals and future data. A framework amenable to data growth will be vital as the body of chemical information grows exponentially with efforts such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) in the European Union (EC 1907/2006), subsequent phases of the U.S. EPA's ToxCast program, and the inclusion of information as it becomes available from the EDSP test batteries.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	In ToxCast™ Database

Vehicle: DMSO, water, culture medium

The stock solutions were diluted daily in culture medium such that the final DMSO concentration was 0.2% to achieve the final dosing concentrations of 0.004, 0.04, 0.4, 4 or 40 µM.

Study Design and Methods:

In-life dates: Not reported.

Data sources: The data used to develop the prioritization profiles for the 309 unique chemicals are housed in U.S. EPA's ToxMiner database, an internal repository for assay data from ToxCast, and have been previously described in detail (***Judson et al. 2010***). Briefly, data were gathered from 467 assays using a variety of technologies, including biochemical HTS and cell-based HTS assays measuring direct molecular interactions with specific protein targets; high-content cell imaging assays measuring complex cellular phenotypes; a multiplexed gene expression assay for XMEs and transporters in human primary hepatocytes; and multiplexed transcription factor reporter assays.

A subset of 90 assays believed to have endocrine relevance and divided into five broad categories:

Androgen signalling pathways (5 assays AR)
Oestrogen signalling pathways (6 assays, ER)
Thyroid signalling pathways (5 assays, TR)
XME/ADME (absorption, distribution, metabolism, and excretion)

Other nuclear receptor (NR) pathways (e.g., glucocorticoid receptor, peroxisome proliferator–activated receptor, pregnane X receptor; 36 assays) included as potentially reflecting either direct (e.g., inhibition of aromatase activity) or indirect (e.g., alterations in metabolism affecting synthesis or degradation of endogenous hormones) effects on the endocrine system in vivo.

For all *in vitro* assays, a characteristic effective concentration (micromolar) for each chemical–assay combination was calculated as described by ***Judson et al. (2010)***. These values were half-maximal activity concentrations (AC_{50}) for all assays except the multiplexed transcription factor assay *in vitro* for which lowest effective concentrations (LECs) were calculated. *In vitro* targets were mapped to human genes as an intermediate connection between assays and pathways. From these assay–gene–pathway connections, chemicals showing activity in at least five assays that mapped to a given pathway were assigned a "pathway perturbation score" as the minimum AC_{50}/LEC value among the assays mapped to that pathway.

Two chemical used were a derived octanol/water partition coefficient (log*P*) and a predicted Caco-2 (cell membrane permeability assay) descriptor, to provide measures of bioavailability (related to gastrointestinal absorption and permeability, respectively, as information that would not have been captured by the *in vitro* assays).

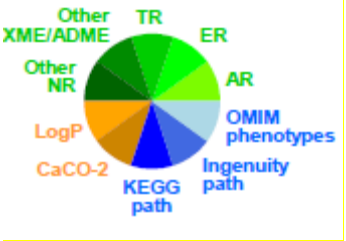
The pathways selected for endocrine relevance were taken from the Kyoto Encyclopedia of Genes and Genomes (KEGG) (***Ogata et al. 1999***) and ***National Center for Biotechnology Information. 2010***. OMIM—Online Mendelian Inheritance in Man.

ToxPi is represented as component slices of a unit circle, with each slice representing one piece (or related pieces) of information.The component data points composing that slice, and the width (in radians) indicates the relative weight of that slice in the overall ToxPi calculation.

Rationale, notation, and definition of ToxPi: Weighted combinations of data were integrated for each chemical from multiple domains, with relative scores represented in ToxPi profiles composed of slices based on one or more components. Domains are basic data types represented by slices of a given colour family: green, *in vitro* assay slices; orange, chemical properties; blue, pathways. Slices represent data from related assays, properties, or pathways, including AR, ER, TR, and seven other slices. Ninety assays, two properties, and 27 pathways make up the 119 components of this endocrine ToxPi (e.g., the ERα transcription factor assay is one of six components in the ER slice).

RESULTS AND DISCUSSION

Cyprodinil is not highlighted as an EDSP chemical.

Cyprodinil	Key
	

AR.assay.hit.potency.normalized =0
ER.assay.hit.potency.normalized =0.137151992
TR.assay.hit.potency.normalized =0.856366914
ADME.assay.hit.potency.normalized = 0.168621582

OtherNR.assay.hit.potency.normalized =0.737183541
KEGG.pathway.hit.potency.normalized =0.617309567
Ingenuity.pathway.hit.potency.normalized = 0.553003268
OMIM.pathway.hit.potency.normalized = 0.2255244

assay.scores = 1.89932403
chemProp.scores = 1.243637849
pathway.scores = 1.395837235
overall.scores =4.538799113

CONCLUSION: Cyprodinil is not highlighted as an EDSP chemical.

The ToxPi profiles developed here provide graphical insight into the relative contributions of multiple data domains considered in this chemical profiling and prioritization. It is amenable to incorporating extant prioritization schemes and relevant data from diverse sources, thereby facilitating meta-analysis across resources from the U.S. EPA and elsewhere. Because ToxPi scores are intended for relative ranking, particular implementations of this framework can be continually updated with new chemicals and future data. A framework amenable to data growth will be vital as the body of chemical information grows exponentially with efforts such as REACH (Registration, Evaluation, Authorisation and Restriction of Chemical substances) in the European Union (EC 1907/2006), subsequent phases of the U.S. EPA's ToxCast program, and the inclusion of information as it becomes available from the EDSP test batteries.

REFERENCES:

Judson RS, Houck KA, Kavlock RJ, Knudsen TB, Martin MT, Mortensen HM, et al. 2010. In vitro screening of environmental chemicals for targeted testing prioritization: the ToxCast project. Environ Health Perspect 118:485–492.

National Center for Biotechnology Information. 2010. OMIM—Online Mendelian Inheritance in Man. Available: http://www.ncbi.nlm.nih.gov/omim/ [accessed by author 27 October 2010].

Ogata H, Goto S, Sato K, Fujibuchi W, Bono H, Kanehisa M. 1999. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 27(1):29–34.

(Reif D., *et al.,* 2010)

Report:	K-CA 5.8.3/03. Rotroff, D., *et al*. (2013). Using *in vitro* high throughput screening assays to identify potential endocrine-disrupting chemicals. *Environ. Health Perspec*. **121(1)**: 7-14. Syngenta File No. NA_13717.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: In this study U.S. EPA ToxCast HTS assays for estrogen, androgen, steroidogenic, and thyroid-disrupting mechanisms were used to classify compounds and compare ToxCast results to *in vitro* and *in vivo* data from EDSP T1S assays.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

Over the past 20 years, an increased focus on detecting environmental chemicals that pose a risk of adverse effects due to endocrine disruption has driven the creation of the U.S. Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program (EDSP). Thousands of chemicals are subject to the EDSP; thus, processing these chemicals using current test batteries could require millions of dollars and decades. A need for increased throughput and efficiency motivated the development of methods using *in vitro* high throughput screening (HTS) assays to prioritize chemicals for EDSP Tier 1 screening (T1S).

An iterative model that optimized the ability of endocrine-related HTS assays to predict components of EDSP T1S and related results was used. Balanced accuracy was used as a measure of model performance.

ToxCast oestrogen receptor and androgen receptor assays predicted the results of relevant EDSP T1S assays with balanced accuracies of 0.91 ($p < 0.001$) and 0.92 ($p < 0.001$), respectively. Uterotrophic and Hershberger assay results were predicted with balanced accuracies of 0.89 ($p < 0.001$) and 1 ($p < 0.001$), respectively. Models for steroidogenic and thyroid-related effects could not be developed with the currently published ToxCast data.

Most chemicals chosen to validate EDSP T1S assays alter estrogen- and/or androgen-related end points through nuclear receptor-mediated mechanisms and are capable of being efficiently detected by the ToxCast HTS assays. For the purpose of prioritization, it is important to establish sufficient confidence that the assays being utilized are specific and sensitive so that chemicals prioritized for EDSP T1S include those most likely to be active. Although further efforts are needed to improve detection of steroidogenic and thyroid-disrupting chemicals with *in vitro* test systems, the authors conclude the results indicate that ToxCast endocrine assays are highly predictive of chemicals with estrogenic and androgenic receptor-based endocrine MOAs, and that their use in predictive models for endocrine testing would allow efficient prioritizing of chemicals for further testing. No specific results for cyprodinil are reported. Cyprodinil was not one of the chemicals with Chemicals Results Overlapping HTS-A and Guideline-A or Chemicals Results Overlapping HTS-E and Guideline-E.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	In ToxCast™ Database

Vehicle: DMSO

Stock solutions (20 mM) were prepared in DMSO and stored in amber vials at -80°C. The stock solutions were diluted to the 0.2 and 2mM working concentrations at the time the assays were conducted.

Study Design and Methods:

In-life dates: Not reported.

Chemical selection: Data from the ToxCast Phase I chemical library, containing data for 309 unique chemical structures (***U.S. EPA 2012a***). Most of these chemicals, including cyprodinil are either current or

former active ingredients in food-use pesticides that were designed to be bioactive or they are industrial chemicals that are environmentally relevant.

Data collection: Data covering guideline endocrine-related *in vitro* and *in vivo* assays was extracted from documents used in EDSP Tier 1 validation or conducted according to OECD guidelines. There were a total of 40 studies covering 154 unique chemicals, resulting in a total of 1,246 captured end points. Data from guideline endocrine-related *in vitro* and *in vivo* studies were extracted from EDSP Tier 1 validation reports from the U.S. EPA EDSP web site (***U.S. EPA 2012b***). Non-guideline studies were obtained from open literature by querying PubMed and Google Scholar using appropriate search terms. Guideline endocrine-related assays gathered from EDSP validation reports and OECD guideline studies were categorized according to whether they tested estrogen-, androgen-, steroidogenesis-, or thyroid-related MOAs (guideline-E, guideline-A, guideline-S, guideline-T, respectively). Additional information captured included study type (e.g., amphibian metamorphosis, reporter gene), assay type (e.g., serum levels, organ weight), species, strain, cell type, target, and whether or not it was an EDSP/OECD guideline study. Chemical potency [e.g., concentration at half-maximum activity (AC50), lowest effective concentration] for a given end point was captured as it was represented in the study report along with the maximum concentration/dose tested.

ToxCast *in vitro* assays. HTS competitive binding, enzyme inhibition, and reporter gene assays representing estrogen-, androgen-, steroidogenesis-, or thyroid-related end points (HTS-E, HTS-A, HTS-S, HTS-T, respectively) were selected as a subset of the > 500 HTS assays generated by the ToxCast program.

Model development: An iterative, balanced optimization analysis was done to determine the ability of ToxCast HTS assays to correctly classify the results of guideline endocrine-related assays while maintaining balance between sensitivity and specificity.

Statistical analysis: To identify statistically significant balanced accuracy (BA) values, a permutation test was performed. The test randomized which ToxCast assays were associated with guideline endocrine studies or biomedical literature for each endocrine MOA in order to determine whether or not a randomly chosen set of assays from the > 500 ToxCast end points would likely produce a similar association. The BA calculation based on random assay associations was performed using the same number of ToxCast assays as the model and with the same threshold criteria. Assays were permuted 10,000 times to build the random BA population distribution, and the percentile where the model BA fell among this distribution was calculated to provide a p-value. A p-value of < 0.01 was considered statistically significant.

RESULTS AND DISCUSSION

No specific results for cyprodinil are reported. Cyprodinil was not one of the chemicals with Chemicals Results Overlapping HTS-A and Guideline-A or Chemicals Results Overlapping HTS-E and Guideline-E (these are concordance between results for high throughput screening and experimental (guideline) data for androgenic and oestrogenic modes of action respectively).

Comparison of HTS and uterotrophic and Hershberger assays. A separate analysis was conducted to determine the predictive capability of the ToxCast HTS-E assays to detect positive and negative chemicals reported in EDSP/OECD guideline uterotrophic assays. Eighteen chemicals were available for comparison, and the optimal thresholds for HTS-E produced a BA of 0.9 ($p < 0.001$), with a sensitivity and a specificity of 0.88 and 0.9 respectively. Because of the wide range of test conditions and assay technologies for non-guideline studies in the biomedical literature, and species present in the open-literature, sensitivity of predictive modelling results was lower than in the guideline studies. Using the criteria of at least one ToxCast assay being positive (ToxCast HTS-E threshold of 1) and a literature threshold of > 50%, there was an overall concordance of 0.7 between the guideline-E assay results and the estrogen-related literature results.

The results of this study demonstrate that ToxCast *in vitro* assays perform adequately to prioritize chemicals for further EDSP T1S for estrogen and androgen activity, and these HTS assays are predictive of the likelihood of a positive or negative finding in more resource-intensive assays. Additional HTS assays will be needed to predict steroidogenic and thyroid activity of chemicals.

CONCLUSION: Most chemicals chosen to validate EDSP T1S assays alter estrogen- and/or androgen-related end points through nuclear receptor-mediated mechanisms and are capable of being efficiently detected by the ToxCast HTS assays. For the purpose of prioritization, it is important to establish sufficient confidence that the assays being utilized are specific and sensitive so that chemicals prioritized for EDSP T1S include those most likely to be active. Although further efforts are needed to improve detection of steroidogenic and thyroid-disrupting chemicals with *in vitro* test systems, the results indicate that ToxCast endocrine assays are highly predictive of chemicals with oestrogenic and androgenic receptor-based endocrine MOAs, and that their use in predictive models for endocrine testing would allow efficient prioritizing of chemicals for further testing. No specific results for cyprodinil are reported. Cyprodinil was not one of the chemicals with Chemicals Results Overlapping HTS-A and Guideline-A or Chemicals Results Overlapping HTS-E and Guideline-E.

REFERENCES:

U.S. EPA (U.S. Environmental Protection Agency). 2012a. TOXCAST: Research Chemical Inventory for EPA's ToxCast TM Program: Structure-Index File Available: http://www.epa.gov/ncct/dsstox/sdf_toxcst.html [accessed by author 9 November 2012].

U.S. EPA (U.S. Environmental Protection Agency). 2012b. Endocrine Disruptor Screening Program for the 21st Century (EDSP21 Work Plan). The Incorporation of *In Silico* Models and *In Vitro* High Throughput Assays in the Endocrine Disruptor Screening Program (EDSP) for Prioritization and Screening. Available: http://www.epa.gov/endo/pubs/edsp21_work_plan_summary%20_overview_final.pdf [accessed by author 12 January 2012]

(Rotroff D., *et al*, 2013)

Report:	K-CA 5.8.3/04. Rotroff, D., *et al*. (2014). Predictive endocrine testing in the 21st century using *in vitro* assays of estrogen receptor signaling responses. *Environ. Sci. Technol.* **48**: 8706-8716. Syngenta File No. NA_13715.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Estrogen receptors (ER) mediate a vast array of physiological responses through a highly complex network of signalling mechanisms Due to the potential consequences of disrupting ER signalling, there is a regulatory need to integrate existing knowledge and to develop prioritization tools capable of testing thousands of chemicals that span a broad range of chemical classes for potential estrogenicity (e.g., industrial chemicals, food additives, cosmetic ingredients, pesticides). Using *in vitro* assays has been proposed as a means to efficiently address the

regulatory requirements; however, the complexity of the ER signalling pathway presents multiple challenges.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

Thousands of environmental chemicals are subject to regulatory review for their potential to be endocrine disruptors (ED). *In vitro* high-throughput screening (HTS) assays have emerged as a potential tool for prioritizing chemicals for ED-related whole-animal tests. In this study, 1814 chemicals including pesticide active and inert ingredients, industrial chemicals, food additives, and pharmaceuticals were evaluated in a panel of 13 *in vitro* HTS assays. The panel of *in vitro* assays interrogated multiple end points related to estrogen receptor (ER) signalling, namely binding, agonist, antagonist, and cell growth responses. The results from the *in vitro* assays were used to create an ER Interaction Score. For 36 reference chemicals, an ER Interaction Score >0 showed 100% sensitivity and 87.5% specificity for classifying potential ER activity. The magnitude of the ER Interaction Score was significantly related to the potency classification of the reference chemicals ($p < 0.0001$). ERα/ERβ selectivity was also evaluated, but relatively few chemicals showed significant selectivity for a specific isoform. When applied to a broader set of chemicals with *in vivo* uterotrophic data, the ER Interaction Scores showed 91% sensitivity and 65% specificity.

An ER Interaction Score was developed by aggregating data from 13 different *in vitro* ER assays based on the known cellular ER signalling pathways. This model produced scores for an overall likelihood of a chemical being estrogenic, and these scores were highly correlated with *in vivo* data and ER reference chemical classifications, indicating that the model is capable of predicting estrogenic likelihood with a high degree of accuracy. Overall, this study provides a novel method for combining *in vitro* concentration response data from multiple assays and, when applied to a large set of ER data, accurately predicted estrogenic responses and demonstrated its utility for chemical prioritization challenges.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Description:	In ToxCast™ Database

Vehicle: DMSO

Test chemicals were diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 20 mM.

Study Design and Methods:

In-life dates: Not reported.

Chemical selection: The present study was conducted using data from the ToxCast chemical library which consisted of ~1800 unique chemicals. This inventory includes pesticide active and inert ingredients, industrial chemicals, food additives, pharmaceuticals, and other chemical classes.

Chemical Quality Control (QC): Chemical samples were commercially procured and analysed to ensure stability during the testing regime.

***In Vitro* Assays:**All *in vitro* assays were selected from the ToxCast assay database. Assays were selected in an effort to maximize coverage of the ER signalling pathway. They were classed according to type of assay.

Table 5.8.3-1: *In vitro* assay annotation and model grouping

Model Group	Assay information
Binding assay	human ER binding assay
	murine ERα binding assay
	bovine ER binding assay
Agonist	Odyssey Thera ERα-ERα dimerization in agonist mode after 1440 min
	Odyssey Thera ERα-ERβ dimerization in agonist mode after 1440 min
	Odyssey Thera ERβ-ERβ dimerization in agonist mode after 1440 min
Antagonist	ERα luciferase reporter gene assay in human BG-1 ovarian cells in antagonist mode
	ERα β-lactamase reporter gene assay in human HEK-293 cells in antagonist mode
Agonist	ERα luciferase reporter gene assay in human BG-1 ovarian cells in agonist mode
	ERα β-lactamase reporter gene assay in human HEK-293 cells in agonist mode
	multiplexed ER reporter gene assay using full length receptor in HepG2 cells
	multiplexed GAL4 reporter construct with human ERα ligand-binding domain in HepG2 cells
Cell growth	cell growth using real-time cell analysis in T47D cells at 80 h

Data Processing: All assay responses were normalized to 17β-estradiol, except those in the antagonist group, which were normalized to 4-hydroxytamoxifen. The normalized data from all chemicals for each assay were fit using nonlinear least squares regression to a Hill-model in a separate analysis.

Composite Concentration Response Curves: The interpolated, aggregated, and linearly transformed data for each of the assigned groups was fit to a single, composite concentration curve using a Hill model and nonlinear least squares regression in the open-source statistical software, R and the *sfsmisc* package

Calculation of the ER Interaction Score: An Efficacy Score (%) was calculated from the maximum value of the lower-window of the boot-strapped confidence interval. If the window did not overlap with zero, then a chemical was considered to have a statistically significant response and given an Efficacy Score > 0. If the maximum value of the lower window overlapped with 0, then the Efficacy Score will equal 0.

Hierarchical Clustering: Two-way hierarchical clustering was performed using the "pheatmap" package in statistical software.

Model Evaluation: The impact of the linear transformation was determined by comparing distributions of R2 values across chemicals in the agonist and antagonist groups, respectively, with and without the linear transformation. Statistical significance was determined using a one-tailed Wilcoxon test, and a false-discovery rate correction was applied to correct for multiple comparisons.

RESULTS AND DISCUSSION

No specific results for cyprodinil are reported. Cyprodinil was not included as one of the chemicals with the Highest ER Interaction Scores

Breakdown of Group-wise ER Assay Activity. Experimental data used in the analysis was obtained from a total of 1814 chemicals tested in the 13 assays. Assays were split into four groups depending on the type of ER-dependent activity reported: receptor binding, agonist mode transactivation, antagonist mode transactivation, and cell growth.

Agonist and Antagonist Comparisons. A broad spectrum of ER activity spanning from potent agonist to potent antagonist was observed among the chemicals tested. The agonist activity of the receptor was further investigated for ERα ERβ selectivity based on the ERα and ERβ Potency Scores. A total of 12 chemicals demonstrated statistically significantly increased ERα Potency.

Comparison to ER Reference Chemicals. The chemical library screened through the *in vitro* assays contained 36 reference compounds that were used to assess the performance of the model.

Comparison to *in vivo* Uterotrophic Assay. Uterotrophic assays are designed to measure estrogenic activity through increased uterine weight in immature or ovariectomized rats. Data from *in vivo* uterotrophic studies were available for a total of 45 chemicals with 23 negatives and 22 actives (12 agonists, 2 antagonists, 8 with both agonist and antagonist activity).

CONCLUSION: An ER Interaction Score was developed by aggregating data from 13 different *in vitro* ER assays based on the known cellular ER signalling pathways. This model produced scores for an overall likelihood of a chemical being estrogenic, and these scores were highly correlated with *in vivo* data and ER reference chemical classifications, indicating that the model is capable of predicting estrogenic likelihood with a high degree of accuracy. Overall, this study provides a novel method for combining *in vitro* concentration response data from multiple assays and, when applied to a large set of ER data, accurately predicted estrogenic responses and demonstrated its utility for chemical prioritization challenges.

(Rotroff D., *et al.,* 2014)

Report:	K-CA 5.8.3/05. Orton F, Rosivatz E, Scholze M and Kortenkamp A, (2011). Widely used pesticides with previously unknown endocrine activity revealed as *in vitro* antiandrogens.. Published paper. *Environ Health Perspect* **119:(6)**794–800. Syngenta File No. NA_13741.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Evidence suggests that there is widespread decline in male reproductive health and that anti-androgenic pollutants may play a significant role. There is also a clear disparity between pesticide exposure and data on endocrine disruption, with most of the published literature focused on pesticides that are no longer registered for use in developed countries. Estimated human exposure data was used to select pesticides to test for anti-androgenic activity, focusing on highest use pesticides.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil are reported within this summary.

The aim of this study was to test the anti-androgenic activity of currently used pesticides, with a view to informing future studies to determine their likely role in causing testicular dysgenesis syndrome (TDS). Compounds for testing, including cyprodinil, were selected based on evidence of human exposure (dietary intake data for Europe) and predicted AR antagonism according to a quantitative structure-activity relationship (QSAR) model. Compounds predicted to be AR antagonists and compounds with high exposure scores were analysed for AR antagonist properties using the MDA-kb2 assay (***Ermler et al. 2010***; ***Wilson et al. 2002***). In addition, the yeast antiandrogen screen (YAS) was used to further test a subset of pesticides that were newly identified as AR antagonists or that had MDA-kb2 assay results that were discordant with QSAR predictions.

Cyprodinil was predicted to be inactive based on the QSAR. It was an AR antagonist in the MDA-kb2 assay but was out of the QSAR prediction range because it was anti-androgenic at a concentration higher than the exclusion criterion of the QSAR (limit of detection, $IC_{25} \leq 10$ µM; IC_{20}: cyprodinil, 15.1 µM). Cyprodinil analysed in the MDA-kb2 assay showed AR agonist activity when tested in the absence of DHT co-exposure with androgenic activity occurring at lower concentrations than anti-androgenic activity. Cyprodinil was a more potent AR agonist (EC_{20} = 1.91) than antagonist (IC_{20} = 15.1) in the MDA-kb2 assay. It was anti-androgenic in the YAS. Cyprodinil was not cytotoxic in the MDA-kb2 assay but was in YAS.

Cyprodinil was classified as anti-androgenic.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Supplied by:	Sigma Aldrich (Poole, Dorset, UK)
Purity:	> 97%

Vehicle: Ethanol.

Study Design and Methods:

In-life dates: Not reported.

MDA-kb2 assay: MDA-kb2 cells are human breast cancer cells stably transfected with a fire-fly luciferase reporter gene that is driven by an androgen-response element-containing promoter. Details of the modified assay have been published (***Ermler et al. 2010***).

Cells were seeded at a concentration of 1×10^5 cells/mL in phenol red–free Leibowitz-15 medium containing 10% foetal calf serum and allowed to attach for 24 hr. Cells were then exposed to eight serial dilutions of cyprodinil with or without dihydrotestosterone (DHT) (0.25 nM). After 24 hr, luciferase activity was determined and measured. The following controls were run on each plate: media, ethanol, DHT coexposure (0.25 nM), DHT serial dilutions (0.002–10 nM), and flutamide (0.013–8 µM) or procymidone (0.005–3.2 µM) serial dilutions. All concentrations were tested in duplicate over two plates, and each pesticide was measured at least twice in separate experiments. Initially each was tested over a concentration range of 0.64 nM–50 µM using 5 dilutions (range-finding). Subsequently, the concentration range was modified to reflect the potency and toxicity of the compound. Because cytotoxic effects could not be distinguished from anti-androgenic effects in the co-exposed treatments, any readings of the

pesticide statistically significantly below the mean ethanol control level (0%) were considered toxic to MDA-kb2 cells, and the corresponding co-exposure data were not classified as anti-androgenic.

Yeast antiandrogen screen (YAS): The methods for the YAS are described (***Sohoni and Sumpter 1998***). Stimulation of the transfected AR causes a colour change in the media, which is measured by absorbance at 540 nm. Plates were also measured at 620 nm to measure cell growth (turbidity) to check for any cytotoxic effects that may have occurred. The pesticide was co-incubated with DHT (6.4 nM). Controls run in each experiment were ethanol, DHT serial dilutions (0.0026–100 nM), and flutamide serial dilutions (0.19–100 µM). The pesticide concentration range varied according to potency observed in MDA-kb2 assay but was between 0.016 and 750 µM. Incubation time was 53 hr at 28°C. Where turbidity readings were significantly depressed, toxicity was indicated and the effect could not be considered anti-androgenic; therefore, these dilutions were removed from analysis. Pesticide serial dilutions were tested in duplicate over two plates and were tested in two separate experiments.

Statistics: To analyse anti-androgenic action, raw luminescence readings were normalized on a plate-by-plate basis to the means of the positive DHT and solvent controls. All data from the same compound was pooled and statistical concentration–response regression analysis using the best-fit approach was performed. Luminescence readings from pesticides tested in the absence of DHT were divided by the mean of the solvent controls from the same plate and analysed for negative and positive trends (suggestive of cytotoxic or androgenic action, respectively) by nonparametric contrast tests. Data considered to be statistically significant at $p < 0.05$ were analysed using the best-fit approach. All statistical analysis was performed using SAS statistical software (SAS Institute Inc., Cary, NC, USA). From the best-fitting model, inhibitory concentrations for anti-androgenicity and effect concentrations for cytotoxicity were derived.

RESULTS AND DISCUSSION

Cyprodinil was predicted to be inactive based on the QSAR . It was an AR antagonist in the MDA-kb2 assay but was out of the QSAR prediction range because it was anti-androgenic at a concentration higher than the exclusion criterion of the QSAR (limit of detection, $IC_{25} \leq 10$ µM; IC_{20}: cyprodinil, 15.1 µM).

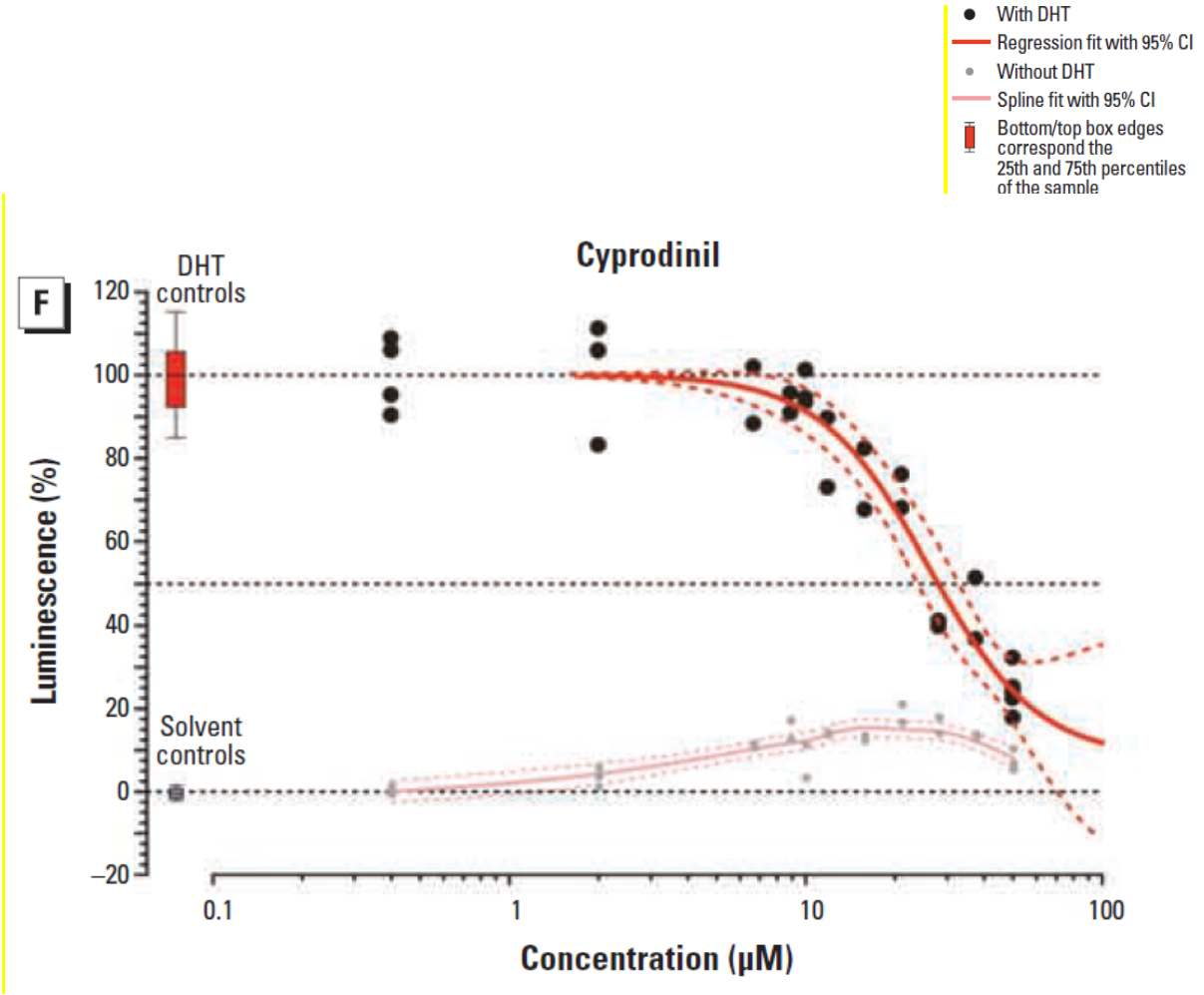
Table 5.8.3-2: Receptor-mediated anti-androgenic activity and cytotoxicity in the MDA-kb2 and YAS assays for cyprodinil

QSAR prediction	Antiandrogen IC20 (µM)		Cytotoxic EC20 (µM)		Androgen EC_{20} (µM)
	MDA-kb2	YAS	MDA-kb2	YAS	MDA-kb
Inactive	15.1	1.34	>50	27.8	1.91

MDA-kb2 assay: Cyprodinil was not cytotoxic in the MDA-kb2 assay. Cyprodinil analysed in the MDA-kb2 assay showed AR agonist activity when tested in the absence of DHT co-exposure with androgenic activity occurring at lower concentrations than anti-androgenic activity. Cyprodinil was a more potent AR agonist (EC_{20} = 1.91) than antagonist (IC_{20} = 15.1) in the MDA-kb2 assay.

Figure 5.8.3-1: Results of the MDA-kb2 assay showing stimulatory activity for cyprodinil

Data for cyprodinil (*F*) demonstrates overlap of AR antagonism (black data points and curves) with receptor agonism (grey curves).



YAS: Cyprodinil was found to be anti-androgenic and cytotoxic.

CONCLUSION: Cyprodinil was classified as anti-androgenic.

REFERENCES:

Ermler S, Scholze M, Kortenkamp A. 2010. The sensitivity of the MDA-kb2 cell in vitro assay in detecting antiandrogenic chemicals - identification of sources of variability and estimation of statistical power. Toxicol in Vitro 24(6):1845–1853.

Sohoni P, Sumpter JP. 1998. Several environmental oestrogens are also antiandrogens. J Endocrinol 158(3):327–339.

Wilson VS, Bobseine K, Lambright CR, Gray LE Jr. 2002. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. Toxicol Sci 66(1):69–81.

(Orton F *et al.*, 2011)

Report:	K-CA 5.8.3/06. Orton F, Rosivatz E, Scholze M and Kortenkamp A, (2012). Competitive androgen receptor antagonism as a factor determining the predictability of cumulative anti-androgenic effects of widely used pesticides. Published paper. *Environ Health Perspect* **120**:1578–1584. Syngenta File No. NA_13820.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Many pesticides in current use have shown to be *in vitro* androgen receptor (AR) antagonists, but there is little information about their combined effects so this paper investigated the combined effects and the competitive AR antagonism of three pesticide mixtures.

EXECUTIVE SUMMARY

The MDA-kb2 assay was used to test three pesticide mixtures: an 8 mix, a 5 mix, and a 13 mix. The 8 mix comprised eight "pure" AR antagonists (fludioxonil, fenhexamid, *ortho*-phenylphenol, tebuconazole, dimethomorph, imazalil, methiocarb, pirimiphos-methyl); the 5 mix comprised five antagonists with additional agonist properties (cyprodinil, pyrimethanil, vinclozolin, chlorpropham, linuron); and the 13 mix comprised the eight "pure" antagonists together with the five "mixed" antagonists. Fixed-mixture ratios were calculated in proportion to the concentrations of the individual mixture components that led to a suppression of DHT effects by 1%, 10%, 20%, or 50% (inhibitory concentrations IC_{01}, IC_{10}, IC_{20}, IC_{50}). The 13 mix was tested at four fixed mixture ratios (IC_{01}, IC_{10}, IC_{20}, IC_{50}), and the 8 mix and 5 mix were tested at two fixed mixture ratios (IC_{01}, IC_{10}). Concentration addition (CA) and independent action (IA) was used to formulate additivity expectations, and Schild plot analyses were used to investigate competitive AR antagonism.

A good agreement between the effects of the mixture of eight "pure" AR antagonists and the responses predicted by CA was observed. Schild plot analysis revealed that the 8 mix acted by competitive AR antagonism. However, the observed responses of the 5 mix and the 13 mix fell within the "prediction window" boundaries defined by the predicted regression curves of CA and IA. Schild plot analysis with these mixtures yielded anomalous responses incompatible with competitive receptor antagonism.

Conclusion: A mixture of widely used pesticides can, in a predictable manner, produce combined AR antagonist effects that exceed the responses elicited by the most potent component alone. Because large populations are regularly exposed to mixtures of anti-androgenic pesticides, these results underline the need for considering combination effects for these substances in regulatory practice.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Supplied by:	Sigma Aldrich (Poole, Dorset, UK)
Purity:	> 97%

Pesticide mixtures:

8 mix: Comprised eight "pure" AR antagonists (fludioxonil, fenhexamid, *ortho*-phenylphenol, tebuconazole, dimethomorph, imazalil, methiocarb, pirimiphos-methyl);

5 mix: Comprised five antagonists with additional agonist properties (cyprodinil, pyrimethanil, vinclozolin, chlorpropham, linuron);

13 mix: Comprised the eight "pure" antagonists together with the five "mixed" antagonists.

Fixed-mixture ratios were calculated in proportion to the concentrations of the individual mixture components that led to a suppression of DHT effects by 1%, 10%, 20%, or 50% (inhibitory concentrations IC_{01}, IC_{10}, IC_{20}, IC_{50}). The 13 mix was tested at four fixed mixture ratios (IC_{01}, IC_{10}, IC_{20}, IC_{50}), and the 8 mix and 5 mix were tested at two fixed mixture ratios (IC_{01}, IC_{10}).

Vehicle: Ethanol.

Study Design and Methods:

In-life dates: Not reported.

MDA-kb2 assay: MDA-kb2 cells are human breast cancer cells stably transfected with a fire-fly luciferase reporter gene that is driven by an androgen-response element-containing promoter. Details of the modified assay have been published (***Ermler et al. 2010***).

Cells were seeded at a concentration of 1×10^5 cells/mL in phenol red–free Leibowitz-15 medium containing 10% foetal calf serum in white luminometer plates. After 28 hr, luciferase activity was determined with SteadyGlo assay reagent and measured. For regression analysis, cells were exposed to eight serial dilutions of selected pesticides with or without DHT (0.25 nM). Subsequent to the initial testing range of 1.17 nM–150 µM, the mixtures' concentrations were modified to reflect the potency and toxicity of each individual mixture. For Schild plot analysis, cells were coexposed with eight serial dilutions of DHT (0.009–20 nM) and fixed concentrations of pesticide mixtures (150–6.25 µM), which varied according to the individual activity/toxicity of each mixture.

Controls were run on each plate: medium, ethanol (0.25%), DHT coexposure (0.25 nM), DHT serial dilutions (0.009–20 nM) with ethanol (0.25%), and procymidone (0.005–3.2 µM) with DHT (0.25 nM).

All concentrations were tested in duplicate over two plates; each mixture stock was measured at least twice in separate experiments, and mixtures were independently tested at least three times (using new stock solutions, in separate experiments) by two experimenters.

For comparative purposes, luminescence was normalized to DHT alone at the coexposure concentration (i.e., maximum response, 100%) and solvent-only (ethanol) controls (i.e., minimum response, 0%).

Cytotoxicity as a confounding factor: Cytotoxicity was determined in treatments without DHT by a reduction in luminescence relative to the ethanol controls. Where agonism in the absence of DHT was observed, the comparison was with the maximal response.

Renilla assay: To eliminate the possible interfering effects of cell proliferation a Renilla luciferase plasmid with a mammalian selection marker and a constitutively active promoter (herpes simplex virus–thymidine kinase (*HSV-Tk*) gene) was constructed. This construct produces luminescence in proportion to cell number, independent of AR activation.

DNA was incubated with TurboFect in serum-free Leibowitz L-15 medium. MDA-kb2 cells were transfected with the *Renilla* construct for 48 hr prior to following the normal procedure for the MDA-kb2 assay.

After 28 hr of incubation, luciferase activity was determined with Dual-Glo Reporter assay reagent which uses the sequential addition of two reconstituted reagents with luminescence measurement after each addition. The first reagent provides the necessary substrate for firefly luciferase, and the second reagent quenches this activity while at the same time activating *Renilla* luciferase.

Cells transfected with the *Renilla* construct were exposed to the 5 mix IC_{10} only; for regression analysis, 5 mix (serial dilutions: 150–5.6 µM) was coexposed with a fixed concentration of DHT (0.25 nM), and for Schild plot analysis, serial concentrations of DHT (0.009–20 nM) with various fixed concentrations of 5 mix (110–13.75 µM).

Schild plot calculations: The applicability of the MDA-kb2 assay to Schild plot analysis, was confirmed

Statistics: Raw luminescence readings were normalized on a plate-by-plate basis to the means of the positive DHT and solvent controls. Luminescence readings from pesticides tested in the absence of DHT were divided by the mean of the solvent controls from the same plate and analyzed for negative and positive trends (suggestive of cytotoxic or androgenic action, respectively). All data from the same test compound were pooled and statistical concentration–response regression analyses were conducted by using the best-fit approach to derive ICs for androgenicity (***Scholze et al. 2001***). Concentration–response data were analyzed by a generalized nonlinear mixed modeling approach with plate as a random effect modifier for individual effect data to control for variations between experiments. If readings in the absence of DHT showed indications for cytotoxic or androgenic action, the nonmonotonic concentration–response relationship was modeled by nonparametric local regression methods (***Cleveland et al. 1988***). All statistical analyses were performed using SAS statistical software version 9.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

All mixtures showed AR antagonist activity in a clear dose-dependent way.

IC_{50}s for the mixtures were once overestimated by both CA and IA (13 mix, IC_{01} mixture ratio, 10% inhibition) and in all other cases were within the range predicted by CA and IA.

Cytotoxicity was only observed at high mixture concentrations (8 mix: EC_{10}, 60-77 µM; 5 mix: EC_{10}, 70-74 µM; 13 mix; EC_{10}, 63-81 µM).

There was good agreement of the mixture composed entirely of “pure” AR antagonists (8 mix) responses with those predicted by CA over the entire concentration range and for both tested mixture ratios. CA, however, consistently overestimated the combined effects of mixtures containing AR antagonists that also showed AR agonistic properties (5 mix and 13mix). For both these mixtures, androgenic activity was seen at low concentrations when tested in the absence of DHT but when tested on their own, none of the individual pesticides in the mixtures showed AR agonistic effects at the concentration present in the mixture. The androgenicity of 5 mix and 13 mix, therefore, appears to be a genuine combination effect.

Competitive receptor antagonism was confirmed by Schild plot analysis with the pure antiandrogen flutamide and DHT. Schild plot analysis of the 8 mix (“pure” AR antagonists) showed that increasing concentrations of the 8 mix shifted the DHT curve progressively towards lower concentrations, but did not affect the maximal response of the agonist. The resulting Schild plot was linear, suggesting that the observed AR antagonistic effect of the mixture was due to competitive AR antagonism without being confounded by multiple binding sites or pharmacokinetic interactions.

For the 5 mix and 13 mix the maximal effects observed at saturating DHT concentrations were higher than normally seen with the agonist on its own (i.e., "supramaximal" effects). These supramaximal DHT responses increased with rising mixture concentrations until 100 µM (5 mix) and 70 µM (13 mix). At even higher concentrations, a downturn of responses was observed corresponding with cytotoxicity values. Schild plots could, therefore, not be constructed for the 5 mix and 13 mix. These results show that the suppression of DHT effects seen with these two mixtures are not solely due to competitive receptor antagonism and suggest that more complex processes are operational at the receptor.

There was no dose–response relationship between rising concentrations of the 5 mix and *Renilla* luminescence of the MDA-kb2 cells and no differences in luminescence between ethanol only (mean ± SD, 3,335 ± 896) and the DHT background concentration only (3,036 ± 756). The same applied to DHT (3,059 ± 689), the positive control procymidone (4,115 ± 820), and to any concentration of the 5 mix with DHT (110 µM: 2,198 ± 418; 55.5 µM: 1,909 ± 399; 27.5 µM: 2,080 ± 359; 13.75 µM: 2,340 ± 379) and the 5 mix on its own (150–5.6 µM: 2,134 ± 322). This indicated that cell proliferation was not the cause of the increased luminescence observed with this mixture and DHT, and that the supramaximal responses were the consequence of phenomena at the receptor.

CONCLUSION: A mixture of widely used pesticides can, in a predictable manner, produce combined AR antagonist effects that exceed the responses elicited by the most potent component alone. Because large populations are regularly exposed to mixtures of anti-androgenic pesticides, these results underline the need for considering combination effects for these substances in regulatory practice.

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Scholze M, Boedeker W, Faust M, Backhaus T, Altenburger R, Grimme LH. 2001. A general best-fit method for concentration-response curves and the estimation of low-effect concentrations. Environ Toxicol Chem 20(2):448–457.

(Orton F, *et al.,* 2012)

Report:	K-CA 5.8.3/07. Medjakovic S, Zoechling A, Gerster P, Ivanova M, Teng Y, Klinge C, Schildberger B, Gartner M and Jungbauer A, (2014). Effect of non-persistent pesticides on estrogen receptor, androgen receptor, and aryl hydrocarbon receptor. Published paper. Wiley Online Library (wileyonlinelibrary.com).DOI 10.1002/tox.21852. Syngenta File No. NA_13826.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Non-persistent pesticides are considered less harmful for the environment, but their impact as endocrine disruptors has not been fully explored. The aim of this study was to determine residual levels in wine and evaluate the ER, AR, and AhR transactivational potential of several pesticides commonly used in fruit and wine cultivation. This information can provide a better understanding and assessment of potential health risks for fruit and wine consumers, as well as pesticide applicators, farm workers, and people living in nearby agricultural areas.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil and its formulated product "Switch" are reported within this summary.

The aim of the present study was to quantify maximum residue concentrations of the active ingredients of formulated pesticides and to assess the transactivation potential of the formulated products and their active ingredients on human estrogen receptor α (ERα), androgen receptor (AR) and arylhydrocarbon receptor (AhR) *in vitro*. Relative binding affinities of the pure pesticide constituent for AR and their effect on human breast cancer and prostate cancer cell lines were evaluated.

The pesticide Switch (375 g/kg cyprodinil and 250 g/kg fludioxinil) was applied to grape vines, and the maximum residue concentration of its active ingredients was quantified. The transactivation potential of the pesticide, Switch, and its active ingredient, cyprodinil, was tested on human estrogen receptor α (ERα), androgen receptor (AR) and arylhydrocarbon receptor (AhR) *in vitro*. Relative binding affinities of cyprodinil for AR and its effect on human breast cancer and prostate cancer cell lines were evaluated.

Residue concentrations of Switch's ingredients were below maximum residue limits. Cyprodinil was an AhR-agonists (EC_{50} of 1.4µM) and showed weak AR binding.

Assuming a total uptake which does not take metabolism and clearance rates into account, the *in vitro* evidence suggests that pesticides could activate pathways affecting hormonal balance, even within permitted limits, thus potentially acting as endocrine disruptors.

Cyprodinil is an arylhydrocarbon receptor (AhR) agonist, a low estrogen receptor α (ERα) agonist and a very weak antagonist for the androgen receptor (AR).

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil (active ingredient)
Supplied by:	Sigma Aldrich
Lot/Batch number:	Not reported
Purity:	Analytical standard purity grade
CAS#:	Not reported

Test Material:	Switch (Formulation)
Supplied by:	Syngenta Agro GmbH (Dielsdorf, Switzerland),
Lot/Batch number:	Not reported
Purity:	375 g/kg cyprodinil and 250 g/kg fludioxinil
CAS#:	Not reported

Vehicle: DMSO.

Study Design and Methods:

In-life dates: Not reported.

Pesticide Application and Residue Analysis: Grüner Veltliner grapes were grown and treated with the active ingredients cyprodinil and fludioxonil. The variants were split into three groups:

- Group 1, observation for a waiting period of at least 5 weeks with cyprodinil and fludioxonil application on July 23, 2008;
- Group 2, no observance over the waiting period (approx. 3.5 weeks) with cyprodinil and fludioxonil application on September 19, 2008;
- Group 3, no final spraying.

Until the final application, the spraying followed the established program. The grapes were harvested on October 13, 2008.

GC-MS analysis: Samples were prepared for GC-MS analysis according to the QuECheRS method (***Lesueur et al., 2008***) and analysed on a Hewlett-Packard GC/MS Model 6890N Series gas chromatograph coupled to 5973N and 5975 mass selective detectors. Agilent Chemstation Software G1701DA (version D.02.00.237) was used for data analysis. Analyses were conducted on the principle of the simultaneous full scan/SIM mode method (***Lesueur and Gartner, 2005***).

HPLC-MS Analysis: The HPLC system was an HP-1100 Series controlled with the Agilent Technologies Chemstation for LC 3D System Software.

Sample Preparation for *In Vitro* Assays: Stock solutions (0.01 M) of the pure compounds in DMSO were prepared and further diluted in 1:10 steps. Pesticide extracts were made by dissolving 20 mg of the preparations in 1 mL DMSO and were further diluted in DMSO for testing.

Yeast Estrogen Assay α (yESα) and Yeast Androgen Assay (yAS): Both assays are described in ***Reiter et al, 2009*** and are two-plasmid systems containing expression plasmids with the appropriate human receptor gene (ERα or AR) and a LacZ reporter plasmid. The expression plasmid is expressed on induction with copper. The presence of a ligand provokes the binding of the hormone receptor as a homodimer to a hormone response element on the reporter plasmid. The gene product β-galactosidase is quantified and measured for the agonistic effect of the ligand.

The YEAST yAhR assay is described in detail in ***Medjakovic and Jungbauer, 2008***. The yeast construct used contains the human AhR and aryl hydrocarbon receptor nuclear translocator (ARNT) genes integrated in chromosome III under the control of a galactose-regulated promoter. After induction with galactose, the genes are expressed equally. On AhR-ligand binding, an AhRARNT complex forms and is able to bind xenobiotic response elements of the lacZ-reporter plasmid. Induced expression of the lacZ gene can be quantified photometrically.

Only extract concentrations that produced a transactivation signal in the linear range of the logistic dose response curve were used for calculations and evaluation.

Competitive LBA for AR: The assay was performed according to the protocol of the test kit. The binding of the receptor to a fluorescent Fluoromone™ ligand results in high polarization. The displacement of the labelled ligand by a test compound results in lower polarization values. The shift in polarization is a measure for the relative binding affinity of a test compound.

Cell culture: Human prostate cancer cell lines LNCaP (androgen sensitive) and DU145 (androgen insensitive) and human breast cancer cell lines MCF-7 (ERα positive) and MDA-MB-231 (ERα negative) were cultured in appropriate media (LNCaP: RPMI 1640 w/o phenol red + 10% fetal calf serum (FCS); DU145:RPMI 1640 + 10% FCS; MCF-7: MEM Earl's Salt + 2 mM L-alanyl glutamin, 1 mM sodium-pyruvate, 1x nonessential amino acid solution, 0.01 mg/mL bovine insulin and 10% FCS; MDA-MB-231: Leibovitz's L-15 + 10% FCS) and incubated at 37°C and 5% CO_2 and humidified atmosphere. MDA-MB-231 cells were incubated in CO_2 free atmosphere. Media were replaced every 2–3 days, depending on growth rate.

Cell Growth Assay (MTT-Assay): 100 µL cell suspension (10^5cells/mL) were applied to each well of sterile 96-well microtiter plates and incubated at 37°C and 5% CO_2 for 24 h after which media were removed and 198 µL of fresh media were added to the adhered cells. A total of 2µL of sample were applied in each well. Staurosporine (10 µM) used as a positive control and DMSO as a blank. Each test consisted of 3 microtiter plates. Cyprodinil was tested at 10 and 100 µM (end concentrations).

After 24 h 20 µL MTT-solution was added to each well of the first plate, incubated for 2 h at 37°C and the media removed. A total of 100 µL DMSO were added to each well and mixed for 5 min. The absorbance of the dissolved purple formazan complex that is a measure of cell growth and proliferation activity was measured with a Tecan Genios Pro plate reader at 570 nm with a reference wavelength of 690 nm. After 48 h, the procedure was repeated with the second microtiter plate of the test run and after 72 h with the third microtiter plate. Results were related to DMSO.

RESULTS AND DISCUSSION

GC-MS Analysis of Switch Residue on Grapes: Both with and without a waiting period, the residue level of cyprodinil was clearly below the MRLs (0.049, 0.074 and 0.038 mg/kg grapes for Switch with waiting period, Switch without waiting period and control, respectively).

Transactivating Potential of Pesticides in the yAhR: Switch exhibited transactivating potential (equivalent β-naphthoflavone concentrations 37.9 µM/g sample). The transactivation of the AhR by the pure compound was consistent with this result. Cyprodinil was able to transactivate AhR in the yeast assay

Transactivating Potential of Pesticides in the yESα: Switch showed transactivation of ERα. The calculated equivalent estradiol concentration was 8.0 nM/g sample. Cyprodinil was not a ERα agonist.

Transactivating Potential of Pesticides in the yAS: No EC_{50} values could be obtained in the yAS because the pesticide did not exhibit high enough agonistic activity to establish a logistic dose response curve.

Relative Binding Affinity in the Competitive AR-LBA: Cyprodinil showed binding affinities in the AR-LBA. It competed only at concentrations >100 µM for the AR binding.

Cell growth: At a concentration of 100 µM cyprodinil inhibited strongly cell growth of all cell lines.

Phenol red that is used as a pH-indicator in cell culture media is a weak estrogen. Therefore, MCF-7 was also cultured in phenol red-free media to evaluate the effect of the pesticides on the ER-positive breast cancer cells in the absence of other estrogenic compounds. The anti-proliferative effects of cyprodinil was slight which could have been due to high variation as the basal growth rate of MCF-7 cells that were incubated with phenol red media was highly reduced.

Discussion: The consumption of 250 g of blackberries or salad containing cyprodinil at the permitted MRL (10 mg/kg) would result in a higher blood concentration (2.2 µM) than the yAhR EC_{50} value of 1.4 µM. It should be noted that concentrations slightly under the EC_{50} value can also have physiological

effects as for cyprodinil where the calculated concentrations for permitted MRLs are slightly below the EC_{50} in the yAhR. Cyprodinil is an AhR and a low ERα agonist. As anti-androgenic endocrine disrupters are environmentally highly relevant, the relative binding affinity of the pesticides were evaluated to show possible antagonistic effects. Cyprodinil seems to be a very weak antagonist for the AR. The effect of the cyprodinil on human breast and prostate cancer cell lines showed high growth inhibiting effects when tested at a concentration of 100 µM, but the effect was lost when tested with a concentration an order of magnitude lower.

Switch consumer exposure is confirmed as below the statutory MRLs. However, there is still a mentionable safety margin when considering metabolism, the unlikelihood of 100% uptake and bioavailability, and the fact that the MRLs are, in most instances, much higher than the defacto residue levels in food.

CONCLUSION: Cyprodinil is an arylhydrocarbon receptor (AhR) agonist, a low estrogen receptor α (ERα) agonist and a very weak antagonist for the androgen receptor (AR).

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Lesueur C, Knittl P, Gartner M, Mentler A, Fuerhacker M. 2008. Analysis of 140 pesticides from conventional farming foodstuff samples after extraction with the modified QuECheRS method. Food Control 19:906–914.

Medjakovic S, Jungbauer A. 2008. Red clover isoflavones biochanin A and formononetin are potent ligands of the human aryl hydrocarbon receptor. J Steroid Biochem Mol Biol 108:171–177.

Reiter E, Beck V, Medjakovic S, Mueller M, Jungbauer A. 2009. Comparison of hormonal activity of isoflavone-containing supplements used to treat menopausal complaints. Menopause 16:1049–1060.

(Medjakovic S *et al.*, 2013)

Report:	K-CA 5.8.3/08. Sipes N, Martin M, Kothiya P, Reif D, Judson R, Richard A, Houck K, Dix D, Kavlock R and Knudsen T (2013). Profiling 976 ToxCast chemicals across 331 enzymatic and receptor signalling assays. Published paper. *Chem. Res. Toxicol.* 26, 878-895. Syngenta File No. NA_13822.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: To analyse 976 chemicals (including failed pharmaceuticals, alternative plasticizers, food additives, and pesticides) in Phases I and II of the U.S. EPA's ToxCast project across 331 cell-free enzymatic and ligand binding high-throughput screening (HTS) assays. The purpose of the analysis was to better understand the potential health risks of chemicals with poorly characterized exposures and mechanisms of toxicities.

EXECUTIVE SUMMARY

Understanding potential health risks is a significant challenge due to the large numbers of diverse chemicals with poorly characterized exposures and mechanisms of toxicities. The present study analyzes 976 chemicals (including failed pharmaceuticals, alternative plasticizers, food additives, and pesticides) in Phases I and II of the U.S. EPA's ToxCast project across 331 cell-free enzymatic and ligand binding high-throughput screening (HTS) assays. Half maximal activity concentrations (AC50) were identified for 729 chemicals in 256 assays (7,135 chemical−assay pairs). Some of the most commonly affected assays were CYPs (CYP2C9 and CYP2C19), transporters (mitochondrial TSPO, norepinephrine, and dopaminergic), and GPCRs (aminergic). Heavy metals, surfactants, and dithiocarbamate fungicides showed promiscuous but distinctly different patterns of activity, whereas many of the pharmaceutical compounds showed promiscuous activity across GPCRs. Literature analysis confirmed >50% of the activities for the most potent chemical−assay pairs (54) but also revealed 10 missed interactions. Twenty-two chemicals with known estrogenic activity were correctly identified for the majority (77%), missing only the weaker interactions. In many cases, novel findings for previously unreported chemical-target combinations clustered with known chemical−target interactions.

No information specific to cyprodinil is included in this article.

Results from this large inventory of chemical-biological interactions can inform read-across methods as well as link potential targets to molecular initiating events in adverse outcome pathways for diverse toxicities.

MATERIALS AND METHODS

Test Material: Cyprodinil

Vehicle: Not applicable

Study Design and Methods:

In-life dates: Not reported.

Chemical library: Phases I and II of the ToxCast chemical library considered in this study contain 1020 diverse compound samples, consisting of 976 unique structures, and 44 replicate samples for quality control purposes. The rationale for chemical selection was based on several criteria, including chemical nominations within the EPA and other federal agencies. Chemical quality control (QC) procedures were applied.

Assay Description: The NVS assays consisted of 331 assays that detect whether a test chemical alters the binding of ligands to receptors (131) or inhibits enzymatic activity (100). The 100 inhibitory enzymatic assays were also assessed for enzymatic activation, resulting in an additional 100 assays.

Screening Strategy: All chemicals were initially screened at a single concentration in duplicate. A single concentration of 10 μM was used for CYP assays and 25 μM for all other assays. Assay-chemical combinations were identified as active from set criteria.

AC50 Calculation: All Phase I and II data were subjected to custom curve-fitting algorithms for processing and computing AC50 values (chemical concentration at which 50% of maximum activity is achieved).

Clustering: Transformed data were clustered an assay-assay and chemical-chemical similarity matrices were built.

Enrichment Score: In order to identify the assays affected by chemicals clustered together in the chemical-chemical similarity matrix, an assay category enrichment score (ES) was calculated for each chemical over the 21 assay categories, showing which categories the chemical is preferentially affecting.

Chemical Structure Enrichment: Univariate analyses between chemical-assay categories and chemical structure fragments were used to determine chemical structure fragment assay category associations. Chemical-assay category ES scores were calculated. Chemical structure fingerprints were determined chemicals to indicate the presence of a particular chemical fragment.

RESULTS AND DISCUSSION

No information specific to cyprodinil is included in the results section of this article.

CONCLUSION: Results from this large inventory of chemical-biological interactions can inform read-across methods as well as link potential targets to molecular initiating events in adverse outcome pathways for diverse toxicities.

(Sipes N, *et al.*, 2013)

Report:	K-CA 5.8.3/09. Prutner W, Nicken P, Haunhorst E, Hamscher G and Steinberg P, (2013). Effects of single pesticides and binary pesticide mixtures on estrone production in H295R cells. Published paper. *Arch Toxicol.* **87**:2201–2214. Syngenta File No. NA_13824.

The relevant data from this paper has been summarised and reviewed as part of the review for potential for endocrine disruption in mammalian species (K-CA 5.8.3/01); in addition, as requested by the RMS a summary and reliability score has been presented below.

KLIMSCH RELIABILITY SCORE: 2 (Reliable with restrictions)

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: The aim of the present study was to determine whether the human adrenocortical carcinoma cell line H295R can be used as an *in vitro* test system to investigate the effects of binary pesticide combinations on estrone production as a biological endpoint.

EXECUTIVE SUMMARY

Note that only the methods and results for cyprodinil and cyprodinil mixtures are reported within this summary.

Ten pesticides, including cyprodonil, were selected according to a tiered approach based on residue data provided by LAVES, Germany. In this study, these ten pesticides were tested individually and in selected binary pesticide mixtures in the human adrenocortical carcinoma cell line H295R to investigate their ability to modulate estrone concentration in the supernatant of H295R cells.

The number of combinations was limited based on exposure-related and toxicology-related considerations. The frequency with which each potential binary pesticide mixture was detected as a real residue combination in single food samples was taken into account. The outcome of the experiments, in which the ten compounds were tested individually in H295R cells, was taken into account, thereby putting special emphasis on the potency of the individual substances to modulate estrone production. The following four

binary pesticide mixtures were selected: (i) cyprodinil + pyrimethanil, (ii) cyprodinil + procymidone, (iii) cyprodinil + myclobutanil, (iv) cyprodinil + azoxystrobin.

Cyprodinil increased estrone concentration in the supernatant of H295R cells. When cyprodinil and pyrimethanil, which belong to the same chemical group and increase estrone production, were combined, in most of the cases the overall effect was solely determined by the most potent compound in the mixture (i.e., cyprodinil). When cyprodinil and procymidone, which belong to different chemical groups but both increase estrone production, were combined, in most cases an additive effect was observed. When cyprodinil, which increased estrone production, was combined with either myclobutanil or azoxystrobin, which decreased estrone production, the overall effect of the mixture was in most cases either entirely determined by myclobutanil or at least partially modulated by azoxystrobin.

Conclusion: In conclusion, cyprodinil increased estrone concentration in the supernatant of H295R cells. When cyprodinil and pyrimethanil, (both anilinopyrimidines and both increased estrone production) were combined, in most of the cases the overall effect was solely determined by the most potent compound in the mixture (i.e., cyprodinil). When cyprodinil and procymidone, (different chemical class but both increase estrone production), were combined, in most cases an additive effect was observed. When cyprodinil, which increased estrone production, was combined with either myclobutanil or azoxystrobin, which decreased estrone production, the overall effect of the mixture was in most cases either entirely determined by myclobutanil or at least partially modulated by azoxystrobin. It was concluded that H295R cells appear to be an adequate *in vitro* test system to study the effect of combining two pesticides affecting estrone production.

MATERIALS AND METHODS

Materials:

Test Material:	Cyprodinil
Supplied by:	Dr. Ehrenstorfer (Augsburg, Germany)
Lot/Batch number:	Not reported
Purity:	97.5-99.5%
CAS#:	121552-61-2

Test Material:	Pyrimethanil
Supplied by:	Dr. Ehrenstorfer (Augsburg, Germany)
Lot/Batch number:	Not reported
Purity:	97.5-99.5%
CAS#:	53112-28-0

Test Material:	Procymidone
Supplied by:	Dr. Ehrenstorfer (Augsburg, Germany)
Lot/Batch number:	Not reported
Purity:	97.5-99.5%
CAS#:	32809-16-8

Test Material:	Myclobutanil
Supplied by:	Dr. Ehrenstorfer (Augsburg, Germany)
Lot/Batch number:	Not reported
Purity:	97.5-99.5%
CAS#:	88671-89-0

Test Material:	Azoxystrobin
Supplied by:	Dr. Ehrenstorfer (Augsburg, Germany)
Lot/Batch number:	Not reported
Purity:	97.5-99.5%
CAS#:	131860-33-8

Vehicle and/or positive control: Dimethyl sulfoxide (DMSO, purity ≥99.5 %) / 4-Androsten-4-ol-3,17-dione (CAS No. 566-48-3) was used as a positive control for the reduction in estrone production and 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt (CAS No. 76939-46-3) as a positive control for the increase in estrone production.

Cell line: The cell line H295R was obtained from ATCC (Manassas, VA, USA).

Culture media: The basal DMEM/Ham's F-12 medium consisted of 12.12 g DMEM/Ham's F-12 powder medium, 20 mL 1 M HEPES buffer solution, 20 mL 7.5% w/v sodium bicarbonate solution and 1 L distilled water.

H295R cells were cultured in 75 cm^2 cell culture flasks using the "maintenance" cell culture medium (500 mL basal DMEM/Ham's F-12 medium, 12 mL NuSerum™, 5 mL penicillin/streptomycin and 5 mL ITS + ™ Premix Universal Culture Supplement).

When H295R cells were incubated with single pesticides or binary mixtures of them, a "treatment" cell culture medium consisting of the basal DMEM/Ham's F-12 medium, 2% Ultroser® SF (steroid-free) and maximally 0.1% (v/v) DMSO was used.

Study Design and Methods:

In-life dates: Not reported.

Treatment of the H295R cells with single pesticides or binary pesticide mixtures: 35,000 cells in 150 µL "maintenance" cell culture medium per well were seeded into a 96-well plate and incubated for 24 h at 37°C and 5% CO_2. The "maintenance" cell culture medium was then substituted by the "treatment" cell culture medium including the compounds to be tested individually at a final concentration of 0.01, 0.1, 0.3, 1, 3, 10, 30 and 100 µM.

The test substance solutions were prepared immediately before adding them to the cell culture medium. The final concentration of DMSO in each well never exceeded 0.1% (v/v). After cell treatment, 70 µL of the supernatant was immediately taken from each well and frozen at −20°C. These samples were used to measure estrone concentration by means of an ELISA kit (DRG Instruments, Marburg, Germany). The remaining supernatant in each well was used to determine the degree of cytotoxicity with the CytoTox-ONE™ Homogenous Membrane Integrity Assay (Promega, Mannheim, Germany).

The procedure to test the binary pesticide mixtures was identical to the one for the individual compounds with one exception: only 25,000 cells per well were seeded. Three concentrations in the 0.1–100 µM range were chosen individually for each compound of the binary combination. In general, these were the highest concentration not leading to a significant change in the estrone concentration (i.e., a no-effect concentration), the concentration leading to an approximately half-maximal change and the concentration leading to a maximal change in the estrone concentration in the cell culture supernatant when compared to the solvent control. Each of the three concentrations of the one compound in the binary mixture was tested with each of the three concentrations of the other compound in the binary mixture, so that nine concentration combinations per binary pesticide mixture were actually tested.

Each 96-well plate included a blank (i.e., no cells), a solvent control with 0.1% (v/v) DMSO and two positive controls (1 µM 4-androsten-4-ol-3,17-dione and 100 µM 8-bromoadenosine 3′,5′-cyclic monophosphate sodium salt).

Each concentration of the individual compounds and each concentration combination of the binary pesticide mixtures were tested in triplicate within each experiment. In the case of the individual compounds and the binary pesticide mixtures, three and five independent experiments, respectively, were performed.

Estrone measurement: The samples consisting of 70 µL supernatant of each well were thawed at room temperature and 50µL per sample was then pipetted into a 96-well estrone ELISA plate. The sample processing and estrone quantification were conducted according to the instructions of the manufacturer with slight modification (using a matrix calibration rather than the manufacturer's estrone standard solutions dissolved in serum). A preliminary control experiment demonstrated that even at 100 µM none of the pesticides tested cross-reacted >0.001 % with the antibodies of the ELISA kit.

Cytotoxicity assay: 40 µL of each supernatant of the H295R cells treated for 24 h with single compounds or binary mixtures was pipetted into a 96-well plate and cytotoxicity determined with the CytoTox-One™ Homogeneous Membrane Integrity Assay from Promega.

RESULTS AND DISCUSSION

Cyprodinil tested individually: Cyprodinil increased the estrone concentration in the supernatant of H295R cells. The maximal alteration in the estrone amount in the supernatant, compared to the corresponding solvent control, was +84% (SD±28) and the concentration of cyprodinil leading to the maximal alteration was 30 µM.

It was not cytotoxic in the concentration range tested (0.01–100 µM).

Cyprodinil tested in binary mixtures:

Cyprodinil + pyrimethanil: When both compounds were added in the lowest concentration, there was no increase in estrone biosynthesis. When only one of the two compounds in the mixture was present at its no-effect concentration, the estrone concentration in the cell culture medium increased depending on the concentration of the second compound. The extent of the estrone increase was similar to that observed when the H295R cells were treated with the single compounds in the corresponding higher concentrations. If cyprodinil was present at a concentration in the mixture that led to a half-maximal increase in estrone biosynthesis and pyrimethanil at the two higher concentrations, the effect of the combination was stronger than that of the individual compounds. If cyprodinil was present in its highest concentration in the mixture, it determined the increase in the estrone amount in the cell culture medium by itself, independently of the pyrimethanil concentration in the mixture.

Cyprodinil + procymidone: When both compounds were added in the lowest concentration, there was no increase in estrone biosynthesis. When only one of the two compounds in the mixture was present at its no-effect concentration, the estrone concentration in the cell culture medium increased depending on the effective concentration of the second compound. The extent of the estrone increase was similar to that observed when the H295R cells were incubated with the single compounds in the corresponding higher concentrations. If cyprodinil was present at a concentration in the mixture that led to a half-maximal increase in estrone biosynthesis and procymidone at the two higher concentrations, an additive effect was observed. If cyprodinil was present in its highest concentration in the mixture, it determined the increase in the estrone amount in the cell culture medium by itself, independently of the procymidone concentration in the mixture.

Cyprodinil + myclobutanil: When both compounds were added in the lowest concentration, there was no increase or decrease in estrone biosynthesis. When myclobutanil was present at its no-effect concentration (0.01 µM) in the mixture, cyprodinil at concentrations of 10 or 30 µM increased the estrone amount in the cell culture medium. At a concentration of 1 or 30 µM, myclobutanil determined the overall effect of the mixture, i.e. it led to a decrease in the estrone levels in the cell culture medium, independently of the amount of cyprodinil added.

Cyprodinil + azoxystrobin: When cyprodinil was present at a no-effect concentration, azoxystrobin determined the overall effect of the mixture, a decrease in the amounts of estrone in the cell culture medium, at all three concentrations tested. When cyprodinil was present at a concentration of 10 or 30 µM in the mixture, azoxystrobin counteracted the effect of cyprodinil in a concentration-dependent manner up to the extent that at a concentration of 30 µM azoxystrobin in the mixture (almost), no net increase in the concentration of estrone in the cell culture medium was measured.

CONCLUSION: In conclusion, cyprodinil increased estrone concentration in the supernatant of H295R cells. When cyprodinil and pyrimethanil, (both anilinopyrimidines and both increased estrone production) were combined, in most of the cases the overall effect was solely determined by the most potent compound in the mixture (i.e., cyprodinil). When cyprodinil and procymidone, (different chemical class but both increase estrone production), were combined, in most cases an additive effect was observed. When cyprodinil, which increased estrone production, was combined with either myclobutanil or azoxystrobin, which decreased estrone production, the overall effect of the mixture was in most cases either entirely determined by myclobutanil or at least partially modulated by azoxystrobin. It was concluded that H295R cells appear to be an adequate *in vitro* test system to study the effect of combining two pesticides affecting estrone production.

(Prutner W, *et al.*, 2013)

Report:	K-CA 5.8.3/10. Go RE and Choi KC, (2014). Antifungal agents as agricultural products, fenhaxamid, fludioxonil and cyprodinil, induced the expression of cytochrome P450 family and cell cycle-related genes in estrogen receptor expressing BG-1 ovarian cancer cells. Published abstract. Reproduction, Fertility and Development 27(1) 201-201. Syngenta File No. NA_13833.

KLIMSCH RELIABILITY SCORE: 4 (Not assignable - only abstract available).

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Not reported.

EXECUTIVE SUMMARY

Note that only the results for cyprodinil are reported within this summary.

The effect of three pesticides, including cyprodinil on cell viability was determined in an MTT assay. Expression levels of mRNA and protein of AhR, CYP1A1 and Cyclin D1 were analysed by RT-PCR and Western blot analysis in BG-1 ovarian cancer cells with estrogen receptors (ER). To evaluate cell viability, BG-1 cells were cultured with 0.1% DMSO (negative control), 17β-oestradiol (positive control; $1x10^{-9}$M), fenhaxamid, fludioxinil or cyprodinil (10^{-5} to 10^{-8}M). To evaluate the expression levels of mRNA and protein, BG-1 cells were cultured with 0.1% DMSO (negative control), 17β-oestradiol ($1x10^{-9}$M) and fenhaxamid, fludioxinil or cyprodinil (10^{-5}M).

17β-oestradiol (positive control), increased BG-1 cell viability approximately 5-fold compared to negative control ($p<0.05$).Treatment with cyprodinil increased BG-1 cell viability (ca.1.5 times) at a concentration of 10^{-8}M and (c.a. x2) at 10^{-5}M ($p<0.05$). When treated with an ER antagonist (ICI182780), BG-1 cell viability was the same as a negative control.

mRNA expression of CYP1A1 was increased by 17β-oestradiol and cyprodinil in a time-dependent manner.

In parallel with their transcriptional levels, protein levels of CYP1A1 and cyclin D1 were induced by 17β-oestradiol and cyprodinil. The level of AhR was not affected by E2 or cyprodinil.

Cyprodinil may have disruptive effects on ER expressing cells or tissues by alteration of CYP1A1 and cyclin D1 via an ER-dependent pathway.

(Go RE and Choi KC, 2014)

CA 5.9 Medical Data

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Cyprodinil is a broad-spectrum anilinopyrimidine fungicide, which was discovered and patented by Syngenta Crop Protection AG. It is sold worldwide under the trademark UNIX® for use in cereals, and as 'CHORUS' and 'SWITCH' for use in apples, grapes and vegetables respectively.

The active substance has been manufactured in our manufacturing plant at Monthey, Switzerland since 1993, with formulation and packing activities also taking place at that site for most Cyprodinil formulations. A 3rd party toller in the US makes Vangard. The key products are water dispersible granule (WG) and emulsifiable concentrate (EC) formulations. The main formulation type is emulsion concentrate (EC).

Three cases of moderate, reversible local irritation (erythema, swelling of eyelids) occurred among laboratory personnel during formulation development in 1992. One case of accidental eye exposure has been reported during the collection of a sample during synthesis of the active ingredient in 2000. The affected person experienced slight conjunctivitis but returned to work immediately after medical assistance. Medical follow up revealed no sequelae. No further cases have been reported. The reported cases give no indication that the eye reactions in humans are different from test animals and that no classification is required.

Since the formation of Syngenta in 2000, the Occupational Health group has maintained a database of incidents involving chemical exposure of workers. A query of the Syngenta internal database in December 2014 resulted in zero records of adverse health reported from the handling of cyprodinil during synthesis and formulation activities.

CONTROL STRATEGY

The principles of good occupational hygiene practice set a clear hierarchy of control which places primacy to removing the hazard or controlling it by engineering or procedural means, before the use of personal protective equipment (PPE) and respiratory protective equipment (RPE).

This hierarchy of control is clearly followed in Syngenta and includes consideration of aspects such as design and construction of the plant, the cleanliness of the workplace and equipment, working practices and personal hygiene.

For exposure to any substance that can be hazardous by ingestion, absorption or inhalation control must be to a standard that eliminates any health effects.

Ingestion: Eating and drinking are forbidden in areas where chemical handling takes place.

Skin contact: The plant design aims to contain, as far as is possible, chemical exposure by use of total or partial enclosure. Suitable PPE is worn by operators where there is potential for skin exposure.

Inhalation: The plant design aims to contain, as far as is possible, chemical exposure by use of total or partial enclosure and appropriate extraction systems. The plant is designed using the Occupational Exposure Limits (OEL) (see below).

ATMOSPHERIC EXPOSURE STANDARD

Occupational Exposure Limits (OELs) are used in pesticide manufacture as a means of monitoring and controlling atmospheric exposure to chemicals during active ingredient synthesis and formulation. The standards are set by the Syngenta OEL Panel as a primary mechanism of control. These are acceptable concentrations in work-place air based on available toxicology data with the application of a suitable safety factor when making the extrapolation from animal data to a human standard.

The OEL Panel considers the toxicology data available from the package of registration studies together with worker experience during the research, development and commercial manufacturing operations. The standard value is kept under review and may be amended in the light of significant new toxicology or hygiene information.

The current Syngenta OEL value for cyprodinil is 5 mg/m^3 for an 8-hour time weighted average (TWA) exposure. A value of 7 mg/m^3 was derived from the no-observed effect level (NOEL) of 3 mg/kg bw/day from the 2-year feeding study in rat, assuming a body weight of 70 kg for an adult worker and a shift inhalation volume of $10m^3$. The limit value was further reduced to 5 mg/m^3, equivalent to the agreed Syngenta maximum concentration for relatively non-toxic ‘nuisance dusts’.

In conclusion, cyprodinil has been handled in large quantities for over 20 years, at a number of manufacturing and formulation sites and with the use of appropriate control strategies, no adverse health effects associated with the material have been reported in the workforce.

CA 5.9.2 Data collected on humans

~~Please refer to original EU review. No new data or assessment is provided.~~

The publications listed below have been taken from the open literature. 5 papers (K-CA 5.9.2/01, K-CA 5.9.2/02, K-CA 5.9.2/03, K-CA 5.9.2/04 & K-CA 5.9.2/05) were found as part of the comprehensive literature search (**M-CA Section 9**).

Report:	K-CA 5.9.2/01. Schummer C., Salquèbre G., Briand O., Millet M., Appenzeller B.M.R. (2012). Determination of farm workers' exposure to pesticides by hair analysis. Toxicology Letters 210;203-210. Syngenta File No. NA_13850.

RELEVANCE AND RELIABILITY EVALUATION: The aim of the study was to develop a multi-residue method allowing the simultaneous monitoring of a large number of agricultural pesticides with high sensitivity. The results presented demonstrate that hair analysis can be used to assess human exposure to pesticides, but not quantitatively in relation to a specific route of exposure.

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: The publication makes no reference to GLP compliance.

JUSTIFICATION FOR TEST SYSTEM SELECTION: Not reported.

EXECUTIVE SUMMARY

In the present work, a highly sensitive method based on solid phase microextraction and gas chromatography tandem (triple quadrupole) mass spectrometry was used to test hair samples for 50 pesticides including 39 molecules from different chemical families currently used in agriculture and 11 organochlorines. The population investigated was composed of 18 farm workers who provided hair samples repeatedly collected during the entire treatment period (from March to November 2009). Among the 62 hair samples that were collected, 33 different target molecules were detected. The most frequently detected agricultural pesticides were Diflufenican and Pyrimethanil, two herbicides which were detected in 13 subjects. The concentration in volunteers' hair matched with agricultural activity and the highest concentration was observed for Cyprodinil (1161 pg/mg), an anilinopyrimidine used as a fungicide. For organochlorines, p,p´-DDE and γ-HCH were the most frequently detected molecules as they were present in at least one of the hair samples provided by each of the 18 volunteers. The highest concentrations detected for these chemicals reached 21.0 pg/mg for p,p´-DDE and 23.5 pg/mg for γ-HCH, but the highest concentration of organochlorine was observed for β-endosulfan (105 pg/mg). The results suggest that farm workers have a weak, though constant exposure to organochlorine pesticides, especially to p,p´-DDE and γ-HCH, while exposure to currently used pesticides is strongly associated with occupation. Observations also suggest that spraying work would not necessarily be the only source of exposure to agricultural pesticides and that worker not directly involved in spraying can also be submitted to significant level of exposure.

(Schummer C., *et al*., 2012)

Report:	K-CA 5.9.2/02. Li Y., Chen L., Chen Z., Coehlo J., Cui L., Liu Y., Lopez T., Sankaran G., Vega H., Krieger R. (2011). Glove Accumulation of Pesticide Residues for Strawberry Harvester Exposure Assessment. Bull Environ Contam Toxicol. 86:615-620. Syngenta File No. NA_13851.

RELEVANCE AND RELIABILITY EVALUATION: Although cyprodinil is mentioned in the paper, it contains no specific quantified data on cyprodinil. Not reliable (no relevant data specific to cyprodinil).

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: The publication makes no reference to GLP compliance.

EXECUTIVE SUMMARY

We investigated the accumulation of pesticide residues on rubber latex gloves that are used by strawberry harvesters to protect their skin, reduce pesticide exposure and promote food safety. Gloves accumulated residues of 16 active ingredients including azoxystrobin, bifenthrin, boscalid, captan, cyprodinil, fenhexamid, fenpropathrin, fludioxonil, hexythiazox, malathion, methomyl, naled, propiconazole, pyraclostrobin, quinoline, and quinoxyfen at different times. Glove residue accumulation (t_ 2.8–3.7 d) was very similar to the dissipation of DFRs (t_ 2.1–3.0 d) during the first 3 weeks after malathion applications. Dermal malathion dose was 0.2 mg/kg at the preharvest interval and declined to trace levels during the following 3 months. Glove accumulation of malathion indicated trace surface residue availability and was used to assess the relationship between dislodgable foliar residues and potential hand exposure.

(Li, Y., *et al*., 2011)

Report:	K-CA 5.9.2/03. Hassan A.A. (2012). Effects reported in farm workers repeatedly exposed to Pesticides. Abstracts / Toxicology Letters 211S; S43-S216. Meeting Info: 48th Congress of the European Societies of Toxicology. Syngenta File No. NA_13849.

RELEVANCE AND RELIABILITY EVALUATION: Full paper is not available – this is included in a journal of abstracts, and the abstract is presented below. Not assignable - "This includes studies or data from the literature, which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.).".

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: The publication makes no reference to GLP compliance.

EXECUTIVE SUMMARY

Purpose: Growing interest has been observed for hair to document human exposure to pesticides. Most of the studies based on hair analysis focused on specific compounds or compounds family such as organochlorines or organophosphorus, and occupational exposure to agricultural pesticides including several chemical families has been few documented. Moreover, studies were based on a single sampling and follow-up of the exposure was generally not performed. *Method:* A sensitive method using liquid–liquid extraction with gas chromatography mass spectrometry was used to test hair samples for 15 pesticides including molecules from different chemical families currently used in agriculture. The population investigated was composed of 9 farm workers who provided repeated hair sampling between March and November 2010. *Results and conclusion:* Among the 54 hair samples that were collected. 12 different target molecules were detected. For agricultural pesticides, the concentration is volunteers' hair matched with agricultural activity and the highest concentration was observed for Cyprodinil, an

anilinopyrimidine used as fungicide. For organophosphorus and organochlorines, malathion and chlordane were the most frequently detected molecules, with concentration ranging from 0.1 to 25.6 pg/mg. and the medical investigations showed that repeated or prolonged exposure to pesticides may result in the same effects as acute exposure including the delayed symptoms. Other effects reported in workers repeatedly exposed include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking and drowsiness or insomnia.

(Hassan A.A., 2012)

Report:	K-CA 5.9.2/04. Appenzeller B.M.R., Schummer C., Salquebre G., Millet M., Briand O., (2011). Hair analysis to document human exposure to agricultural and non-agricultural pesticides. Abstracts / Toxicology Letters 205S; S60-S179. Meeting Info.: 47th Congress of the European-Societies-of-Toxicology. Syngenta File No. NA_13852.

RELEVANCE AND RELIABILITY EVALUATION: Full paper not available (this abstract relates to the paper referenced in K-CA 5.9.2./01 above. Not assignable - "This includes studies or data from the literature, which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature (books, reviews, etc.).".

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: This was a non-guideline study published in the open literature. The publication makes no reference to GLP compliance.

EXECUTIVE SUMMARY

Purpose: Growing interest has been observed for hair to document human exposure to pesticides. Along with sampling easiness, the possibility to reach extended windows of detection and to obtain information on the average level of molecules entrance into the body makes hair particularly suitable for the biomonitoring of chronic exposure. Although agriculture is considered a major source of exposure to pesticides, non-agricultural pesticides are also increasingly pointed out. Nevertheless, most of the studies based on hair analysis focused on specific compounds or compounds family such as organochlorines or organophosphorus, and occupational exposure to agricultural pesticides including several chemical families has been few documented. Moreover, studies were based on a single sampling and the follow-up of the exposure was generally not performed. *Methods:* In the present work, a highly sensitive method using solid phase microextraction coupled with gas chromatography tandem mass spectrometry was used to test hair samples for 50 pesticides including 39 molecules from different chemical families currently used in agriculture and 11 organochlorines. The population investigated was composed of 18 farmworkerswhoprovided repeated hair sampling between March and November 2009. *Results of the study:*Amongthe 62 hair samples that were collected, 34 different target molecules were detected. For agricultural pesticides, the concentration is volunteers' hair matched with agricultural activity and the highest concentration was observed for Cyprodinil (1161 pg/mg), an anilinopyrimidine used as fungicide. For organochlorines, o,p´-DDE and γ-HCH were the most frequently detected molecules, with concentration ranging from 0.1 to 23.5 pg/mg.

(Appenzeller B.M.R., *et al*., 2011)

Report:	K-CA 5.9.2/05. Rapagnani M.R, Rossi R., Caffarelli V., Mazzinni F. (2007). Evaluation of potential health risks for operators in Emilia Romagna region (Italy) using exposure models. XIII Symposium Pesticide Chemistry - Environmental Fate & Ecological Effects. Syngenta File No. NA_13848.

RELEVANCE AND RELIABILITY EVALUATION: This paper presents a comparison of exposure models in place at the time of writing. Predicted exposures for a WG formulation of cyprodinil demonstrated Risk Ratio of <1 (i.e. <100% AOEL) with the use of PPE in both models at an application rate of 1.2 kg ai/ha on pears, higher than the rate presented in the representative use on apples. It is not a study so no reliability evaluation can be given.

GUIDELINES: This was a research study with no applicable guidelines.

COMPLIANCE: The publication makes no reference to GLP compliance.

EXECUTIVE SUMMARY

In this study, the potential dermal and inhalation exposure of the operators in the Emilia-Romagna region was estimated using the exposure models UK-POEM and German Model. In particular, the risk related to the techniques used for the treatment with 11 fungicides, 11 insecticides and 19 herbicides as applied to Pear and Sugar-beet culture was assessed. For this purpose, the amount of exposure estimated using the exposure models was compared to an Acceptable Operator Exposure Level (AOEL). The study has evidenced the different capability of the models to assess the operator exposure at different stages of the treatment process with or without the use of PPE (Personal Protective Equipment).

(Rapagnani M.R., *et al*., 2007)

CA 5.9.3 Direct observations

Syngenta has kept detailed records of exposure and poisoning incidences on marketed products for many years. Incident data in Syngenta are collected in two different databases. Reports on cases reported in the USA and Canada are collected in the PROSAR database; all other cases are reported into the Adverse Health Incident Database (AHI-DB).

A review of the exposure incidences of Cyprodinil formulations reported between 2003 and 2014 has been conducted and is presented in the tables below. Within this 12 years period exposure to Cyprodinil after occupational, accidental, intentional and uncertain exposure did only cause health effects of transient nature with almost minor severity or below.

In total a comparatively low number of only 20 cases have been reported in this period. 2 (10%) cases were related to intentional misuse. The other incidents were caused by occupational (8 cases, 40%) and accidental (10 cases, 50%) exposure. Exposure happened predominantly via the dermal route (30%), followed by ingestion (25%), inhalation (15%) and eye exposure (20%).

The majority of reported incidents were of very low severity grade[2] (minor and none), representing 80% of all reported incidents. All other incidents were assigned to moderate (20%) severity grade. The highest

[2] **Severity Grades:**

NONE (0):	No symptoms or signs related to poisoning
MINOR (1):	Mild, transient and spontaneously resolving symptoms
MODERATE (2):	Pronounced or prolonged symptoms
SEVERE (3):	Severe or life-threatening symptoms
FATAL (4):	Death

severity grade reported was moderate assigned to 4 cases. One of these cases was caused by deliberate ingestion. The second case of deliberate ingestion was leading to health effects of minor severity grade.

Occupational and accidental exposure predominantly happening via the dermal route, were causing mainly temporary health effects of minor or none severity grade (occupational: 88% of all occupational cases, accidental: 80% of all accidental cases).

The following summary tables of exposure related to Cyprodinil have been compiled from the PROSAR (USA/Canada) and AHI-DB[3] databases for the period 2004-2013:

exposure/severity	none	minor	moderate	severe	fatal	total
occupational	4	3	1	0	0	8
accidental	4	4	2	0	0	10
intentional	0	1	1	0	0	2
uncertain	0	0	0	0	0	0
Total	**8**	**8**	**4**	**0**	**0**	**20**

Suspected route of exposure/ severity	none	minor	moderate	severe	fatal	total
Dermal	1	2	3	0	0	6
Eye	0	2	0	0	0	2
Ingestion	2	2	1	0	0	5
Inhalation	1	2	0	0	0	3
Other	0	0	0	0	0	0
Unknown	4	0	0	0	0	4
Total	**8**	**8**	**4**	**0**	**0**	**20**

CA 5.9.4 Epidemiological studies

The company has not performed an epidemiological study. The public literature does not report on investigations indicating health effects on the general population due to exposure to cyprodinil.

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

Please refer to original EU review. No new data or assessment is provided.

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

Please refer to original EU review. No new data or assessment is provided.

Clinical Toxicology Jan 1998, Vol. 36, No. 3: 205–213.

[3] Countries included in AHI-DB: Albania, India, Algeria, Argentina, Armenia, Australia, Azerbaijan, Belarus, Belgium, Bosnia-Herzegovina, Brazil, Bulgaria, Canada, Chile, China, Colombia, Costa Rica, Croatia, Cuba, Czech Republic, Denmark, Ecuador, Egypt, El Salvador, Fiji, France, Georgia, Germany, Greece, Guatemala, Hungary, India, Indonesia, Iraq, Ireland, Italy, Japan, Jordan, Kazakhstan, Kenia, Kenya, Korea Republic of, Kosovo, Kuwait, Kyrgyzstan, Lebanon, Lithuania, Macedonia, Malawi, Malaysia, Mauritius, Mexico, Moldova, Morocco, Mozambique, New Zealand, Nicaragua, Oman, Pakistan, Peru, Philippines, Poland, Portugal, Qatar, Romania, Russian Federation, Serbia, Slovakia, Singapore, Slovenia, Spain, Switzerland, Syrian Arab Republic, Taiwan, Tajikistan, Thailand, Turkey, Turkmanistan, Ukraine, United Arab Republic, United Kingdom, USA, Uzbekistan, Venezuela, Vietnam, Yemen, Zimbabwe.

CA 5.9.7 Expected effects of poisoning

Please refer to original EU review. No new data or assessment is provided.