Bayer CropScience

Document Title

Summary of the toxicological and metabolism studies for

Thiacloprid

Data Requirements

EU Regulation 1107/2009 & EU Regulation 283/2013

Document MCA

Section 5: Toxicological and metabolism studies

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

Date

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Author(s)

[redacted]

Bayer CropScience

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Version history

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

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

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CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

INTRODUCTION

Thiacloprid was included in Annex I of Directive 91/414/EEC on 01/01/2005, as notified in Directive 2004/99/EC dated 1 October 2004 wherein there is no specific provision under Part B which needs to be considered related to toxicological data.

The Monograph prepared by the Rapporteur Member State United Kingdom in the context of the inclusion of thiacloprid in Annex 1 of the Council Directive 91/414/EEC, the Review Report for thiacloprid (SANCO/4347/2000-Final – 13th May 2004), as well as the Evaluation table of thiacloprid (SANCO/4346/2000 rev.3.1 (11.03.2004) are considered to provide the relevant scientific information for the review of the active substance.

Comments with respect to the Annex I renewal process

This supplemental dossier contains only summaries of studies, which were not available at the time of the first Annex I inclusion of thiacloprid and were therefore not evaluated during the first EU review of this compound. The summaries on the different toxicological endpoints (information is taken from the Monograph/Review Report (July 2003)/Evaluation table (March 2004)) were supplemented and adapted with the new information. In order to facilitate discrimination between new information and original paragraphs, the new information is written in bold and italic letters. All other studies, which were already submitted by Bayer for the first EU review, are contained in the Monograph/Review Report (May 2004)/ Evaluation table (March 2004)) and in the baseline dossier provided by Bayer CropScience.

Synonymous names for thiacloprid used at several locations in this supplemental dossier is YRC 2894, AE F158944, BCS-AA56362 or Calypso Ftech..

The following table provides an overview on the batches of thiacloprid used in all toxicological studies on this compound. Studies not evaluated during the first EU review are written in bold and italic letters. For the mixed batch 290894 the impurity profile is available.

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

Annex Point	Report / Document No.	Study	Thiacloprid – Batch No.	Purity [%]*
KCA 5.2.1/01	23876 / M-000796-01-1	YRC 2894 - Study for acute oral toxicity in rats	290894	97.3
KCA 5.2.1/02	23891 / M-000703-01-4	YRC 2894 - Pilot toxicity study on rats - acute oral toxicity to non-fasted animals - subacute oral toxicity with gavage administration over 2 weeks	NLL-3351-3	98.3
KCA 5.2.2/01	24879 / M-000808-01-1	YRC 2894 - Study for acute dermal toxicity in rats	290894	97.3

Annex Point	Report / Document No.	Study	Thiacloprid – Batch No.	Purity [%]
KCA 5.2.3/01	24775 / M-000815-01-1	YRC 2894 - Study on acute inhalation toxicity in rats according to OECD no. 403	290894	97.2
KCA 5.2.4/01	24217 / M-000708-03-1	YRC 2894 - Study for skin and eye irritation/ corrosion in rabbits	290894	97.3
KCA 5.2.5/01	24217 / M-000708-03-1	YRC 2894 - Study for skin and eye irritation/ corrosion in rabbits	290894	97.3
KCA 5.2.6/01	24641 / M-003836-02-1	YRC 2894 - Study for the skin sensitization effect in guinea pigs (guinea pig maximization test method according Magnusson and Kligman)	290894	97.3
KCA 5.2.7/01	1609500 / M-480557-01-1	*Thiacloprid: Cytotoxicity assay in vitro with BALB/c 3T3 cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight*	PH-CA-2013-07-01	98.9
KCA 5.3.1/01 & KCA 5.2.1/02	23861 / M-000703-01-4	YRC 2894 - Pilot toxicity study on rats - acute oral toxicity to non-fasted animals - subacute oral toxicity with gavage administration over 2 weeks	NLL 3351-3	98.3
KCA 5.3.1/02	27201 / M-000785-02-1	YRC 2894 - Study for subacute oral toxicity in rats (feeding study over 2 weeks)	NLL 3351-13	98.6
KCA 5.3.1/03	29674 / M-030427-03-1	YRC 2894 (c.n. Thiacloprid) Special study for subacute oral toxicity in rats (feeding study for 5 weeks)	290894	96.8
KCA 5.3.1/04	26017 / M-000821-01-1	YRC 2894 - Study for subacute oral toxicity in mice (feeding study over 2 weeks)	NLL 3351-13	98.6
KCA 5.3.1/05	23437 / M-000688-01-1	YRC 2894 - Pilot study on subacute toxicity in B6C3F1 mice (administration in feed over 3 weeks)	NLL 3351-13	98.6
KCA 5.3.1/06	27137 / M-003816-02-1	YRC 2894 - Subacute toxicity study in Beagle dogs (dose range finding study by feed admixture over at least 10 weeks) - revised final version -	NLL 3351-13	98.6

Annex Point	Report / Document No.	Study	Thiacloprid – Batch No.	Purity [%w/w]
KCA 5.3.2/01	26239 / M-000863-01-1	YRC 2894 - Investigations of subchronic toxicity in Wistar rats (feeding study over 12 weeks with a subsequent recovery period over 5 weeks)	NLL 3351-13	98.6
KCA 5.3.2/02	23834 / M-000697-02-1	YRC 2894 - Subchronic range-finding study for a two-year study in B6C3F1 mice (administration in feed over about 14 weeks)	NLL 3351-13	98.6 – 98.7
KCA 5.3.2/03	27464 / M-003814-01-1	YRC 2894 - Subchronic toxicity study in Beagle dogs (feeding study for about 15 weeks)	290894	96.8-97
KCA 5.3.2/04	27563 / M-003818-01-1	YRC 2894 - Chronic toxicity study in beagle dogs (52 week feeding study)	290894	96.8-97.1
KCA 5.3.3/01	24248 / M-000725-02-1	YRC 2894 - Pilot study on subacute inhalation toxicity in rats (exposure: 5 x 6 hours)	290894	97.2
KCA 5.3.3/02	27688 / M-241815-01-1	YRC 2894 Subacute inhalation toxicity on rats (Exposure for 6 hour/week for 4-weeks)	290894	96.8 - 97.2
KCA 5.3.3/03	25950 / M-000824-01-1	YRC 2894 - Study for subacute dermal toxicity in rats (four-week treatment and two-week recovery period)	290894	97.2
KCA 5.4.1/01	23781 / M-000694-01-1	YRC 2894 - Salmonella/microsome test-plate incorporation and preincubation method	290894	97.2
KCA 5.4.1/02	RA 6011 / M-000903-01-1	YRC 2894 - Reverse mutation assay (Salmonella typhimurium and Escherichia coli)	290894	96.8
KCA 5.4.1/06	NR97220 / M-009203-01-1	*YRC 2894 - DNA repair test in bacterial system*	290894	96.9
KCA 5.4.1/03	25143 / M-000799-01-1	YRC 2894 - Mutagenicity study for the detection of induced forward mutations in the V79/HPRT assay in vitro	290894	96.8-97.2
KCA 5.4.1/04	24577 / M-000772-01-1	YRC 2894 - In vitro mammalian chromosome aberration test with chinese hamster V79 cells	290894	96.8-97.2

Annex Point	Report / Document No.	Study	Thiacloprid – Batch No.	Purity [%]
KCA 5.4.1/05	25429 / M-000790-01-1	YRC 2894 - Test on unscheduled DNA synthesis in rat liver primary cell cultures in vitro	290894	97.2
KCA 5.4.2/01	24515 / M-000775-01-1	YRC 2894 - Micronucleus test on the mouse	290894	96.8
KCA 5.5/01	27480 / M-003817-02-1	YRC 2894 - Combined chronic toxicity/carcinogenicity study in Wistar rats (dietary administration over 2 years)	290894	96.8-97.2
KCA 5.5/02	27247 / M-003819-02-1	YRC 2894 - Carcinogenicity study in B6C3F1-mice (administration in the food over 2 years)	290894	96.8-97.2
KCA 5.6.1/01	24084 (107043)/ M-000911-01-1	A two-generation reproduction range-finding study with YRC 2894 technical in rats	LLL 2951-12	98.6
KCA 5.6.1/02	BC8385 (107628) / M-001304-01-1	A two-generation dietary reproduction study in rats using technical YRC 2894	290894	96.7 - 97.5
KCA 5.6.2/01	26132 / M-000892-01-1	YRC 2894 - Developmental toxicity in rats after oral administration	290894	97.0 - 97.3
KCA 5.6.2/02	26132 / M-031344-01-1	YRC 2894 - Development toxicity study in rats after oral administration (report no. 26132 of March 27, 1997) - additional information on dysplasia of limb bones in fetuses	290894	97.3
KCA 5.6.2/03	24709 / M-000780-01-1	YRC 2894 - Developmental toxicity study in rabbits after oral administration	290894	97.3
KCA 5.7.1/01	BC8828 / M-000894-03-1	An acute oral neurotoxicity screening study with technical grade YRC 2894 in Fischer 344 rats	290894	96.8-97.0
KCA 5.7.1/02	107419 / M-003815-01-1	A subchronic dietary neurotoxicity screening study with technical grade YRC 2894 in Fischer 344 rats	290894	96.6 - 97.5
KCA 5.7.1/03	110734 / M-088059-01-1	Oral (diet) developmental neurotoxicity study of YRC 2894 in CRL:CD(SD) IGS BR VAF/PLUS	898013001	99.2

Annex Point	Report / Document No.	Study	Thiacloprid – Batch No.	Purity [%]
KCA 5.8.1/	SA 13306 M-495981-01-1	*Thiacloprid / Thiacloprid-thiadiazine - 7-day toxicity study in the rat by dietary administration*	EDFL021010	98.8
KCA 5.8.1/25	SA 13334 M-490186-01-1	*Evaluation of thiacloprid in the H295R steroidogenesis assay*	PFHCA-2013-07-01	98.9
KCA 5.8.2/01	27717 / M-003766-03-1	YRC 2894 - Mechanistic studies on aromatase induction and toxicokinetics in rats (4-week feeding studies)	290894	98.2-97.2
KCA 5.8.2/02	27657 / M-003821-01-1	YRC 2894 - Special study for subacute oral toxicity in rats (Toxicokinetics in pregnant and non-pregnant rats)	290894	97.2
KCA 5.8.2/14	M-075786-01-2	*YRC 2894 - Plasma protein binding*	290896	n.r.
KCA 5.8.2/15	24572 M-000760-01-1	*YRC 2894 - Concentration of YRC 2894 in the plasma of dogs in a subchronic feeding study*	290894	97.2-96.8
KCA 5.8.2/16	SA 10362 / M-428958-01-1	*Thiacloprid - 28-day immunotoxicity study in the female Wistar rat by dietary administration*	EDE0011099	98.7
KCA 5.8.2/05	27716 / M-003764-01-1	YRC 2894 - Mechanistic studies on aromatase induction in mice (Feeding study for 13 weeks)	290894	97.0 - 97.2
KCA 5.8.2/06	23495 / M-000690-02-1	Studies on the inhibition of thyroid peroxidase-catalyzed reactions by YRC 2894 and its metabolites in vitro	NLL 3351-13	98.6
KCA 5.8.2/07 & 5.3.1/03	29674 / M-030427-03-1	YRC 2894 (c.e. Thiacloprid) - Special study for subacute oral toxicity in rats (feeding study for 5 weeks)	290894	96.8
KCA 5.8.2/17	SA 06252 / M-293209-01-1	*Thiacloprid - Evaluation in the immature rat - Uterotrophic assay*	EDE0011099	99.0
KCA 5.8.2/18	SA 07125 / M-359235-01-1	*Thiacloprid - Investigation of effects on hormone levels in adult female wistar rats following a single oral dose*	EDE0011099	99.0
KCA 5.8.2/19	SA 07011 / M-360362-01-1	*Thiacloprid - Evaluation of hormone levels in female rats 2 and 8 hours after 4 days exposure by oral gavage*	EDE0011099	99.0
KCA 5.8.2/20	SA 07010 / M-360349-01-1	*Thiacloprid - Evaluation of hormone levels in female rats 24 hours after 4 days exposure by oral gavage*	EDE0011099	99.0

Annex Point	Report / Document No.	Study	Thiacloprid – Batch No.	Purity [%]
KCA 5.8.2/21	SA 08054 / M-360757-02-1	*Thiacloprid - Exploratory 28-day toxicity study in the rat by dietary administration*	EDE0011099	98.7
KCA 5.8.2/22	SA 08327 / M-359926-01-1	*Thiacloprid - Exploratory 28-day toxicity study in the aged female rat by dietary administration*	EDE0011099	98.7
KCA 5.8.2/23	SA 08351 / M-361492-01-1	*Thiacloprid: Investigation of in vitro effects on steroidogenesis using H295R cells*	EDE0011099	98.7
KCA 5.8.2/24	SA 09062 / M-361609-01-1	*Thiacloprid: In vitro investigation of steroid sex hormone secretion in rat ovarian preantral follicles*	EDE0011099	98.7
KCA 5.8.2/09	BC8489 (107641) / M-003820-01-1	A one-generation dietary reproduction study in rats using technical grade YRC 2894 to evaluate the reproducibility of Dystocia and an increase in stillbirths in the P generation of a two-generation dietary reproduction study in rats	290894	96.7 - 97.0
KCA 5.8.2/10	BC 8601 (107746) / M-004291-01-1	An experimental study to investigate the cause of dystocia and stillbirths in rats treated with technical grade YRC 2894	290894	96.7 - 97.0
KCA 5.8.2/11	BC 8681 (107640) / M-002127-01-1	A reproduction study in rats to determine administration of technical YRC 2894 from gestation days 18 to 21 will cause dystocia (Study number II)	290894	97.0
KCA 5.8.2/12	BC 8555 (107639) / M-016564-02-1	A reproduction study in rats to determine administration of technical YRC 2894 from gestation days 18 to 21 will cause dystocia	290894	96.7 - 97.0
KCA 5.8.2/13	BC 8604 (108360) / M-004253-01-1	Further examination of the increased occurrence of dystocia and stillbirths observed in a reproductive bioassay with an experimental cyanamide (YRC 2894)	290894	97.0
KCA 5.8.2/25	SA 10007 / M-403763-01-1	*Thiacloprid - A special one-generation dietary reproduction study in Sprague-Dawley rats*	EDE0011099	98.7

* Purity as stated in the study report

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

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

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

According to the new data requirements (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013) (1), the conduct of a "comparative *in vitro* metabolism study shall be performed on animal species to be used in pivotal studies and on human material (microsomes or intact cell systems) in order to determine the relevance of the toxicological animal data and to guide in the interpretation of findings and in further definition of the testing strategy". For this data requirement no test guideline is available yet. Therefore, the study described below was conducted as described in the Materials and Methods section to what the sponsor believes could be considered as the current state of scientific standard.

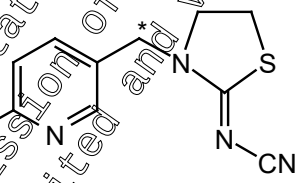
Report: KCA 5.1.1 /01; [redacted], J ;2014;M-489777-01
Title: [Methylene-^{14}C]Thiacloprid: Metabolic Stability and Profiling in Liver Microsomes from Rats and Humans for Inter-Species Comparison.
Report No: S48310 / EnSa-14-0245
Document No: M-489777-01-1
Data Requirement: **Regulation (EC) No 1107/2009 amended by the Commission Regulation (EU) No. 283/2013, US EPA OCSPP 870.SUPP**
Deviations: none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test materials:

Radiolabelled test item: [Methylene-^{14}C]Thiacloprid
Chemical structure



*denotes position of radiolabel

Batch Number (Sample ID):	KML-0707
Radiochemical Purity:	99% (HPLC, radioactivity detector
Chemical purity:	>99% (HPLC, UV-detector, 210 nm)
Specific radioactivity:	3.77 MBq/mg = 102 µCi/mg
Total radioactivity:	31.45 MBq = 0.85 mCi
Non-radiolabelled test item:	Thiacloprid
Company experimental name:	YRC 2894 / BCS-AA56362
Lot/Batch code:	AE F1 58944-PU-01
Methods of identification:	MS, IR, ^{1}H-NMR, ^{13}C-NMR, UV
Description:	Beige powder
Chemical purity:	99.0%

2. Positive control:

Identification:	6ß-hydroxytestosterone
Batch code	0446800-2
Description:	White powder
Purity:	99.7%

3. Internal Standard:

Identification:	Dexamethasone Vetranal
Batch code	SZBB118XV
Description:	White colourless powder
Purity:	99.5%

4. Test system:

Liver microsomes: Pooled liver microsomes from male Wistar rats (RLM, batch 101026, pool of 200 individuals and humans (HLM, batch 1210097, pool of 50 donors from both genders) were purchased from Xenotech, LLC (USA)

Microsome characterisation: Each microsome batch was characterised at least according to:

batch number, microsomal protein concentration, storage conditions, total cytochrome P450 content, microsome drug metabolising enzyme activity.

5. Experimental Procedures:

Sample preparation and incubation: ^{14}C-thiacloprid was incubated separately with rat and human liver microsomes (n=3) at 37±1°C in a final volume of 500 µl. Incubations were performed in a thermomixer device with shaking at 1000 rpm.

After 2 minutes pre-heating at 37±1°C with shaking at 1000 rpm, the reactions were started by the addition of 50 µl of 10 mM NADPH and were stopped after 0.5 and 1 hour incubation with 0.5 mL of AcN at room temperature.

Final concentrations of the incubates were: 5 mM $MgCl_2$; 1 mg/mL microsomal protein; 10 µM ^{14}C-thiacloprid (0.129 µCi/incubate); 1 mM reduced NADPH.

Triplicate samples at T=0 (not incubated) were prepared by adding the same components as test samples but in different order (i.e. AcN was added prior to NADPH and ^{14}C-thiacloprid).

Two stability control samples were prepared by mixing 490 µL of 100 mM sodium-phosphate buffer pH 7.4 with 10 µL of 500 µM ^{14}C-thiacloprid solution. These samples were also incubated for 0.5 and 1 h, respectively. As for the test samples, 500 µL of AcN at room temperature was added at the end of the incubation period.

Finally on reference sample was prepared mixing 450 µL of 100 mM sodium phosphate buffer pH 7.4 with 50 µL of 0.1 mM unlabelled thiacloprid and 500µL of AcN. This sample was used to assess the correct performance of the HPLC system, and to determine the retention time of unchanged thiacloprid.

Sample processing for analysis: The microsomal incubates were centrifuged at 16.000 x g for 15 minutes at 20°C. After centrifugation, 100 µL of each

	supernatant were diluted with 250 µL of HPLC mobile phase A. The samples were directly analysed by HPLC without any further extraction procedure. For details of the chromatographic and radioactivity measurement conditions see report.
Positive control	The metabolic activity of the microsomes was determined by measurement of 6ß-hydroxytestosterone that was formed from testosterone by testosterone 6ß-hydroxylase. This biochemical reaction is well-known for CYP3A microsomal enzyme activity. In this measurement, dexamethasone was used as an internal standard. After 2 minutes pre-heating at 37±1°C with shaking at 1000 rpm, the reactions were started by the addition of 40 µl of 1 mM NADPH and were quenched after 5 minutes incubation with 0.4 mL of AcN at room temperature. Final concentrations of the incubates were: 5 mM $MgCl_2$; 0.15 mg/mL microsomal protein; 150 µM testosterone; 1 mM reduced NADPH. After incubation, 32 µL of dexamethasone solution + 32 µL MeOH was added to each incubate. The samples were put in a tray with ice until the end of the experiment and further stored at -80°C±10°C until analysis. After thawing at room temperature, the samples were centrifuged at 16.000 x g for 15 minutes at 4°C. After centrifugation, 100 µL of each sample supernatant was placed into a conical 1 -mL glass HPLC vial and diluted with 100 µL of 0.1 % acetic acid. Samples were analysed by LC-MS/MS without further extraction process for 6ß-hydroxytestosterone (details in the report).
Expression of the results:	The relative percentages were calculated from the radiochromatographic profiles at the different incubation times according to:

$$\%\ \text{Relative Pi} = \frac{\text{Area Pi}}{\sum \text{Area P}} \times 100$$

where Area Pi is the mean area of the unchanged [14]C-thiacloprid (or metabolites) peak in the radiochemical chromatogram of a test sample, and ∑Area P is the sum of the total radioactive mean peak areas in the chromatogram. Peak areas of compounds detected below the lower limit of quantitation (BLLOQ) were considered as zero for further calculations (mean peak area and relative percentages).
The results from the HPLC-radioactivity analysis were expressed also as percentage of unchanged [14]C-Thiacloprid transformed as a function of incubation time.
The results from the positive metabolism control incubations are expressed as testosterone 6ß-hydroxylase enzyme activity (CYP3A):

$$A = \frac{(\text{6ß-hydroxytestosterone conc.}\left(\frac{\text{pmol}}{\text{mL}}\right))}{(\text{t x Pr})}$$

where A is the testosterone 6ß-hydroxylase enzyme activity (pmol/min/mg), t is the incubation time (min) and Pr is the microsomal protein concentration (mg/mL).

II. Results and discussion

1. Qualification of the HPLC-Method

Two aliquots of ^{14}C-thiacloprid testing solution were analysed prior to the initiation of the experiments for linearity and lower limit of quantification (LLOQ) estimation and microsomal incubations. The retention time of the parent compound was found to be approximately 20.5 min, and the radiochemical purity amounted to 99.23% (calculated from peak area values). The LLOQ value was set at the 293 dpm level for radioactivity detection (cv < 20%). These results indicate that after analysis of test samples, compounds showing radioactive peak areas below the mean peak area value obtained for the LLOQ (peak area of 1245.1) were not considered for quantification

Although no upper limit of detection-quantitation is actually defined for radioactivity detection systems at the low activity levels used in the experiments of the present study (i.e. 0.281 µCi/incubate), which are considered linear, the linearity of the methodology was determined as a tool to assess the correct performance of the radioactivity flowthrough detector. It was determined at the radioactivity levels above the LLOQ in terms of injected dpm (*i.e.* 7 levels for radioactivity detection). Mean peak area values at each level were plotted versus the respective nominal injected dpm, and linear regression was carried out. The correlation coefficient obtained was 0.999764 (see Table 5.1.1-1).

Table 5.1.1.-1: Qualification of the HPLC-RAD method: Linearity of response and determination of LLOQ

Solution	Nominal injected (dpm)	Peak area ^{14}C thiacloprid					
		Replicate			Mean	SD	cv (%)
		A	B	C			
STD-1	**48832**	178497	166435	175044	173325.2	6211.7	3.6
STD-2	**24416**	86673	88956	94834	90154.0	4210.4	4.7
STD-3	**9766**	37410	34957	37189	36518.5	1356.9	3.7
STD-4	**4883**	20636	20349	19508	20164.6	586.0	2.9
STD-5	**977**	4246	3613	4037	3965.3	322.5	8.1
STD-6	**488**	2304	2201	2206	2237.0	57.9	2.6
STD-7	**293**	1013	1277	1445	**1245.1**	217.9	17.5

r^2: 0.999764
LLOQ: 1245.1 (peak area)

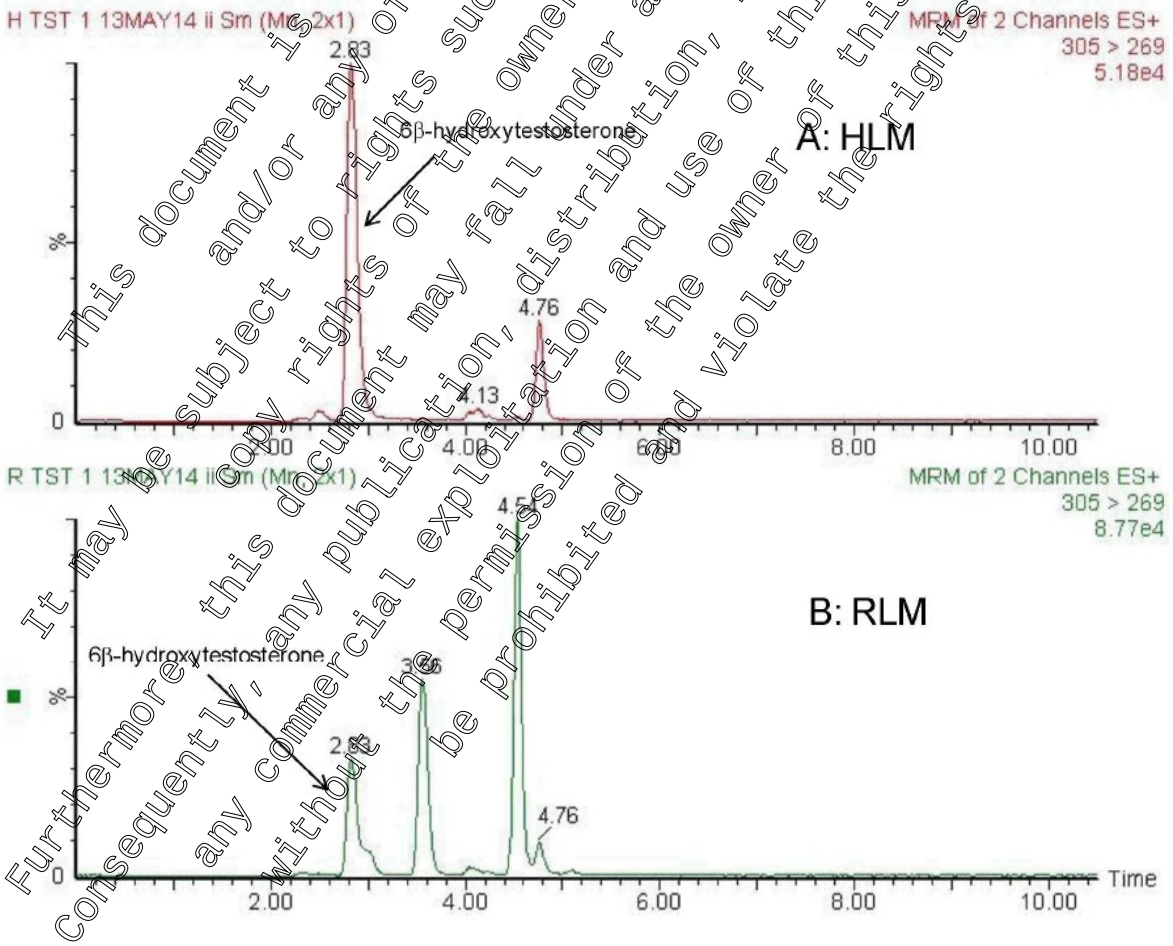
2. Positive Metabolism Controls

The results of the positive metabolism control incubations in human and rat liver microsoms are shown in Table 5.1.1.-2. Formation of 6ß-hydroxytestosterone from testosterone demonstrated sufficient metabolic capability of the microsome batches used in the study. Testosterone 6ß-hydroxylase activities were found to be 1694.6 pmol/mg/minute in male rat liver microsomes and 3238.7 pmol/mg/minute in pooled human liver microsomes. Representative LC-MS/MS profiles of testosterone metabolism by RLM and HLM are depicted in Figure 5.1.1-1.

Table 5.1.1.-2: Activity in liver microsomes (positive control)

Sample	6ß-hydroxytestosterone (pmole/mL)		Testosterone-6ß-hydroxylase (pmol/mg/min)
Rat liver microsomes			
Replicate 1	1411.42		1881.9
Replicate 2	1190.14		1586.9
Replicate 3	1211.30		1615.1
		Mean	1694.6
		cv (%)	9.6
Human liver microsomes			
Replicate 1	2222.62		2963.5
Replicate 2	2594.16		3458.9
Replicate 3	2470.28		3293.7
		Mean	3238.7
		cv (%)	7.8

Figure 5.1.1.-1: LC-MS/MS profiles of testosterone metabolism by rat (bottom) and human (top) liver microsomes (positive control)



3. Recovery of radioactivity

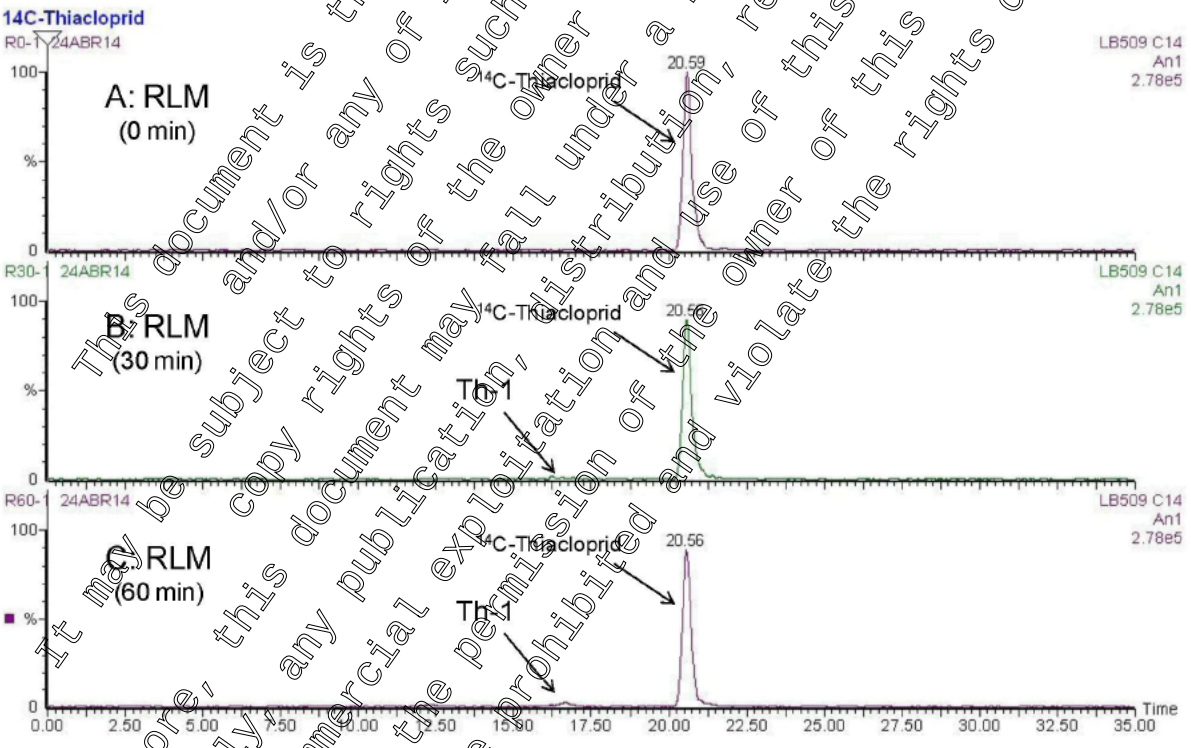
The mean recovery of radioactivity after microsome incubations and sample preparation (*i.e.* protein precipitation with AcN and centrifugation) at T=0 hours was found to be 106.5% and 89.0% in rat (RLM) and human liver microsomes (HLM), respectively, after 0.5 hour incubation the recoveries were 91.3% in RLM and 94.9% in HLM, after 1 hour incubation the recoveries were 111.5% in RLM and 102.3% in HLM

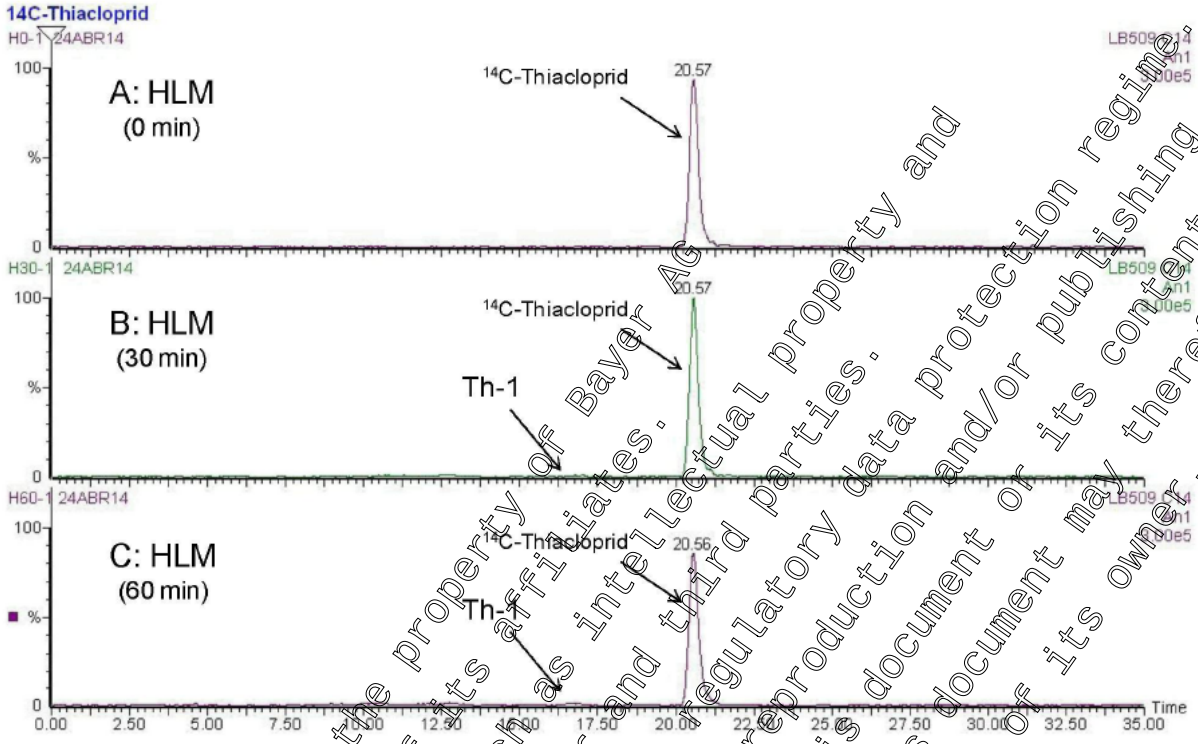
4. Metabolite profile of ^{14}C thiacloprid

The *in-vitro* metabolite profile of 10 µM ^{14}C-Thiacloprid was determined in pooled liver microsomes (1 mg/mL) from male Wistar rats and humans (pool from both genders), incubated for 0, 0.5 and 1 hour in the presence of 1 mM NADPH.

^{14}C-Thiacloprid was found to be stable in the incubation buffer at 37±1°C after 0.5 and 1 h incubation and in the rat and human microsome incubations at 0 hours. A small ^{14}C-labelled product (Th-1) was found after 0.5 and 1 hour in RLM incubates and after 1 hour in HLM incubates. The radioactive peak corresponding to Th-1 was found below the lower limit of quantification in RLM incubated for 30 minutes, and accounted for 1.7% and 0.5% of the radioactivity in RLM and HLM incubated for 1 hour, respectively (see Figure 5.1.1.-2).

Figure 5.1.1.-2: HPLC-profiles of ^{14}C thiacloprid metabolism by rat liver microsomes (top) and human liver microsomes (bottom)





III. Conclusion

From the results of this study, the following conclusions can be drawn:

- ^{14}C-thiacloprid is highly metabolically stable after *in-vitro* incubations with liver microsomes from either rats or humans.
- The *in-vitro* metabolism of ^{14}C-thiacloprid when incubated with liver microsomes was found to be comparable between rats and humans; there is no indication of the formation of a unique human metabolite.
- Only one metabolite was detected in very low amounts of the relative percentage (<1.7%) after ^{14}C-thiacloprid in-vitro incubations with both, rat and human liver microsomes.
- The results of this study demonstrate that phase I metabolism plays a very moderate role in the biotransformation of thiacloprid in rat and human liver microsomes. In addition, no differences with respect to the metabolic pattern were found in both *in-vitro* test systems.

In the *in-vivo* rat metabolism studies (reported in the baseline dossier) thiacloprid is intensively metabolised. On average, only ca. 6% of the administered radioactivity was identified as unchanged parent compound, while more than one third of the given dose consisted of 6-chloronicotinic acid and its glycine conjugate. Altogether 13 metabolites were identified, but most of them at amounts below 5% of the administered radioactivity. These metabolites were either formed by ring-opening of the thiazolidine ring and/or by conjugation, i.e. by a phase II reaction. This may be the reason that they were not found in the *in vitro* microsomal system.

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

Not required because the toxicity following dermal exposure is lower compared to that following oral exposure. (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013) (1).

CA 5.2 Acute toxicity

Summary of acute toxicity studies

Thiacloprid displayed moderate acute oral toxicity in male and female Wistar rats, but was toxic after acute oral administration to Fisher 344 rats in a pilot study for the acute oral neurotoxicity study (LD_{50}: 177 mg/kg bw, calculated from 100% mortality after 244 mg/kg bw and 0% mortality after 109 mg/kg bw in both sexes). The results obtained with fasted and non-fasted Wistar rats suggest that dietary status can influence the toxicity of thiacloprid. Thiacloprid was moderately toxic after inhalation (LC_{50}: > 2535 / ~ 1223 mg/m^3 air in males / females) and of low toxicity after dermal application (LD_{50} > 2000 mg/kg bw). Sex differences are evident in rats when exposed *via* the oral or inhalative route, females appear to be more sensitive than males. Thiacloprid does not cause skin or eye irritation and is no skin sensitizer.

In addition, an acute oral toxicity study in mice (conducted in 1999 for the registration of thiacloprid in Japan) as well as an acute oral toxicity study in rats and a skin irritation study in rabbits (conducted in 2004 for the registration in India) are available, which were performed according to local guideline requirements. Since the results of these studies confirm the results of the previously submitted studies on thiacloprid, they are - in agreement with the rapporteur - not part of this delta dossier.

Furthermore, thiacloprid was tested in an in vitro 3T3 NRU phototoxicity test. The test did not give any indication for a phototoxic potential of thiacloprid.

Table 5.2-1: Summary of acute toxicity studies*

Route/Study	Species	Sex	Results [mg/kg bw or mg/m³ air, resp.] NSD#	LLD##	LD_{50} /LC_{50}	Reference
Oral[1)]	Rat (Wistar)	M F	62.5 62.5	700 300	~ 832 ~ 424	[redacted], 1995 M-000796-01-1
Oral[2)]	Rat (Wistar)	M F	< 140 < 100	600 370	621 396	[redacted] 1995 M-000703-01-4
Oral[1)]	Rat (Fischer 344)	M F	11 11	244 244	177** 177**	[redacted] & [redacted], 1997, and [redacted], 1998 M-000894-03-1
Dermal	Rat (Wistar)	M F	2000 2000	> 2000 > 2000	> 2000 > 2000	[redacted], 1995 M-000808-01-1
Inhalation (aerosol, 4h)	Rat (Wistar)	M F	1.80 1.80	> 2.53 2.53	> 2.53 ~ 2.53	[redacted], 1996 M-000815-01-1
Skin irritation	Rabbit	F	Not irritating			[redacted], 1995 M-000708-03-1
Eye irritation	Rabbit	F	Not irritating			[redacted], 1995 M-000708-03-1
Skin sensitisation M&K method	Guinea pig	M	Not sensitising			[redacted], 1996 M-003836-02-1
In vitro 3T3 NRU phototoxicity test	BALB/c 3T3 cells		Not phototoxic			[redacted], 2014 M-480557-01-1

*: New studies, i.e. studies previously not submitted, are written in bold and italic
**: Data stem from a range finding study for the acute neurotoxicity study. The LD_{50} of 177 mg/kg bw was calculated from 100% mortality at 244 mg/kg bw and 0% mortality at 109 mg/kg bw.
M: male F: female [1)] animals were fasted (overnight) [2)] animals were not fasted
#: NSD: no-symptoms dose ##: LLD: lowest lethal dose
Results for acute oral and dermal toxicity are given in mg/kg bw; results for acute inhalation toxicity in mg/m³ air.

CA 5.2.1 Oral

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

CA 5.2.2 Dermal

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

CA 5.2.3 Inhalation

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

CA 5.2.4 Skin irritation

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

CA 5.2.5 Eye irritation

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

CA 5.2.6 Skin sensitization

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

CA 5.2.7 Phototoxicity

According to the new data requirements (COMMISSION REGULATION (EU) No 283/2013 of 1 March 2013; Official Journal of the European Union, L 93/1, 3.4.2013) (1), the conduct of a phototoxicity study is required under certain conditions. As the ultraviolet/visible molar extinction coefficient of thiacloprid of 98 L x mol^{-1} x cm^{-1} at 290 nm (determined in the photodegradation study on thiacloprid by [redacted], 1995, M-000677-01-2) exceeds the trigger of 10 L x mol^{-1} x cm^{-1} for the conduct of a phototoxicity study, a cytotoxicity assay in vitro with BALB/c 3T3 cells has been performed.

Report: [redacted]; 2014; M-480557-01-1
Title: Thiacloprid: Cytotoxicity assay in vitro with BALB/c 3T3 cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No: 1609500
Document No: M-480557-01-1
Guidelines: Commission Regulation (EC) No. 440/2008, B41; Committee for Proprietary Medicinal Products (CPMP), Note for Guidance on Photosafety testing, EMEA, CPMP/SWP/398/01; OECD 432;
Deviations: none
GLP: yes

I. Materials and methods

A. Materials

1. Test materials:

Name:	thiacloprid
Synonyms:	AE F158944; YRC2894
Description:	light grey solid
Lot/Batch no:	PFHCA-2013-07-01
Purity:	98.9% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2015-01-11

2. Vehicle and or positive control: vehicle: dimethylsulfoxide (DMSO), 1% (v/v) in Earle´s

Balanced Salt Solution (EBBS)
Solvent control: EBSS containing 1% (v/v) DMSO
Positive control: chlorpromazine (Sigma) dissolved in EBSS

3. Test system:

Culture medium: Dulbecco´s Minimal Essential Medium (DMEM) supplemented with 10% (v/v) Newborn calf Serum (NCS).

Cell cultures: BALB/c 3T3 cell clone 31 (supplied by [redacted], Germany).

Large stocks (Master Cell Stock) of the BALB/c 3T3 31 cell line are stored in liquid nitrogen in the cell bank of Harlan CCR. A working cell stock is produced by multiplying from the master cell stock. Thawed stock cultures were propagated at 37 ± 1.5 °C in 75 cm^2 plastic flasks. Seeding was done with about 1 x 10^6 cells per flask in 15 mL DMEM, supplemented with 10 % NCS. Cells were sub-cultured twice weekly. The cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

B. Study design and methods

1. Treatment:

Dose:

Test item	+/– UV	Final concentrations in µg/mL
Thiacloprid	+/–	3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500
Positive control	–	6.25, 12.5, 25, 37.5, 50, 75, 100, 200
	+	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0
Solvent control	+/–	EBSS containing 1 % (v/v) DMSO

The test item thiacloprid was dissolved in DMSO. The final concentration of the solvent in EBSS was 1 % (v/v). The highest applied concentration of the test item was 500 µ/mL in accordance with the OECD Guideline. At higher concentrations often false positive results are produced.

Solar simulator: Irradiation was performed with a Dr. Hönle Sol 500 solar simulator. The filter H1 was used to keep the UVB irradiation as low as possible. The produced wavelength of the solar simulator with the filter was > 320 nm. Due to the heterogenous distribution of irradiation intensity the UVA intensity was measured at the complete area with a UV-meter. The homogeneous area was marked and the cultures were irradiated in this area. The solar simulator was switched on about 30 minutes prior to the start of experiment. The absorption spectrum of the test item was determined in the range from 270-800 nm. The test item showed absorption maxima at 285.0 and 287.9 nm.

Seeding of cultures: 2 x 10^4 cells per well were seeded in 100 µL culture medium in two 96-well plates

Replicates:	2 (one for exposure to irradiation, one for treatment in the dark)
Treatment & irradiation:	24 h after seeding the cultures were washed with EBSS. 100 µL of the dissolved test item were added/well and the plates were pre-incubated for 1 hour in the dark. Afterwards one plate was irradiated at 1.65 mW/cm^2 (4.95 J/cm^2) for 50 min at 26 °C, the other plate was stored for 50 min at 26°C in the dark. The test item was removed and both plates were washed twice with EBSS. Fresh culture medium was added and the plates were incubated overnight at 37 ± 1.5 °C and 7.5 ± 0.5 % CO_2.
Cytotoxicity determination:	For measurement of Neutral Red uptake the medium was removed and 0.1 mL serum-free medium containing 50 µg Neutral Red / mL were added to each well. The plates were incubated for another 3 hours at 37°, before the medium was removed completely and the cells were washed with EBSS. For extraction of the dye 0.15 mL of a solution of 49% (v/v) deionized water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approximately 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.
Number of measurements:	Thiacloprid and positive control: 6 times per concentration Solvent control: 12 times

2. Evaluation

The mean absorption (OD_{540}) value per concentration was calculated. The ED_{50} values were determined by curve fitting by software. The Photo-irritancy factor (PIF)[1], as well as the Mean Phototoxic effect (MPE)[2] was calculated according to OECD guideline 432.

Evaluation criteria:	PIF < 2 or MPE < 0.1 => no phototoxic potential
	PIF > 2 and < 5 or
	MPE > 0.1 and < 0.15 => probable phototoxic potential
	PIF > 5 or MPE > 0.15 =>phototoxic potential

[1] PIF: Photo-Irritancy-Factor, is calculated according to the following equation:
PIF = ED_{50} (-UV) / ED_{50} (+UV)

[2] MPE: Mean Phototoxic Effect, is based on the complete concentration response curves. It is defined as the weighted average across a representative set of photo effect values.

II. Results and discussion

In the range finding experiment (RFE) no cytotoxic effects were observed after exposure of the cells to the test item thiacloprid, neither in the presence nor in the absence of irradiation to artificial sunlight. Therefore, ED_{50}-values and PIF could not be calculated. The resulting MPE value was -0.003.

In the main experiment (ME) no cytotoxic effects were observed after exposure of the cells to thiacloprid, neither in the presence nor in the absence of irradiation with artificial sunlight. Therefore, ED_{50}-values and PIF could not be calculated. The resulting MPE-value was 0.015.
Thus, thiacloprid does not possess any phototoxic potential.
The mean of solvent control values of the irradiated versus the non-irradiated group met the acceptance criteria. The positive control chlorpromazine induced phototoxicity in the expected range in the presence of irradiation.
The results are summarised in the tables below.

Table 5.2.7/01-1: Optical density at 540 nm (OD_{540} values) in the Neutral Red assay of the range finding experiment (RFE)

Con-centration [µg/mL]	OD_{540} with artificial sunlight Mean	SD	% of solvent control	Con-centration [µg/mL]	OD_{540} without artificial sunlight Mean	SD	% of solvent control
Treatment with thiacloprid							
Solvent control	1.0006*	0.0704	100.00	**Solvent control**	1.1467*	0.0836	100.00
3.91	1.0612	0.0735	106.06	3.91	1.1909	0.1015	100.86
7.81	1.0029	0.0753	100.23	7.81	1.2048	0.0796	105.07
15.6	1.0971	0.1513	109.65	15.6	1.2008	0.1116	104.71
31.3	1.0774	0.1295	107.67	31.3	1.2566	0.1639	109.58
62.5	1.0305	0.1408	102.99	62.5	1.1929	0.0720	104.03
125.0	1.0523	0.1480	105.17	125.0	1.1901	0.0781	103.79
250.0	1.0256	0.0951	102.50	250.0	1.1627	0.0869	101.40
500.0	1.0782	0.0665	107.76	500.0	1.1163	0.0578	97.35
Treatment with positive control chlorpromazine							
Solvent control	0.9786*	0.0869	100.00	**Solvent control**	0.9984*	0.0873	100.00
0.125	1.0175	0.0512	103.97	6.25	0.9926	0.0933	99.41
0.250	0.9742	0.0197	99.54	12.50	0.4825	0.0538	48.32
0.500	0.8497	0.0685	86.82	25.00	0.1158	0.0069	11.60
0.750	0.6390	0.1086	65.29	37.50	0.0575	0.0024	5.76
1.000	0.5750	0.0675	58.75	50.00	0.0521	0.0017	5.21
1.500	0.1552	0.0249	15.86	75.00	0.0527	0.0064	5.28
2.000	0.0828	0.0171	8.46	100.00	0.0507	0.0027	5.07
4.000	0.0779	0.0089	7.96	200.00	0.0498	0.0010	4.99

* mean OD_{540} out of 12 wells

Table 5.2.7/01-2: Optical density at 540 nm (OD_{540} values) in the Neutral Red assay of the main experiment (ME)

	OD_{540} with artificial sunlight				OD_{540} without artificial sunlight		
Con-centration [µg/mL]	Mean	SD	% of solvent control	Con-centration [µg/mL]	Mean	SD	% of solvent control
Treatment with thiacloprid							
Solvent control	0.8315*	0.0450	100.00	**Solvent control**	0.9081*	0.0542	100.00
3.91	0.8331	0.0322	100.19	3.91	0.9206	0.0325	101.38
7.81	0.8200	0.0421	98.61	7.81	0.9495	0.0462	104.56
15.6	0.8514	0.0221	102.39	15.6	0.9579	0.0474	105.41
31.3	0.8311	0.0238	99.95	31.3	0.9322	0.0237	102.66
62.5	0.8555	0.0443	102.88	62.5	0.9292	0.0501	103.32
125.0	0.8455	0.0350	101.67	125.0	0.8841	0.0533	97.35
250.0	0.8306	0.0414	99.88	250.0	0.9157	0.0417	100.83
500.0	0.8075	0.0406	97.14	500.0	0.9321	0.0470	102.64
Treatment with positive control chlorpromazine							
Solvent control	0.9282*	0.0477	100.00	**Solvent control**	0.9590*	0.0753	100.00
0.125	0.8850	0.0436	95.34	6.25	0.8463	0.0689	88.25
0.250	0.8630	0.0304	93.19	12.50	0.4726	0.0429	49.29
0.500	0.7066	0.0330	76.12	25.00	0.3188	0.0142	33.25
0.750	0.4173	0.0386	45.00	37.50	0.2952	0.0283	30.79
1.000	0.3597	0.0242	38.75	50.00	0.3045	0.0243	31.75
1.500	0.3682	0.0119	39.66	75.00	0.3079	0.0223	32.11
2.000	0.3656	0.0256	39.38	100.00	0.3055	0.0256	31.85
4.000	0.4134	0.0398	44.53	200.00	0.3091	0.0153	32.23

*: mean OD_{540} out of 12 wells

Table 5.2.7/01-3: Summary of the results of the Neutral Red assay

	Substance	ED_{50} (+UV) [µg/mL]	ED_{50} (–UV) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated *vs.* non-irradiated plate
Range finding experiment	Thiacloprid	--	--	--	-0.003	87.3
	Positive control	1.07	12.18	11.48	0.396	98.0
Main experiment	Thiacloprid	--	--	--	0.015	91.6
	Positive control	0.68	12.28	18.10	0.368	96.8

PIF: Photo-Irritancy-Factor MPE: Mean Phototoxic Effect

--: No cytotoxic effects occurred after exposure of thiacloprid to the cells, neither with nor without irradiation with artificial sunlight. Therefore, ED_{50}-values or a PIF could not be calculated.

III. Conclusions

Based on the study results thiacloprid does not possess any phototoxic potential.

CA 5.3 Short-term toxicity

Summary of short-term toxicity studies

Short-term toxicity studies have been conducted in rats, mice and dogs.

The main target in rodents proved to be the liver. There was no evidence of accumulation in the short-term studies at dose levels that did not overload the metabolic capacity of the liver. A dose-dependent liver enzyme induction occurred in rats and mice. This enzyme induction was associated with increased liver weight, centrilobular hypertrophy and changes in the cytoplasm of the hepatocytes.

In rats, the enzyme induction was believed to be responsible for the secondary effects observed in the thyroid glands (e.g. increased weight, increased mitotic rate and hypertrophy of follicular epithelium). A comparison of the enzyme induction seen at the top dose levels in the 14 day rat study and 13 week rat study indicate that some but not all of the enzyme levels increased with the duration of exposure. The cytochrome P-450 (males) and UDP-glucuronyl-transferase (males and females) levels appeared to increase with time. Liver enzyme induction and the related morphological changes were also observed in the inhalation and dermal studies. Following oral (13 weeks) and dermal administration (22 applications), the enzyme induction and increased liver weight were shown to be reversible or at least partly reversible. The thyroid follicular cell hypertrophy was also shown to be at least partly reversible following dermal administration and a 2-week recovery period. There was evidence of an effect on circulating thyroid hormone levels and biochemical parameters. Body weight and food intake effects were also observed in rats.

In mice, the liver effects also included an increase in the lipid content of the hepatocytes. A dose-related increase in fatty vacuolation and hypertrophy of the adrenal X-zone was also detected in female mice. A NOAEL was not established for this finding.

In dogs the liver was also a target, but the effects were less pronounced, and although an enzyme induction was observed, it was weaker than in rodents. Re-evaluation of the thyroid hormone data in dogs with the adequate historical control data of the year of study conduct revealed, that there is no effect on the thyroid in this species. The mean prostate weights were increased in the 10- and 15-week dog studies at dose levels ≥ 1000 ppm. Microscopy revealed slight to moderate hypertrophy of the prostate at dose levels ≥ 1000 ppm in the 15-week study only. In the 52-week dog study, there was no evidence of increased prostate weights at 26 weeks but the mean prostate weight was increased at 1000 ppm on termination. Microscopic and ultrasonographic investigations of the prostates did not detect any treatment-related effects at week 26 or week 52. Therefore, the report regarded the prostate effects seen at 1000 ppm as incidental and possibly related to high individual variation in growing dogs. It was noted that six treated dogs had individual prostate weights that were noticeably higher than the cited historical control data.

Based on a toxicokinetic evaluation of thiacloprid concentrations in blood plasma of dogs after sub-chronic dietary exposure for 13 weeks (see [redacted] & [redacted] 1998 and 5.8.2/15 [redacted], 1995) an efficient absorption of thiacloprid was demonstrated. Therefore, the less pronounced toxicity observed in dogs as compared to rodents are not due to inefficient or low absorption of the test substance.

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

Study *Doses tested*	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL (mg/kg bw/day)	Main findings seen at LO(A)EL	Reference
Rat 2-weeks, oral (gavage) *0-5-10-20-60-120 mg/kg bw/day*	M F	20 20	60 60	↓ bw, food consumption & excretion of faeces (F only), ↑ water consumption (F), ↑ alkaline phosphatase (F) & cholesterol levels, liver enzyme induction,↑ liver weight, changed structure of hepatocellular cytoplasm (F)	[redacted], 1995 M-000703-01-4
Rat 2-weeks, oral (diet) *0-25-100-500-2000 ppm*	M F	11.2 9.6 *(100 ppm)*	49.2 49.5 *(500 ppm)*	trend towards ↓ bw gain (F), ↑ cholesterol levels (M), liver enzyme induction, distinct liver lobulation (F), ↑ liver weights, hepato-cellular hypertrophy with slight cytoplasmatic changes, thyroid: ↑ mitotic index (M)	[redacted], 1995, amended 1999 M-000785-02-1
Rat 3-weeks, oral (diet) *0-25-100-400-1600 ppm*	M F	9.0 12.3 *(100 ppm)*	36.9 44.6 *(400 ppm)*	marked liver enzyme induction (UDP-GT), ↑ liver weight (M), minimal to slight thyroid follicular cell hypertrophy (M)	[redacted], 2000, amended 2000 & 2000 M-030427-03-1
Mouse 2-weeks, oral (diet) *0-50-200-2000-10000 ppm*	M F	84.3 113.2 *(200 ppm)*	765.1 1201.2 *(2000 ppm)*	↓ water intake (M), liver enzyme induction, ↑ liver weights, hypertrophy & slightly ↑ lipid content of hepatocytes	[redacted], 1997 M-000821-01-1
Mouse 3-weeks, oral (diet) *0-100-1000-10000 ppm*	M F	30.1 63.9 *(100 ppm)*	267.8 559.3 *(1000 ppm)*	↓ food consumption (F), ↑ liver weights	[redacted], 1994 M-000688-01-1
Dog 10-weeks, oral (diet) *0-100-300-1000* (1250-1600-2500)-2500 (4 weeks)*	M + F	9.6 *(300 ppm)*	80.0 - 65.7 *(up to 2500 ppm 10 weeks / 2500 ppm 4 weeks)*	↓ bw gain, slightly ↓ food consumption, slightly ↑ ALAT, urea & creatinine levels (mostly in the 2-s range of HCD), marginal liver enzyme induction, slight cytoplasmatic changes in the liver in single animals, ↑ prostate weights	[redacted] & [redacted], 1998, amended 1999 M-003816-02-1

Study *Doses tested*	Sex	NO(A)EL	LO(A)EL	Main findings seen at LO(A)EL	Reference
		(mg/kg bw/day)			
Rat 12-weeks, oral (diet) + 5 weeks recovery *0-25-100-400-1600 ppm, 0-1600 ppm (rec.)*	M F	7.3 7.6 *(100 ppm)*	28.6 35.6 *(400 ppm)*	↓ bw , liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy with cytoplasmatic changes	[redacted] & [redacted], 1997 M-000863-01-1
Mouse 14-weeks, oral (diet) *0-50-250-1250-6250 ppm*	M F	19.1 < 27.2 *(50 ppm)*	102.6 27.2 *(250 ppm M, 50 ppm F)*	102.6 mg/kg bw (M): liver enzyme induction, hepatocellular hypertrophy 27.2 mg/kg bw (F): ↑ vacuolisation, hypertrophy & enlargement of the adrenal X-zone	[redacted] & [redacted]-[redacted], 1995, amended 1998 M-000697-02-1
Dog 15-weeks, oral (diet) *0-250-1000-(4000-0)**-2000 ppm*	M F	8.5 *(250 ppm)* 65.3 *(2000 ppm)*	34.9 *(1000 ppm)* > 65.3 *(> 2000 ppm)*	M, 34.9 mg/kg bw: ↑ weight & slight to moderate hypertrophy of the prostate (M)	[redacted] & [redacted], 1998 M-003814-01-1
Dog 52-weeks, oral (diet) *0-40-100-250-1000 ppm (52 weeks)* *0-100-1000 ppm (26 weeks, M, investigation of prostata effects)*	M F	8.88 *(250 ppm)* 33.80 *(1000 ppm)*	34.42 *(1000 ppm, 52 weeks)* > 33.80 *(> 1000 ppm)*	↓ food consumption (F), ↑ liver weight & hepatocellular cytoplasmatic change (M, 26 weeks), slightly ↑ prostatic size & weights without sonographical & histopathological correlates (52 weeks)	[redacted] & [redacted], 1998 M-003818-01-1
Rat 5-days (6 h/d) inhalation (2-week recovery) *0-1.97-19-205 mg/m³*	M F	19 mg/m³ air *(ca. 5 mg/kg bw/day)*	205 mg/m³ air *(ca. 57 mg/kg bw/day)*	clinical signs, hypothermia, transient ↑ in grip strength, ↓bw & food consumption, ↑ liver size & weight, liver enzyme induction, changes of clinicochemical parameters indicative of first signs of hepatotoxicity, ↓ thymus size & weight (M), dark spleen (F)	[redacted], 1995, amended 1999 M-000725-02-1
Rat 4-weeks (5 x 6 h/week) inhalation *0-2.0-18.2-143.4# mg/m³*	M F	18.2 mg/m³ air *(6.6 mg/kg bw/day)*	143.4 mg/m³ air *(52 mg/kg bw/day)*	clinical signs, hypothermia, ↓bw, changes of clinicochemical parameters indicative of first signs of hepatotoxicity, liver enzyme induction, minimal to slight hepatocellular hypertrophy, slight thyroid follicluar cell hypertrophy (2/10 M)	[redacted], 1998 M-241815-01-1

Study *Doses tested*	Sex	NO(A)EL (mg/kg bw/day)	LO(A)EL (mg/kg bw/day)	Main findings seen at LO(A)EL	Reference
Rat 4-weeks, dermal (2-week recovery) *0-100-300-1000, 0-1000 (rec.) mg/kg bw/d*	M F	100 300	300 1000	300 mg/kg bw, M: hepatocellular hypertrophy with cytoplasmic change, 1000 mg/kg bw/day, F: ↑ liver weight, hepatocellular hypertrophy with cytoplasmic change, thyroid folliclular cell hypertrophy	[redacted] & [redacted], 1997 M-000824-01-1

M: male F: female ↑: increase(d) ↓: decrease(d) bw: body weight
HCD: historical control data
rec.: recovery groups
*: Since no toxic signs were observed at 1000 ppm the high-dose was increased to 1250 from day 19 on wards, to 1600 ppm from day 26 onwards, and to 2500 ppm from day 38 onwards. An additional 2500 ppm treatment group was added from day 38 to day 66.
**: Due to vomitus, slight tremor, feed refusal and reduced body weights the high dose of 4000 ppm was set to 0 ppm from day 5 to 14 and then to 2000 ppm from day 15 throughout the study.
#: The study commenced with a high concentration of ca. 200 mg/m³. Due to severe respiratory distress and reduced body weight it was reduced to ca. 100 mg/m³ from the second exposure week onwards.

CA 5.3.1 Oral 28-day study

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of thiacloprid. (M-000703-01-4; M-000785-02-1, M-030427-03-1; M-000821-01-1; M-000688-01-1

CA 5.3.2 Oral 90-day study

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of thiacloprid. (M-000863-01-1; M-000697-02-1; M-003816-02-1)

CA 5.3.3 Other routes

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of thiacloprid. (M-000725-02-1 , M-241815-01-1 ,M-000824-01-1)

CA 5.4 Genotoxicity testing

Summary of genotoxicity testing

Guideline genotoxicity studies conducted with thiacloprid were consistently negative. They consisted of point mutation assays in bacteria and mammalian cells, an in vitro cytogenetic study, an unscheduled DNA synthesis assay on primary rat hepatocytes as well as a micronucleus test in vivo.

Furthermore, an additionally available bacterial DNA-repair test (rec-assay) revealed no indication for a DNA-damaging effect of thiacloprid.

In addition, three publications emerged from public literature between 2012 and 2013, which described genotoxic effects of thiacloprid in different test designs in vitro and in vivo. All three of them were based on non-GLP studies, which according to different deficiencies were considered to be non reliable and, thus, not relevant.

Table 5.4-1: Summary of genotoxicity testing*

Study	Test system	Concentration / Dose	Results	Reference
		In vivo		
Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA1535, TA1537 +/-S9 mix	up to 5000 µg/plate	negative	[redacted], 1995 M-000694-01-1
Bacterial reverse mutation assay	*S. typhimurium* TA98, TA100, TA1535, TA1537 *E.coli* WP2/uvrA +/-S9 mix	up to 5000 µg/plate	negative	[redacted], 1995 M-000903-01-1
Bacterial DNA-repair test	***Bacillus subtilis H17 (Rec$^+$) and M45 (Rec$^-$) +/-S9 mix***	***up to 6660 µg/disk***	***negative***	[redacted], ***1998 M-009213-01-1***
Mammalian cell gene mutation test (HGPRT)	Chinese hamster lung fibroblasts V79 +/-S9 mix	up to 500 µg/mL	negative	[redacted], 1996 M-000799-01-1
Mammalian chromosome aberration test	Chinese hamster lung fibroblasts V79 +/-S9 mix	75, 300, 750 µg/mL	negative	[redacted], 1995 M-000772-01-1
Unscheduled DNA synthesis (UDS) assay	Primary rat hepatocytes +/-S9 mix	up to 500 µg/mL	negative	[redacted], 1996 M-000790-01-1
		In vivo		
Micronucleus test	Mouse bone marrow	60 mg/kg bw i.p.	negative	[redacted], 1995 M-000775-01-1

*: New studies, i.e. studies previously not submitted / evaluated on EU level, are written in bold and italics.

CA 5.4.1 In vitro studies

In addition to the genotoxicity studies already contained in the Monograph and Baseline Dossier of thiacloprid a bacterial DNA-repair test / Rec-assay on bacillus subtilis is available, which had to be conducted for the registration of thiacloprid in Japan.

Report: [redacted]p;[redacted];1998;M-009213-01
Title: YRC 2894 – DNA repair test in bacterial system
Report No: NR 97220
Document No: M-009213-01-1
Guidelines: JMAFF 59 Nohsan: No. 4200 (1984);
Deviations: none
GLP: yes

I. Materials and methods

A. Materials

1. Test material: YRC 2894

Synonym:	thiacloprid
Description:	light yellow powder
Lot/Batch no:	290894
Purity:	96.9%
Stability of test compound:	guaranteed for study duration; expiry date: not reported

2. Vehicle and/or positive control: vehicles: dimethyl sulfoxide (DMSO) for solvent control, YRC 2894 and positive control 2-AA
distilled water for positive control MMC and negative control KM
positive controls:
Mitomycin C (MMC)
2-aminoanthracene (2-AA)
negative control:
Kanamycin sulfate (KM)

3. Test system:

Test strains: *Bacillus subtilis* H17 (Rec^+) and M45 (Rec^-)

Spore preparation: The spores of both strains, which were pre-incubated with liquid broth medium, were shaked and cultured with the modified Schaffer's medium. The cultured spores were washed with minimal salt (MM) solution and treated with lysozyme and sodium lauryl sulfate (sodium dodecyl sulfate). Subsequently, the spores were washed and resuspended in distilled water to prepare suspensions of about 2 x 10^7 / mL for storage at 4°C.

Metabolic activation: S9 mix prepared from liver homogenate of phenobarbital and 5,6-benzoflavon-induced male Sprague-Dawley rats (7 weeks of age)

B. Study design and methods

1. Treatment:

Dose: 0-416-833-1665-3330-6660 µg/disk

Positive controls:	Without metabolic activation: MMC: 0.005 µg/disk for H17 0.01 µg/disk for M45 With metabolic activation: 2-AA: 5 µg/disk for H17 20 µg/disk for M45
Negative control:	Without metabolic activation: KM: 0.5 µg/disk for H17 1 µg/disk for M45
Application volume:	20 µL/disk
Treatment without S9:	10 mL of stock spore suspension for each strain was added to 1 L of molten nutrient agar medium (about 45°C) and mixed well. Then 10 mL of these mixtures each was poured into a sterilized plate and solidified at room temperature. Two round paper disks (∅ 8 mm), which were impregnated with 20 µL of the test substance or control substance solution, were put on the plate.
Treatment with S9:	0.1 mL of S-9-mix was poured into a sterilized plate and then the same procedure as for the non-metabolic activation system was conducted. The plates were solidified in the refrigerator. Furthermore, each round paper disk was first impregnated with 20µL of co-factor solution, and then the same procedure as for the non-metabolic activation system was conducted.
Incubation time:	24 hours, at 37°C
Replicates:	1 plate per test substance concentration and controls
Measurements:	After the incubation, the diameters of the inhibition zones appearing around the disks were measured.
Evaluation:	A difference of more than 5 mm between the diameters of the growth inhibition zones between the two strains is indicative for DNA-damaging effects.
Test solution analysis:	The test substance solutions were analysed by high pressure liquid chromatograph (HPLC) for confirmation of the contained concentrations, homogeneity after preparation and stability for 5 hours at room temperature after preparation.
Analysed solutions:	Theoretical concentration: 20.2-80.7-323 mg/mL

II. Results and discussion

A. Analysis for achieved concentration, homogeneity and stability of test solutions

The concentrations of the analysed dosing solutions were within 94.6 ~ 98.1 % of the theoretical concentrations (coefficient variances: within 1.0 %). These data show that the test substance distribution in the test solutions proved to be homogeneous.

Analysis of the dosing solutions kept at room temperature for 5 hours after preparation revealed remaining rates of minimum (20.8 mg/mL) and maximum (333 mg/mL) concentrations of 101 % and 99.7 %, respectively. These data indicate that the test substance was stable in the dosing solutions.

B. Evaluation for DNA damage

With regard to YRC 2894 growth inhibition was neither observed in strain H17 (Rec+) nor in strain M45 (Rec-) up to the highest concentration of 6660 µg/disk (limit dose for solubility) with and without metabolic activation.

In contrast, the positive control substances 2-aminoanthracene and Mitomycin C caused growth inhibition in strain M45 (Rec^-), but not in strain H17 (Rec^+), indicating that both positive control substances cause a DNA damaging effect.

The negative control substance kanamycin sulfate caused growth inhibition in both strains but the difference of the diameters of the growth inhibition zone was less than 5 mm. This result shows that the negative control substance does not have a DNA-damaging effect.

Based on these results in control substances, it was confirmed that the test system employed in this study was a proper system for detecting DNA-damaging effects.

Table 5.4.1/06-1: Results of the Rec^+ assay on *Bacillus subtilis* strains H17 (Rec^+) and M45 (Rec^-)

Substance	Concentration (µg/disk)	-S9 mix Growth inhibition zone (mm)		Difference * (mm)	+S9 mix Growth inhibition zone (mm)		Difference* (mm)
		H17	M45		H17	M45	
YRC 2894	416	0	0	0	0	0	0
	833	0	0	0	0	0	0
	1665	0	0	0	0	0	0
	3330	0	0	0	0	0	0
	6660	0	0	0	0	0	0
2-AA	5	0	0	0	0	11	11
	20	0	0	0	0	12	12
MMC	0.005	0	11	11	--	--	--
	0.01	1	18	17	--	--	--
KM	0.5	10	11	1	--	--	--
	1	12	15	3	--	--	--
DMSO	20 µL	0	0	0	0	0	0

2-AA 2-aminoanthracene MMC Mitomycin C
KM Kanamycin sulfate DMSO dimethyl sulfoxide (= vehicle)
*: DNA damaging effects are indicated by a difference of the growth inhibition zones between H17 and M45 of > 5 mm.

III. Conclusion

Thiacloprid (YRC 2894) had no DNA-damaging effect in this bacterial DNA repair assay.

Publications)

Report: [redacted] ∃ ; [redacted] ; [redacted] ; [redacted] ; 2013; M-491849-01-1

Title: *In vitro* investigation of the genotoxic and cytotoxic effects of thiacloprid in

cultured human peripheral blood lymphocytes

Reference: Environmental Toxicology (2012 Jun 22); electronic publication

Document No: M-491849-01-1

Guidelines: None
Deviation(s): none

GLP: no

I. Materials and methods

A. Materials

1. Test material:

Name: thiacloprid

Description: thiacloprid (Fluka 37905)

CAS No.: 111988-49-9

Source: [redacted]

Lot/Batch no: not reported

Purity: not reported

Stability of test compound: not reported

2. Vehicle / positive control: vehicle: 50% ethanol
positive controls:
- without metabolic activation:
  mitomycin C (MMC): 0.25 µg/mL
- with metabolic activation:
  cyclophosphamide (CPA): 28.0 µg/mL

3. Test system: human peripheral blood lymphocytes (PBLs)

Cell cultures: 0.2 mL of whole blood from four healthy, non-smoking donors (21-23 years old, two males and two females) were diluted in 2.5 mL of chromosome medium PB Max (Gibco) supplemented with 10 µg/mL of bromodeoxyuridine. Cultures were incubated at 37°C for 72 h for the determination of chromosome aberrations (CA) and sister chromatid exchanges (SCE) and for 68 h for the cytokinesis-block micronucleus assay (CBMN).
The PB Max medium contains the mitogen PHA.

Culture conditions: 37°C

Metabolic activation: S9 mix
Albino male rats (Rattus norvegicus var. albinos) weighing 200 g were pretreated with 80 mg/kg 3-methylcholanthrene dissolved in sunflower oil for 5 days. For the preparation of the S9 fraction and the S9 mix, the method described by Maron and Ames (1983) was used.

Test concentrations: 75-150-300 µg/mL thiacloprid (300 µg/mL thiacloprid leads to a reduction of the mitotic index (MI) of ~ 50%)

B. Study design and methods

Chromosome aberration (CA) and Sister chromatide exchange (SCE) test: Without metabolic activation the cells were treated with 75, 150, and 300 µg/mL thiacloprid dissolved in 50% ethanol for 24

	(thiacloprid was added 48 h after initiating the culture) and 48 h (thiacloprid was added 24 h after initiating the culture). An untreated control, a solvent control (50% ethanol, 4 µL/mL) and a positive control (MMC, 0.25 µg/mL) were also used. For treatment with metabolic activation the lymphocytes were cotreated with 75, 150, and 300 µg/mL thiacloprid and 0.5 mL S9 mix for 3 h. Thiacloprid and S9 mix were added 48 h after initiating the culture. A control, a solvent control (50% ethanol, 4 µL/mL), and a positive control (CPA, 28 µg/mL) were also performed. Test chemical and S9 mix were removed from the culture by centrifugation for 4 minutes at 2500 rpm. The pellet of lymphocytes was washed twice with 2.5 mL RPMI 1640 medium (Biochrom AG, F 1215) and resuspended in fresh complete medium (chromosome medium PB Max). The cultures were incubated for a total of 72 h at 37°C for the CA and SCE assays.
Slide preparation:	To arrest the cells in metaphase, cells were exposed to 0.06 µg/mL colchicine 2 h before harvesting. The whole blood was centrifuged at 2000 rpm for 5 minutes and the supernatant was removed. Subsequently the cells were treated with a hypotonic solution (0.4% KCl) for 15 minutes at 37°C to lyse red blood cells and centrifuged at 1200 rpm for 10 minutes. The supernatant was removed and replaced with 10-mL cold fixative consisting of methanol:glacial acetic acid (3:1 v/v) at room temperature (22°C ± 1°C). The cell suspension was then centrifuged again at 1200 rpm for 10 minutes. The supernatant was removed, and the cell pellet was washed further two times in 10 mL of cold fixative. Finally, the centrifuged cells were dropped onto clean slides.
Staining of slides for the CA test:	Staining of the air-dried slides was performed following the standard methods using 5% Giemsa stain for chromosome aberration analysis (i.e., with 5% Giemsa in Sorensen Buffer, pH 6.8, 15 minutes).
Staining of slides for the SCE test:	Staining: according to the modified Fluorescence Plus Giemsa method (FPG) (Speit and Haupter, 1985): 1-day-old slides were covered with Sorensen Buffer (pH 6.8) and subsequently irradiated with a 30 W, 254-nm UV lamp at 15 cm distance for 30 minutes. After irradiation, slides were incubated in 1 x SSC (standard saline citrate) at 58–60°C for 60 minutes and then stained with 5% Giemsa prepared with Sorensen buffer for 20 minutes.
Treatment duration:	With (+) S9 mix: 3 hours Without (-) S9 mix: 24 and 48 hours (Thiacloprid was added 48 and 24 hours after culture initiation)

Preparation interval:	72 hours after initiation of culture
Number of evaluated cells:	CA: 400 well-spread metaphases / concentration (100 / culture) SCE: 100 well-differented second-division metaphases / concentration (25 / culture)
Replicates:	4 parallel cultures / dose
Cytotoxicity assessment (CA):	The mitotic index (MI) was calculated from the number of metaphases in 3000 cells analyzed per culture for each donor (12000 cells per concentration).
Cytotoxicity assessment (SCE):	A total of 400 cells (100 cells from each donor) were scored for the proliferation index (PI). PI was calculated according to formula as follows: PI 5 [(1 x M1) + (2 x M2) + (3 x M3)]/N, where M1, M2 and M3 represent those metaphases corresponding to first, second and third divisions and N is the total number of metaphases scored (Lamberti et al., 1983).
Cytokinesis-block micronucleus assay (CBMN):	0.2 mL of fresh blood was used to establish the cultures, which were incubated for 68 h. The cells were treated with 75, 150 and 300 µg/mL thiacloprid for 24 and 48 h treatment periods (thiacloprid was added 44 and 20 h after culture initiation, respectively). Cytochalasin B (cytB) (final concentration: 6 µg/mL) was added after 44 h of incubation in order to block cytokinesis and obtain binucleated (BN) cells. After additional 24 h incubation at 37°C, the cells were harvested by centrifugation and the pellet was resuspended in a hypotonic solution of 0.4% KCl for 5 minutes at 37°C. The cells were fixed in a cold fixative (methanol : glacial acetic acid: 0.9% NaCl, 5:1:6 v/v/v). After centrifugation, the cells were fixed further two times with methanol : glacial acetic acid (5:1 v/v). For treatments with metabolic activation, the lymphocytes were cotreated with 75, 150, and 300 µg/mL thiacloprid and 0.5 mL S9 mix for 3 h, which were both added 48 h after initiating the culture. A control, a solvent control (50% ethanol, 4 µL/mL), and a positive control (CPA, 28 µg/mL) were also performed. Thiacloprid and S9 mix were removed from the culture by centrifugation for 4 minutes at 2500 rpm. The pellet of lymphocytes was washed twice with 2.5 mL RPMI 1640 medium (Biochrom AG, F 1215) and resuspended in fresh complete medium (chromosome medium PB Max). The cultures were incubated for a total of 68 h at 37°C. The cells were harvested and fixed as described above.
Slide preparation:	Slides were prepared by dropping and airdrying.
Staining of slides CBMN assay:	The slides were stained with 5% Giemsa stain solution (diluted with Sorensen buffer, pH 6.8) for 15 minutes (Fenech, 2000; Kirsch-Volders et al., 2003).
Treatment duration:	With (+) S9 mix: 3 hours

	Without (-) S9 mix: 24 and 48 hours (Thiacloprid was added 44 and 20 hours after culture initiation)
Preparation interval:	68 hours after initiation of culture
Evaluation:	Micronuclei (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (NBUDs) were scored in BN cells according to the scoring and identification criteria of Fenech et al. (2003) and Fenech (2007) to determine MN and other nuclear anomalies.
Number of evaluated cells:	4000 binucleated cells (1000/culture)
Replicates:	4 parallel cultures / dose
Cytotoxicity assessment:	Cytostaticity was calculated by using the nuclear division index (NDI). To this aim, 1000 lymphocytes per donor were analyzed. The numbers of cells with one to four nuclei were determined in 1000 cells. NDI was calculated using the following formula: NDI = [(1 x M1) + (2 x M2) + (3 x M3) + (4 x M4)]/N, where M1 through M4 represent the number of cells with one to four nuclei and N is the total number of cells scored (Eastmond and Tucker, 1989; Fenech, 2000, 2007).

II. Results and discussion

A. Chromosome aberration assay

Thiacloprid increased the percentage (%) of cells with structural chromosome aberrations (CAs) and with chromosome aberrations significantly for all concentrations and treatment periods when compared with the control and solvent control in the absence and presence of S9 mix ($p < 0.001$). As shown in Table 5.4.1/07-1, both with and without S9 mix, the chromatid-type aberrations were more common than the chromosome-type aberrations. However, the aberrations were significantly lower when compared with the respective positive control. In addition, thiacloprid generally did not induce the numerical CAs in human peripheral blood lymphocytes.
The mitotoc index (MI) decreased significantly at all thiacloprid concentrations of the 24- and 48-h treatment periods when compared with the respective controls without S9 mix.
The test compound also significantly decreased the MI at 300 µg/mL thiacloprid of the 48-h treatment period when compared with the positive control MMC. At this concentration cytotoxicity was approximately 80%. Similarly, with metabolic activation, thiacloprid caused a statistically significant reduction in the MI when compared with the control and the solvent control at all concentrations. Furthermore, 75 and 150 µg/mL thiacloprid also decreased the MI to the same extent as or, at 300 µg/mL, more than the positive control, in the presence of the S9 mix (Table 5.4.1/07-1). Thus, it can be said that the highest concentration of thiacloprid showed a higher cytotoxic effect than cyclophosphamide (after 3 h exposure) and also mitomycin C (for 48 h treatment period).

Table 5.4.1/07-1: Summary of results of the *in vitro* chromosome aberration test (CA) in human peripheral blood lymphocytes with thiacloprid

Test substance	Concentration (µg/mL)	Structural CA		Poly-ploid cells	% cells with		Mitotic index (MI) (mean ± SD)
		Chromatid-type	Chromosome-type		Structural CA (mean ± SD)	CA (mean ± SD)	

Test substance	Concentration (µg/mL)	Structural CA: Chromatid-type	Structural CA: Chromosome-type	Polyploid cells	% cells with: Structural CA (mean ± SD)	% cells with: CA (mean ± SD)	Mitotic index (MI) (mean ± SD)
24 h exposure without S9 mix							
Control	--	17	1	--	4.50 ± 0.57	4.50 ± 0.57	3.75 ± 0.32
Solvent control	4 µL/mL	17	1	1	4.50 ± 1.00	4.75 ± 0.50	3.71 ± 0.29
MMC	0.25	100	17	1	25.00 ± 1.41 a3b3	25.25 ± 1.25 a3b3	1.01 ± 0.11 a3b3
TCP	75	38	5	--	10.00 ± 1.63 a3b3c3	10.00 ± 1.63 a3b3c3	3.31 ± 0.23 a1b1c3
	150	58	5	--	13.75 ± 0.95 a3b3c3	13.75 ± 0.95 a3b3c3	2.44 ± 0.12 a3b3c3
	300	61	8	--	16.00 ± 0.81 a3b3c3	16.00 ± 0.81 a3b3c3	1.88 ± 0.15 a3b3c3
48 h exposure without S9 mix							
Solvent control	4 µL/mL	17	4	--	5.25 ± 0.95	5.25 ± 0.95	3.19 ± 0.15
MMC	0.25	890	98	--	81.50 ± 3.00 a3b3	81.50 ± 3.00 a3b3	1.02 ± 0.10 a3b3
TCP	75	45	5	1	11.50 ± 1.29 a3b3c3	11.75 ± 1.70 a3b3c3	2.67 ± 0.25 a3b2c3
	150	45	13	--	13.25 ± 1.89 a3b3c3	13.25 ± 1.89 a3b3c3	*1.33 ± 0.20* a3b3c1
	300	85	12	--	22.25 ± 2.50 a3b3c3	22.25 ± 2.50 a3b3c3	*0.63 ± 0.14* a3b3c1
3 h exposure with S9 mix							
Control	--	16	2	1	4.00 ± 1.41	4.25 ± 0.95	4.71 ± 0.38
Solvent control	4 µL/mL	16	1	1	4.25 ± 0.95	4.50 ± 0.57	4.70 ± 0.57
CPA	28	34	19	--	15.75 ± 1.25 a3b3	15.75 ± 1.25 a3b3	4.28 ± 0.22
TCP	75	31	9	1	9.75 ± 0.95 a3b3c3	10.00 ± 0.81 a3b3c3	4.14 ± 0.50 a1b1
	150	31	9	2	10.00 ± 2.30 a3b3c3	10.50 ± 1.73 a3b3c3	3.99 ± 0.35 a1b1
	300	38	14	--	11.75 ± 2.75 a3b3c2	11.75 ± 2.75 a3b3c2	3.39 ± 0.13 a3b3c2

Mean: mean value of 4 cultures
CA: chromosome aberrations
TCP: thiacloprid
control: untreated cells
Solvent control: 50% ethanol
MMC: mitomycin C
CPA: cyclophosphamide
a significantly different from untreated control
b significantly different from solvent control (ethanol 50%)
c significant from positive control (MMC, CPA).
^{a1b1c1}: $p < 0.05$ ^{a2b2c2}: $p < 0.01$ ^{a3b3c3}: $p < 0.001$

Values in italics refer to > 50 % cytotoxicity

B. Sister chromatid exchange assay

Thiacloprid induced the SCE frequency significantly at all concentrations (75, 150, and 300 µg/mL) when compared with the control and solvent control with and without S9 mix. The increase was concentration-dependent only for the 48 h treatment without S9 mix ($r = 0.999$, $p < 0.05$). Thiacloprid decreased the proliferation index (PI) dose-dependently at all concentrations both with and without S9 mix when compared with the control and solvent control. In addition, at the highest concentration (300 µg/mL) thiacloprid decreased the PI as much as the positive control both with and without S9 mix (Table 5.4.1/07-2).

Table 5.4.1/07-2: Summary of results of the *in vitro* sister chromatid exchange test (SCE) in human peripheral blood lymphocytes with thiacloprid

Test substance	Concentration (µg/mL)	SCE Min	SCE Max	SCE / cell (mean ± SD)	M1	M2	M3	Proliferation index (PI) (mean ± SD)
24 h exposure without S9								
Control	--	1	12	4.00 ± 0.29	80	152	168	2.19 ± 0.15
Solvent control	4 µL/mL	1	12	4.30 ± 0.14	76	201	123	2.11 ± 0.07
MMC	0.25	13	90	49.25 ± 4.54 a3b3	234	155	11	1.44 ± 0.08 a3b3
TCP	75	2	15	6.73 ± 0.78 a1b1c3	111	209	180	1.92 ± 0.08 a2b1c3
	150	3	15	7.53 ± 0.66 a1b1c3	179	180	41	1.65 ± 0.21 a3b3c1
	300	4	17	8.09 ± 0.24 a2b2c3	251	138	11	1.40 ± 0.06 a3b3
48 h exposure without S9								
Solvent control	4 µL/mL	2	12	4.81 ± 0.14	101	159	140	2.09 ± 0.05
MMC	0.25	35	149	89.55 ± 4.23 a3b3	315	85	0	1.21 ± 0.12 a3b3
TCP	75	1	11	5.71 ± 0.39 a2b1c3	132	186	82	1.87 ± 0.09 a3b2c3
	150	2	19	9.32 ± 0.54 a3b2c3	245	146	9	1.41 ± 0.07 a3b3c1
	300	5	18	12.05 ± 1.21 a3b3c3	364	34	2	1.09 ± 0.03 a3b3
3 h exposure with S9								
Control	--	1	8	2.90 ± 0.14	51	176	173	2.30 ± 0.04
Solvent control	4 µL/mL	1	13	4.28 ± 0.05	70	234	96	2.06 ± 0.05
CPA	28	10	47	20.08 ± 0.78 a3b3	121	265	14	1.73 ± 0.10 a3b3
TCP	75	1	10	4.86 ± 0.75 a1c3	75	240	85	2.02 ± 0.12 a3c3
	150	2	12	6.65 ± 0.33 a3b3c3	105	219	76	1.92 ± 0.10 a3b1c2
	300	3	16	7.62 ± 0.44 a3b3c3	134	224	42	1.77 ± 0.04 a3b3

Mean: mean value of 4 cultures
A total of 100 cells were scored per concnetration for the SCE assay, 400 cells were scored for the PI.
CA: chromosome aberrations TCP: thiacloprid control: untreated cells
solvent control: 50 % ethanol MMC: mitomycin C CPA: cyclophosphamide

M1, M2, M3: metaphases corresponding to the first, second and third divisions
a significantly different from untreated control
b significantly different from solvent control (ethanol 50%)
c significantly different from positive control (MMC, CPA)
a1b1c1: $p < 0.05$ a2b2c2: $p < 0.01$ a3b3c3: $p < 0.001$
Values in italics refer to > 50 % cytotoxicity

A. Cytokinesis-block micronucleus test (CBMN)

In the CBMN thiacloprid induced a statistically significant increase in micronucleated binuclear cells (MNBN %) when compared with the control and solvent control at all concentrations tested both with and without S9 mix. However, binuclear (BN) cells could not be detected sufficiently in the highest concentration of thiacloprid (300 µg/mL) after 48 h treatment in the absence of S9 mix. In addition, the MN formation increased linearly as thiacloprid concentration increased in the absence ($r = 0.999$, $p < 0.05$ and $r = 1.000$, $p < 0.001$ for the 24 and 48 h, respectively) as well as presence ($r = 1.000$, $p < 0.001$) of S9 mix.

The results of the present study indicate that thiacloprid statistically significantly increased the BN cells with nuclear plasmic bridges (NPBs, ‰) when compared with the control and the solvent control at all concentrations for 24 and 48 h (except 300 mg/mL: due to the excessive cytostaticity, BN cells could not be determined sufficiently) treatment periods in the absence of S9 mix. However, the NPBs were significantly lower in comparison with the positive control MMC. The increase of NPBs formation was concentration-dependent only for the 48-h treatment ($r = 1.000$, $p < 0.001$). In the presence of S9 mix, the test compound induced a concentration-dependent increase in BN cells with NPBs (%) when compared with the controls at the all concentrations ($r = 1.000$, $p < 0.001$). Furthermore, a statistically significant correlation was observed between micronucleated, binucleated cells (MNBN, %) and BN cells with NPBs % for the 48-h treatment period in the absence and presence of S9 mix ($r = 1.000$, $p < 0.001$ in both cases).

Thiacloprid also induced a statistically significant increase in BN cells with nuclear buds (NBUDs, %) when compared with the control and solvent control at 300 µg/mL for 24 h and at 150 µg/mL for 48-h treatment periods in the absence of the metabolic activator. In addition, NBUD formation

Table 5.4.1/07-3: Summary of results of the cytokinesis-block micronucleus test (CBMN) in human peripheral blood lymphocytes with thiacloprid

Test substance	Concen-tration (µg/mL)	Distribution of BN cells according to no. of micronuclei					Mean of binucleated cells with (‰)			
		0	1	2	3	>3	MN	NPBs	NBUDs	NPB / MN
24 h exposure without S9 mix										
Control	---	3984	16	0	0	0	4.00	0.50	1.50	
Solvent control	4 µL/mL	3982	18	0	0	0	4.50	0.75	1.25	
MMC	0.25	3881	109	10	0	0	29.75 a3b3	3.50 a3b3	5.00 a3b3	
TCP	75	3971	28	1	0	0	7.25 a1b1c3	1.50 a1c3	2.00 c3	0.20
	150	3968	28	4	0	0	8.00 a2b1c3	2.00 a2b1c2	2.50 c2	0.25

Test substance	Concen-tration (µg/mL)	Distribution of BN cells according to no. of micronuclei					Mean of binucleated cells with (‰)			
		0	1	2	3	>3	MN	NPBs	NBUDs	NPB/ MN
	300	3961	30	8	1	0	9.75 [a3b3c3]	2.25 [a2b2c1]	3.50 [a1b2c1]	0.23
48 h exposure without S9 mix										
Solvent control	4 µL/mL	3985	15	0	0	0	3.75	0.75	1.25	
MMC	0.25	3746	222	30	2	0	63.50 [a3b3]	3.75 [a3b3]	10.25 [a3b3]	
TCP	75	3958	38	4	0	0	10.50 [a3b3c3]	1.75 [a1b1c3]	2.00 [c3]	0.16
	150	3939	53	8	0	0	15.25 [a3b3c3]	2.50 [a3b2c1]	3.25 [a1b1c3]	0.16
	300	#					#	#	#	
3 h exposure with S9 mix										
Control	---	3991	9	0	0	0	2.25	0.50	0.75	
Solvent control	4 µL/mL	3989	11	0	0	0	2.75	0.25	1.25	
CPA	28	3960	35	4	0	1	10.00 [a3b3]	3.00 [a3b3]	4.00 [a3b3]	
TCP	75	3982	17	1	0	0	4.50 [a2b1c3]	1.25 [b1c2]	1.75 [c3]	0.27
	150	3982	15	3	0	0	5.00 [a1b2c3]	1.50 [a1b1c2]	2.25 [a2c2]	0.30
	300	3976	23	1	0	0	6.00 [a3b3c3]	2.00 [a2b2c1]	3.00 [a3b2]	0.33

Mean: mean value of 4 cultures
A total of 4000 cells were scored per concentration for micronuclei and other abnormalities in binuclear cells.
TCP: thiacloprid control: untreated cells solvent control: 50 % ethanol
MMC: mitomycin C CPA: cyclophosphamide
MN: micronucleated cells NPB: nuclear plasmic bridges NBUDS: nuclear buds
a significantly different from untreated control
b significantly different from solvent control (ethanol 50%)
c significant from positive control (MMC, CPA)
a1b1c1: $p < 0.05$ a2b2c2: $p < 0.01$ a3b3c3: $p < 0.001$
#: insufficient binuclear cells

Cytotoxicity assessment for the CBMN

Cytostatic effects of thiacloprid were measured by calculating the nuclear division index (NDI). Thiacloprid decreased the NDI significantly for all concentrations and treatment periods when compared with the control groups both in the absence and presence of the S9 mix. In addition, at the highest concentration (300 µg/mL) for 24 h and two higher concentrations (150 and 300 µg/mL) for 48-h treatment periods, thiacloprid caused a significant reduction in the NDI to the same extent as positive control, MMC, in the absence of the S9 mix. Similarly, in the presence of the S9 mix, at all concentrations (75, 150, and 300 µg/mL) of thiacloprid reduced the NDI to the same extent as the positive control CPA. In addition, the decrease of the NDI was concentration-dependent in the presence of the S9 mix ($r = -0.997$, $p < 0.05$).

Table 5.4.1/07-4: Effect of thiacloprid on the nuclear division index (NDI) in human peripheral blood lymphocytes

Test substance	Concen-tration (µg/mL)	Distribution of cells according to number of nuclei 1	2	3	4	NDI (mean ± SD)	CBPI* (mean)	RI* (%)
24 h exposure without S9 mix								
Control	--	3253	661	38	48	1.22 ± 0.02	1.17	--
Solvent control	4 µL/mL	3429	507	40	24	1.16 ± 0.01	1.14	75.7
MMC	0.25	3808	189	2	1	1.04 ± 0.01 a3b3	1.05	23.3
TCP	75	3437	526	17	20	1.15 ± 0.01 a3c3	1.14	71.4
	150	3450	523	8	19	1.14 ± 0.00 a3c3	1.13	68.6
	300	3802	188	5	5	1.05 ± 0.01 a3b3	1.05	24.8
48 h exposure without S9 mix								
Solvent control	4 µL/mL	3567	395	23	15	1.12 ± 0.02	1.11	56.2
MMC	0.25	3870	129	1	0	1.03 ± 0.01 a3b3	1.01	15.7
TCP	75	3723	272	4	1	1.07 ± 0.00 a3b3c1	1.03	33.8
	150	3831	169	0	0	1.04 ± 0.00 a3b3	1.07	20.0
	300	3949	51	0	0	1.01 ± 0.00 a3b3	1.04	6.19
3 h exposure with S9 mix								
Control	--	3106	878	10	6	1.22 ± 0.03	1.22	--
Solvent control	4 µL/mL	3211	778	7	4	1.20 ± 0.06	1.20	87.7
CPA	28	3479	520	1	0	1.13 ± 0.03 a2b1	1.13	57.5
TCP	75	3385	595	14	6	1.16 ± 0.01 a1	1.15	69.7
	150	3451	541	5	3	1.14 ± 0.02 a2c1	1.14	61.0
	300	3552	444	3	1	1.11 ± 0.02 a3b2	1.11	49.6

NDI: nuclear division index
CBPI*: cytokinesis-block proliferation index (calculated from reported values; not provided in publication)
RI*: Replication index (calculated from reported values; not provided in publication)

For the evaluation of cytotoxicity OECD 487 recommends to calculate the CBPI or RI.

CBPI = the proportion of second-division cells in the treated population relative to the untreated control. The CBPI indicates the average number of nuclei per cell, and may be used to calculate cell proliferation. It is calculated according to the following formula:

$$CBPI = \frac{(No.\ mononucleated\ cells) + (2 \times No.\ of\ binucleated\ cells) + (3 \times No\ multinucleated\ cells)}{Total\ number\ of\ cells}$$

$$\% \text{ cytostasis} = 100 - 100\{(CBPI_{Treated}-1)/(CBPI_{Control}-1)\}$$

RI = the proportion of cell division cycles completed in a treated culture, relative to the untreated control, during the exposure period and recovery

The RI indicates the relative number of cell cycles per cell during the period of exposure to cytoB in treated cultures compared to control cultures. Calculation according to:

$$RI = \frac{\{(No.\ binucleated\ cells)_T + (2x\ No\ multinucleated\ cells)_T\} / (Total\ No\ of\ cells)_T}{\{(No.\ binucleated\ cells)_C + (2x\ No\ multinucleated\ cells)_C\} / (Total\ No\ of\ cells)_C} \times 100$$

T = treated; C = control
Cytostasis = 100-RI

Mean: mean value of 4 cultures
A total of 4000 cells were scored per concentration for the NDI.

TCP:	thiacloprid	control:	untreated cells	solvent control:	50 % ethanol
MMC:	mitomycin C	CPA:	cyclophosphamide		

a significantly different from untreated control
b significantly different from solvent control (ethanol 50%)
c significant from positive control (MMC, CPA).

^{a1b1c1}:	$p < 0.05$	^{a2b2c2}:	$p < 0.01$	^{a3b3c3}:	$p < 0.001$

III. Conclusion

The authors conclude that thiacloprid most probably causes genotoxic effects by inducing the formation of chromosome aberrations, sister chromatid exchanges, micronucleated cells, nuclear plasmatic bridges and nuclear buds. In addition, it displayed a cytotoxic effect by decreasing the mitotic index and proliferation index and also a cytostatic effect by decreasing the nuclear division index at the tested concentrations of 75, 150, and 300 µg/mL in human peripheral blood lymphocytes both in the absence and presence of a metabolic activation system.

BCS conclusion: This non-GLP study is considered to be not reliable (see reliability assessment below). In addition, tthe described genotoxic effects were observed in vitro at high concentrations of 75 to 300 µg/mL thiacloprid, which is equal to 75 – 300 mg/L. Total plasma concentrations of thiacloprid observed after treatment of rats with 1000 ppm in the diet (the high dose in the 2-year rat study, which clearly exceeded the MTD) were 5.7-11.5 mg/L. This shows that the concentrations tested in this in vitro study are by factors of 6.5 to 52.6 higher than the total plasma concentrations of rats in the high dose of the 2-year rat study. Therefore, it is concluded that the results of this study, besides the fact of its lack of reliability, have no impact on the situation in vivo.

The reliability assessment is shown below.

Klimisch evaluation

Reliability of study	Not reliable (Klimisch code 3)
General comment on reliability:	The study was not conducted according to GLP. Thiacloprid was not purchased from Bayer/BCS, the purity and the impurity profile of the used material is not reported. Culture conditions are not reliable. No CO_2-concentration is reported. No information on pH and osmolality of treatment medium is reported. No historical positive and negative control data are provided.
Comment on reliability CA & SCE:	The study was conducted according to IPCS guidelines (Albertini et al., 2000). However, the IPCS guidlines refer to *in vivo* exposure (i.e. *ex vivo – in vitro* tests). There are no treatment durations given. For result evaluation the IPCS guidelines consider only statistical significances. According to OECD 473 a short-term treatment +/– S9 mix is recommended. Here a short-term treatment was only done with

	S9 mix. The percentage of cells with chromosme aberarrations and structural chromosome aberrations in thiacloprid-groups were statistically significantly different from both positive and negative controls. Since no historical control data (HCD) for both positive and negative controls are available, it is not possible to evaluate / draw a conclusion from these results. In addition, no individual culture data are provided that could help for the evaluation. According to the draft OECD 473 reduction in mitotic index at the highest concentration should be 45 ± 5% of solvent control. In the 48 hour treatment without S9 mix the mitotic index was only about 20% of solvent controls.
Comment on reliability CBMN:	Test was conducted according to accepted protocols (Fenech 2000, Kirsch-Volders et al., 2003), but with major methodical and reporting deficiencies. According to Fenech 2007 nuclear division index (NDI)-values of untreated controls are expected to be in the range of 1.3 – 2.2. Here NDI values of all untreated controls are below 1.2. Furthermore, Fenech reported normal ranges for untreaded cells of 0-30 micronucleated (MN) cells/1000 binucleated cells (BN) cells, 0-10 nuclear plasmatic bridges/1000 BN cells, and 0-5 nuclear buds/1000 BN cells (Fenech, 2007). Except for the positive control in the 48-hour treatment without S9 mix the mean number of miconucleated BN cells for untreated and treated groups and positive controls is below 30. Although no individual culture data were provided, from the mean values it can be concluded that the values observed in the thiacloprid-treated cultures are in the ranges expected for untreated lymphocytes. According to OECD 487 solvent control MN-frequencies should be within 95% control limits of historical control In this publication no historical control data are provided. Without historical control data evaluation of these results are difficult. According to the acceptability criteria of OECD 487 the maximum tested concentration should cause 55±5% cytotoxicity (i.e. reduction of CBPI or RI to 45±5%). In the long-term treatments without metabolic activation the highest concentrations caused more than 55±5% cytotoxicity.
Relevance of study	**Not relevant due to lack of reliability: test system not adequate and not sufficiently described.**

References stated in the publication:
Fenech M. 2000; The in vitro micronucleus technique; Mutat Res 455:81–95
Fenech M. 2007; Cytokinesis-block micronucleus cytome assay; Nature Prot 2:1084–1104
Kirsch-Volders M, Sofuni T, Aardema M, Albertini S, Eastmond D, Fenech M, Ishidate M Jr, Kirchner S, Lorge E, Morita T, Norppa H, Surralle´s J, Vanhauwaert A, Wakata A. 2003; Report from the in vitro micronucleus assay working group. Mutat Res 540:153–163

Report:	[redacted]1; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; [redacted]; 2012; M-486783-01-1
Title:	Evaluation of genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed *in vitro* to neonicotinoid insecticides news
Reference:	Journal of Toxicology, Vol 2012, Art ID 612647, 11 p
Document No:	M-486783-01-1
Guidelines:	None Deviation(s): not applicable
GLP:	No

I. Materials and methods

A. Materials

1. Test material:

Name:	Calypso 480 SC
Source:	[redacted], México
Lot/Batch no:	RSCO-INAC-0102T-301-064-040
Content:	480 g/L thiacloprid
Stability of test compound:	guaranteed for study duration; expiry date: 2014-09-11

2. Vehicle / positive control: - vehicle: deionized water
- positive controls: none

3. Test system: human peripheral blood lymphocytes

Lymphocyte preparation: Heparinized venous blood (20 mL) obtained from three healthy volunteer donors were centrifuged at 2500 rpm for 20 minutes. The cellular layer was diluted 1:1 with HBSS, placed over a Ficoll-Paque layer and centrifuged at 1500 rpm for 10 minutes. Lymphocytes were collected and washed twice in RPMI 1640 medium by centrifugation at 1500 rpm for 10 minutes. The lymphocyte pellet was kept in RPMI 1640 medium supplemented with 1% penicillin/streptomycin at 37°C and was immediately assessed for changes in cellular viability using a Neubauer chamber.

Metabolic activation: none

B. Study design and methods

Cytotoxicity assessment: Cell viability was estimated before and after treatments using the trypan blue exclusion method. A mix of 10 µL cell pellet and 10 µL trypan blue was incubated for 3 minutes. Subsequently the number of dead cells out of 100 consecutive cells was counted in duplicate.

Cytotoxicity was determined in preliminary assays at concentrations of 0.06, 0.09, 0.12, 0.13, 0.14, 0.2 and 0.28 M.

Exposure concentrations for Comet assay:	0.06, 0.09, 0.12, 0.13, 0.14 M
Negative control:	human peripheral lymphocytes in RPMI 1640 medium
Exposure conditions:	Human peripheral blood lymphocytes (5×10^5 cells) with a viability > 92% were incubated with 0.06, 0.09, 0.12, 0.13 and 0.14 M Calypso in 1 mL of 1640 RPMI medium at 37°C for 2 h. The controls consisted of human peripheral lymphocytes (5×10^5 cells) in RPMI 1640 medium under the same conditions. After treatments, the cells were washed twice with RPMI 1640 medium and subjected immediately to the cell viability or alkaline comet assays.
Treatment duration:	2 hours
Replicates:	3 parallel cultures / dose (1 culture / donor)
Comet assay and slide preparation:	The Comet assay was conducted according to Tice et al., 2000 and Singh et al., 1988. Lymphocytes (2500 cells) were mixed with 90 µL of low-melting-point agarose (0.5%) at 37°C, placed on fully frosted slides (Fisher) coated with a thin layer of normal-melting-point agarose (1%) and covered with a coverslip. Two slides were made for each treatment. The slides were kept at 4°C for 5 minutes to allow the agarose to solidify. The coverslip was then carefully removed, and the slides were immersed in a Coplin staining jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100, and 10% DMSO, pH = 10) at 4°C for 1 h. The slides were placed in a horizontal electrophoresis chamber containing freshly prepared cold electrophoresis alkaline buffer (300 mM NaOH, 1 mM EDTA, pH = 13) for 20 minutes to unwind the DNA. Electrophoresis was carried out at 25 V and 300 mA for 20 minutes in darkness to prevent additional DNA damage. The slides were then washed three times with freshly prepared neutralization buffer (0.4 M Tris, pH 7.5) for 5 minutes, fixed with cold absolute methanol for 5 minutes, and air-dried at room temperature. Next, 50 µL of ethidium bromide (20 mg/mL) was added to each slide to stain the DNA. The slides were coded before evaluation.
Number of evaluated cells:	50 nuclei / slide; 2 slides / culture
Parameters assessed:	- comet frequency (nuclei with DNA damage) - comet tail length (DNA fragmentation)
Statistics:	Comet frequency, tail length and cell viability are reported as the mean ± standard error of the mean (SEM) obtained from three independent experiments for each treatment. An analysis of variance (ANOVA) and the Newman-Keuls test were used to

determine significant differences between the treatment groups. Significance was defined as $p < 0.001$. The relationship between comet frequency and comet tail length was evaluated using linear regression analysis.

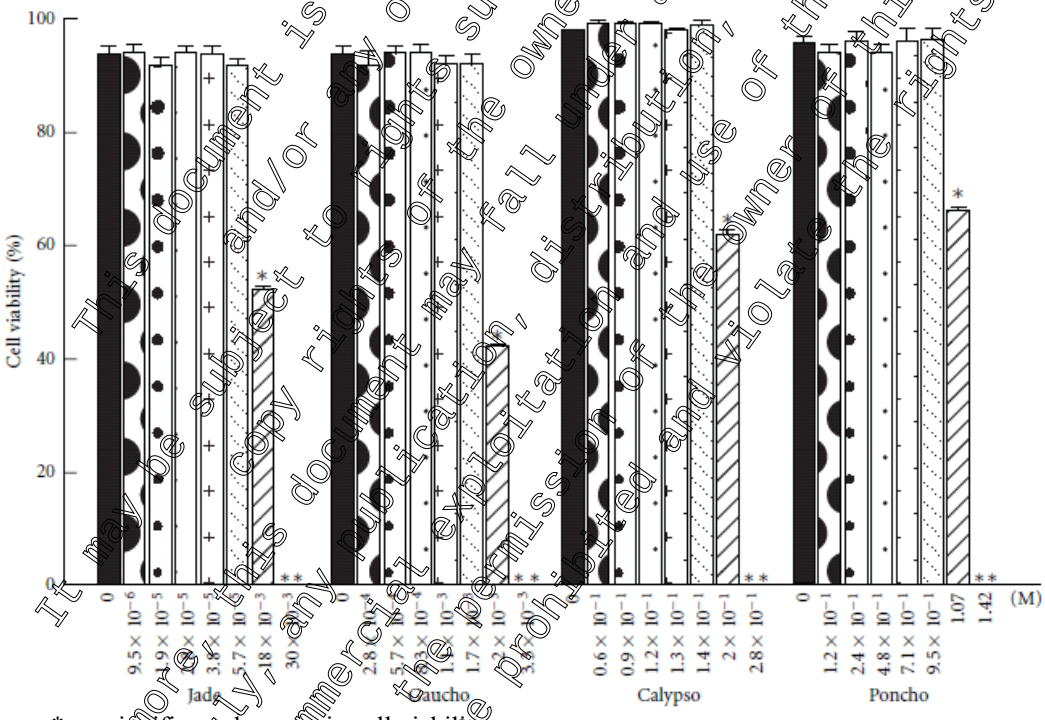
II. Results and discussion

A. Cytotoxicity

In the preliminary experiments, human peripheral blood lymphocytes were exposed to different concentrations of Calypso SC 480 for 2 h. After treatment, cell viability was evaluated by trypan blue dye-exclusion staining. The data indicate that concentrations of 0.06, 0.09, 0.12, 0.13 and 0.14 M did not produce statistically significant differences in cell viability when compared to controls ($p < 0.001$). These concentrations were then used for the alkaline comet assay.

However, when the human lymphocytes were exposed to 0.2 M Calypso cell viability was significantly decreased in relation to the control values ($p < 0.001$). Cell death occurred following exposure to 0.28 M Calypso.

Figure 5.4.1/08-1: Mean viability of human peripheral blood lymphocytes exposed *in vitro* to neonicotinoid insecticides including Calypso. The bars represent the mean values ± SEM from three independent experiments



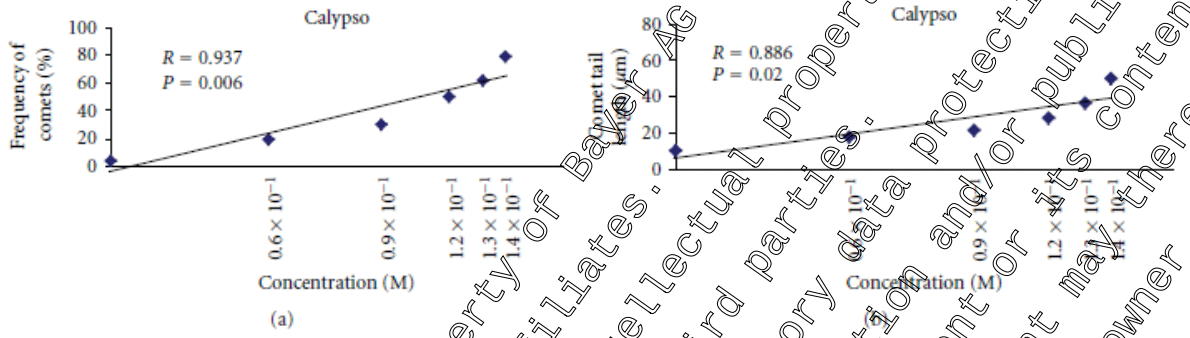
*: significant decrease in cell viability
**: cell death

B. Comet assay

Exposure of human peripheral lymphocytes with Calypso SC 480 for 2 h caused significant increases in the percentages of comets and tail length when compared to controls ($p < 0.001$). In cells exposed to 0.06, 0.09, 0.12, 0.13 and 0.14 M Calypso SC 480 the mean comet frequency concentration

dependently increased from 20 ± 0.6 to 79 ± 0.7 %, while the tail length increased from 17.9 ± 0.1 to 50.3 ± 1.0 µm (see Figure 5.4.1/8-01 below).

Figure 5.4.1/08-2: Regression lines of the frequency of comets (a) and comet tail length (b) in human peripheral blood lymphocytes exposed *in vitro* to Calypso



III. Conclusion

The authors conclude that under the experimental conditions reported, the results indicate that the formulation Calypso SC 480 (480 g/L thiacloprid) at concentrations in the range of 0.06 to 0.14 M induced an increase in DNA damage with a concentration-dependent relationship.

BCS conclusion: This non-GLP study is considered to be not reliable (details see in the reliability assessment below). Furthermore, the described DNA damage was observed in vitro at extremely high concentrations of 60 to 140 mM of Calypso, which exceed not only the maximum concentration of 10 mM recommended for in vitro assays by a factor of 6-14, but even more, by a factor of 600-2800, the total plasma concentrations of thiacloprid (50-100 µM or 0.05 to 0.1 mM) observed after treatment with 1000 ppm in the diet. This dietary concentration was tested as the high dose of the 2-year rat study, which clearly exceeded the MTD. Therefore, it is concluded that the results of this study, besides the fact of its missing reliability, have no impact on the situation in vivo.
Additional comment: In the publication it is stated the application rates of the tested formulation for the intended uses are in the range of 22 to 30 mL product/100 L. This corresponds to concentrations of 0.42 to 0.57 mM thiacloprid. It was stated that these concentrations are higher than the concentrations tested in the comet assay.
If the stated concentrations used in the assays are correct (i.e. 60-140 mM), the real exposure concentrations of thiacloprid (i.e. 0.42 – 0.57 mM) are much lower than the tested concentrations. Furthermore, the applied concentrations refer to external exposure concentrations. Systemic exposure, i.e. the amount / concentration that is internally available will be much lower.

The reliability assessment is presented below.

Klimisch evaluation

Reliability of study	Not reliable (Klimisch code 3)
Comment on reliability:	Test was conducted according to accepted methods (Tice et al., 2000),

	but with major methodical and reporting deficiencies. No concurrent positive control and no solvent control was used. No historical positive and negative control data were provided. Culture conditions not reliable. The used culture medium requires a 5-10% CO_2-concentration to maintain physiological pH. No CO_2-concentration is reported. No information on pH and osmolality of the treatment medium is reported. No information if test substance concentrations affect the pH and osmolality of the culture medium is provided. The tested concentrations (i.e. 60 to 140 mM of Calypso) clearly exceeded the maximum concentration of 10 mM recommended for *in vitro* assays. The authors refer always to concentrations of Calypso. It is not clear if the stated concentrations refer to the active substance or the formulated product Calypso SC 480. In addition, the used concentrations are clearly above the actual plasma concentrations of 60 µM thiacloprid (unbound concentration = 45.5 µM) achieved in *in vivo* rat studies at the high dose of 1000 ppm in the diet (high dose of the 2-year rat study, clearly exceeding the MTD). No metabolic activation system was used. No individual culture data were reported.
Relevance of study	**Non relevant: Test system not sufficiently described and not adequate (see above). Furthermore, a commercial formulation was tested; this is non-relevant for the assessment of thiacloprid technical material. Regarding the investigated formulation, the study is non-relevant due to lack of reliability.**

CA 5.4.2 In vivo studies in somatic cells

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

Publication(s)

Report:	[redacted]; [redacted]; [redacted]; [redacted]; 2013; M-468127-01-1
Title:	Cytogenetic effects of commercial formulations of deltamethrin and/or thiacloprid on wistar rat bone marrow cells
Reference:	Environmental Toxicology (2013), 28(9), 524-531
Document No:	M-468127-01-1
Guidelines:	none Deviation(s): none
GLP:	no

I. Materials and methods

A. Materials

1. Test material:	Clypso OD 240; Decis 2.5 EC
Sorce:	[redacted] (Turkey)
Content of a.s.:	Clypso OD 240: 240 g/L thiacloprid Decis 2.5 EC: 25 g/L deltamethrin
Lot/Batch no:	not reported
Purity:	not reported
Stability of test compound:	not reported, but given in BCS marketed products during shelf life
2. Vehicle / positive control:	vehicle: corn oil positive control: cyclophosphamide (CPA) in sterile water
3. Test animals:	
Species:	rat
Strain:	Wistar albino
Age:	7-8 weeks
Sex:	male
Weight at dosing:	250 – 300 g
Source:	[redacted], Turkey
Acclimatisation period:	not reported
Diet:	pelleted diet, *ad libitum*
Water:	fresh tap water, *ad libitum*
Housing:	in polycarbonate cages with steel wire tops and rice husk bedding

B. Study design and methods

1. Animal assignment and treatment:

Dose:	single application: vehicle control: corn oil Thiacloprid alone: 112.5 mg/kg bw/day Deltamethrin alone: 15 mg/kg bw/day Thiacloprid / deltamethrin mixture: 112.5 / 15 mg/kg bw/day repeated applications (30 days): vehicle control: corn oil Thiacloprid alone: 22.5 mg/kg bw/day Deltamethrin alone: 3 mg/kg bw/day Thiacloprid / deltamethrin mixture: 22.5 / 3 mg/kg bw/day
Positive control:	cyclophosphamide 50 mg/kg bw, i.p.

Application route:	oral gavage except for positive control
Application volume:	2 mL/kg bw
Duration:	single application and repeated dosing for 30 days
Group size:	10 rats (6 for the chromosome aberration assay; 4 for the cytokinesis-block-micronucleus test)
Examinations:	none reported
Chromosome aberration assay (CA):	An aqueous solution of colchicine (2 mg/kg b.w.) was injected intraperitoneally 2 h prior to scheduled euthanasia by cervical dislocation. Both femurs were dissected and bone marrow was flushed from the femoral cavity with isotonic NaCl (0.9%) solution. The material was centrifuged at 500 x g for 10 minutes. The pellet was resuspended in 0.56% KCl and incubated at 37°C for 25 minutes. Cells were recentrifuged and fixed in chilled Carnoy's fixative (acetic acid: methanol, 1:3, v/v) three times. Fixed cells were resuspended and dropped onto chilled slides, air dried, and stained on the following day in 5% buffered Giemsa (pH 6.8).
Cytokinesis-block-micronucleus (CBMN):	Cytochalasin-B (Cyt-B) (3 mg/kg) was injected intraperitoneally 4 h before euthanasia by cervical dislocation to arrest cytokinesis and obtain binucleated (BN) cells. Both femurs were dissected and bone marrow was flushed from the femoral cavity with isotonic NaCl (0.9%) solution. The material was centrifuged at 500 x g for 10 minutes, the supernatant was discarded and the cell pellets were mixed by gentle agitation. The cells were fixed chilled with Carnoy's fixative (acetic acid: methanol, 1:3, v/v). Before the preparation of slides, the fixed material was again centrifuged and resuspended in a small volume of fixative by gentle flushing. Fixed cells were dropped onto chilled slides and air dried, and finally stained on the following day in 5% buffered Giemsa (pH 6.8) for 15 minutes.

2. Evaluation:

Chromosome aberration (CA) assay:	The mitotic index (MI) was determined by scoring 2000 metaphase cells per animal for a total of 12000 cells for each group. Mitotic index, total chromosome aberrations and abnormal metaphases with chromosome aberrations were calculated for each animal. Structural and numerical chromosomal aberrations (CA) were scored in 100 metaphases per animal (a total of 600 metaphases for each group). The scoring and classification of aberrations were also done as described by Preston et al. (1987). Gaps were not considered CA.
Cytokinesis-block micronucleus (CBMN) test:	The criteria used for binucleate (BN) cells and micronucleus (MN) evaluation were those suggested by Titenko-Holland et al. (1997). For the BN cells analysis, the number of BN cells in 2000 bone marrow cells was scored per animal for a total of 8000 cells for each group. To determine MN formation, 2000 BN cells with well preserved cytoplasm were scored for each animal (a total of 8000 BN cells for each group).

II. Results and discussion

A. Chromosome aberration (CA) assay

The major two types of aberrations observed for the thiacloprid-deltamethrin mixture group were breaks and fragments. Aberrant cells with multiple CAs were also observed frequently. The mitotic index (MI) decreased significantly ($p < 0.001$) for all treatment periods and doses of thiacloprid and thiacloprid-deltamethrin mixtures, as compared with their vehicle controls. The results of CA analysis showed that all independent and combined treatments of thiacloprid and/ or delamethrin significantly induced chromosome aberrations compared with their vehicle controls ($p < 0.001$). The frequencies of chromosome aberrations and aberrant cells obtained after 24 h exposure to higher doses of thiacloprid and/or deltamethrin were higher than for the 30 day exposure with lower doses. Furthermore, the frequencies of chromosome aberrations and aberrant cells in combined treatments of thiacloprid and deltamethrin for both treatment times were higher than the independent treatments. The lowest MI value and the highest chromosome aberration frequency were observed in combined treatment of the very high single doses of thiacloprid and deltamethrin for 24 h. Moreover, irrespective of the exposure period of 24 h or 30 days, the total of chromatid and chromosome-type breaks was the predominant type of chromosome aberrations in all treatment groups.

The positive control caused a significant decrease in MI, and a significant increase in chromosome aberrations and frequency of aberrant cells.

The results are summarised in the following tables.

Table 5.4.2/02-1: Summary of the chromosome aberration (CA) assay

Compound and dose [mg/kg bw/day]	MI, mean± SE [%]	Total CA [number]	CA, mean ± SE [%]	% cells with CA, mean ± SE [%]
Single exposure (24 hours)				

Compound and dose [mg/kg bw/day]	MI, mean± SE [%]	Total CA [number]	CA, mean ± SE [%]	% cells with CA, mean ± SE [%]
Control (corn oil)	4.40 ± 0.06	4	0.66 ± 0.21	0.66 ± 0.21
CPA 50	1.70 ± 0.06**	125	20.83 ± 0.94**	18.33 ± 0.88**
TCP 112.5	3.80 ± 0.07**	73	12.16 ± 1.13**	10.66 ± 0.66**
DEL 15	3.53 ± 0.06**	75	12.50 ± 0.76**	11.33 ± 0.33**
DEL + TCP 15 + 112.5	1.95± 0.06**	98	16.33 ± 1.14**	15.16 ± 0.79**
Repeated exposure (30 days)				
Control (corn oil)	4.70 ± 0.04	5	0.83 ± 0.16	0.83 ± 0.16
TCP 22.5	3.20 ± 0.05**	70	11.66 ± 0.76**	10.00 ± 0.57**
DEL 3	3.92 ± 0.03**	54	9.00 ± 0.89**	8.00 ± 0.57**
DEL + TCP 3 + 22.5	2.93 ± 0.05**	90	15.00 ± 1.50**	14.16 ± 1.24**

CPA: cyclophosphamide (positive control) TCP: thiacloprid DEL: deltamethrin
CA: chromosome aberration MI: mitotic index
*: statistically significant different from vehicle control, $p < 0.05$
**: statistically significant different from vehicle control, $p < 0.001$

Table 5.4.2/02-2: Types of observed chromosome aberrations

Compound and dose [mg/kg bw/day]	Number of chromosome aberration P	SCU	B´	B´´	F	CF	E
Single exposure (24 hours)							
Control (corn oil)	--	1	1	1	1	--	--
CPA 50	10	19	22	24	35	7	8
TCP 112.5	17	11	13	10	13	4	5
DEL 15	14	13	13	11	17	4	3
DEL + TCP 15 + 112.5	6	22	18	16	23	7	6
Repeated exposure (30 days)							
Control (corn oil)	1	--	2	1	--	--	1
TCP 22.5	11	13	10	15	15	3	3
DEL 3	3	9	9	10	11	8	4
DEL + TCP 3 + 22.5	8	18	16	18	19	6	5

CPA: cyclophosphamide (positive control) TCP: thiacloprid DEL: deltamethrin
P: polyploidy SCU: sister chromatid unions
B´: chromatid breaks B´´: chromosome breaks
F: fragments CF: centric fusions E: end to ends

B. Cytokinesis-block-micronucleus assay (CBMN)

All thiacloprid and/or deltamethrin treatments decreased the frequency of binucleated (BN) cells significantly ($p < 0.05$ or $p < 0.001$).

The combined treatment of thiacloprid and deltamethrin increased a significant ($p < 0.05$) frequency of micronucleated binucleated (MNBN) cells for both treatment times. The highest frequency of MNBN cells was also observed in the combined treatment of thiacloprid and deltamethrin for 24 h. When

thiacloprid and deltamethrin were administered separately, a significant ($p < 0.05$) increase in MNBN cells was only observed in the thiacloprid treatment for 30 days.

Table 5.4.2/02-3: Summary of the cytokinesis-block micronucleus (CBMN) assay

Compound and dose [mg/kg bw/day]		BN cells %± SE	MNBN cells %± SE
Single exposure (24 hours)			
Control (corn oil)		2.87 ± 0.05	0.02 ± 0.01
CPA	50	0.97 ± 0.09**	0.30 ± 0.03**
TCP	112.5	2.47 ± 0.07*	0.07 ± 0.01*
DEL	15	2.17 ± 0.08**	0.10 ± 0.02
DEL + TCP	15 + 112.5	1.23 ± 0.08*	0.16 ± 0.02*
Repeated exposure (30 days)			
Control (corn oil)		2.97 ± 0.07	0.02 ± 0.01
TCP	22.5	2.02 ± 0.07**	0.11 ± 0.01*
DEL	3	2.61 ± 0.08*	0.06 ± 0.01
DEL + TCP	3 + 22.5	1.73 ± 0.09**	0.12 ± 0.01*

CPA: cyclophosphamide (positive control) TCP: thiacloprid DEL: deltamethrin
BN: binucleated MNBN: micronucleated binucleate
*: statistically significant different from vehicle control, $p < 0.05$
**: statistically significant different from vehicle control, $p < 0.001$

III. Conclusions

The authors state that under the experimental conditions reported commercial formulations of thiacloprid and deltamethrin showed increases of chromosome aberrations and micronucleus formation. The treatment with combinations of the thiacloprid and deltamethrin formulations increased the cytotoxicity and genotoxicity as compared to treatment with individual formulations.

BCS conclusion: This non-GLP study is considered to be not reliable. The deficiencies are described in the reliability assessment below). The fact that two compounds, which were negative for genotoxicity in all regulatory guideline studies, both appear to be positive for genotoxicity under the experimental conditions of this study, raises even more doubts about the reliability of the data.

The reliability assessment is given below.

Klimisch evaluation

Reliability of study:	**Not reliable (Klimisch code 3)**
Comment on the CA assay:	Non GLP study. Experiment was performed in general accordance with OECD 475 (2014) but with the following major deviations / reporting deficiencies: For the repeated administration for 30 days no positive control was used. Regarding single and repeated exposure only one

	dose level was used instead of three. Signs of toxicity were not assessed or reported. Sampling was done only once after 24 h exposure. No individual animal data were presented and GAPs* were not assessed. In addition, criteria for positive and negative results were not provided. The evaluation based solely on statistical analyses of group mean data. No historical positive and negative control data were provided.
Comment to the CBMN assay:	Non-GLP study. There exists no validated guideline or protocol for the *in vivo* CBMN assay. Available protocols or guidelines are either for the *in vitro* or the *ex vivo /in vitro* test. It is stated that this was the first time that the *in vivo* CBMN was conducted in the laboratory. No validation data (historical positive / negative controls) were provided. In the following further deficiencies are summarised: • The group sizes used for the bone marrow MN assay were too small. In the available guidelines it is recommended to use at least 5 animals/group. • At least three dose levels of each compound should have been evaluated. • In the OECD guidelines for *in vivo* evaluation of micronucleus formation polychromatic erythrocytes (i.e. anucleate cells) in the bone marrow (taken from the femurs) are evaluated following acute exposure. Following sub-acute exposure the micronucleated population is evaluated in normochromatic erythrocytes in blood samples. This was not the case in this paper and was without justification. • The use of cytochalasin B to block nucleated cells seems to be strange as this technique is generally used for *in vitro* evaluation of the MN formation. As the authors state that this is the first time that the cytokinesis block method has been used in the *in vivo* bone marrow MN assay, it would be useful to see validation data with positive and negative controls. In addition, no justification for performing such a technique is provided. The bone marrow micronucleus assay has been developed so that only anucleate cells are scored for MN, i.e. those, which have already gone through several cell divisions. • The use of Giemsa to stain the bone marrow samples is not an appropriate stain to use in the rat bone marrow MN assay. Rat bone marrow contains fragile mast cells, which can burst upon manipulation of the bone marrow sample and release basophilic granules. These granules are difficult to distinguish from micronuclei due to their shape and the fact that they also stain blue with Giemsa. Consequently the use of DNA specific dyes have been recommended by the American (1991),

	Canadian (1989) and Japanese (1987; 1990) authorities to differentiate MN from mast cell granules when using the rat model. In this context it is questionable, whether in Fig 2 I (in the publication) the MN highlighted in the image on the right-hand side is truly a micronucleus or a granule. It is not perfectly round and there appears to be other "granules" over the top of the nuclei in the image. Unfortunately the image is not very good to be sure.
Relevance of study	**Non-relevant: test method insufficiently validated (*in vivo* CBMN assay). Test method not sufficiently described. Test system does not follow relevant guidelines completely (CA assay). Furthermore, a commercial formulation was tested; this is non-relevant for the assessment of thiacloprid technical material. The study is also non-relevant for the tested formulation due to lack of reliability.**

* GAP = an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

CA 5.4.3 In vivo studies in germ cells

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

CA 5.5 Long-term toxicity and carcinogenicity

Summary of long-term studies

Long-term studies have been conducted in the rat and mouse.

In rats, body weight effects were observed in both sexes with more pronounced effects in females. The main target was the liver. Hepatic enzyme induction was evident in males at dose levels > 50 ppm and in females at dose levels > 500 ppm. Liver changes were observed which were probably caused by the chronic induction of hepatic phase I and II enzymes. These liver changes included increased weight, hepatocellular hypertrophy, altered hepatocellular foci and cytoplasmic changes in the hepatocytes. NOAELs of 25 ppm (1.6 mg/kg bw/day) and 50 ppm (3.3 mg/kg bw/day) were determined for enzyme induction in males and females, respectively.

Thyroid changes were observed and included hypertrophy and hyperplasia of the follicular epithelium, colloid alteration and follicular cell adenoma. These changes were also considered to be a consequence of the liver enzyme induction. It has been proposed that the increased enzyme activities enhance the capacity of the liver to deactivate and excrete the circulating thyroid hormones. Alterations of the hormone levels trigger a compensatory increase in TSH, which induces the morphological changes in the thyroid gland. At 1000 ppm (69.1 / 51.7 mg/kg bw/day (males /

females)), ***re-evaluation of the data with the appropriate historical controls revealed a trend for an increase in TSH in high dose females in weeks 26 and 105 (statistically significant and pronounced increases in comparison to concurrent controls, but values still in the 2-s range of historical controls; therefore considered as a trend for an increase and not as an adverse effect).*** Although the expected decreases in circulating T3/T4 levels were not detected in this study, such decreases were seen in the short-term rat studies. It has been assumed that the expected decreases in T3/T4 levels are masked by the rapid compensatory reactions of the thyroid system.

There were increased incidences of uterine adenocarcinomas and ***reduced incidences of lacteal cysts and*** galactocele in the mammary glands, which were again considered to be secondary to the liver enzyme induction. ***Special mechanistic studies seemed to indicate an induction of aromatase resulting in increased estradiol levels and continuous stimulation of the uterine endometrium and, after 2 years, in an increased incidence of uterine adenocarcinomas in the old and acyclic rats. However, more recent investigations showed that the apparent increase in aromatase was an artefact caused by the unspecificty of the assay used at that time and that thiacloprid is not an aromatase inducer (see under 5.8.2, [redacted], 2009, M360757-02-1). More recent mode of action work on uterine adenocarcinoma in female rats is presented and discussed in chapters 5.8.2. and 5.8.3 as well as in the summary of mammalian toxicology.***
An additional histopathological investigation of the uteri of females of all dose groups after 1 year of treatment revealed a slightly increased incidence of slight to moderate uterine glandular hyperplasia after 500 and 1000 ppm. This lesion is a spontaneous finding. While it occurred as a reactive change in some animals up to and including 500 ppm due to endometritis, or stromal polyp or both conditions, it could be treatment related in 2 or 4 out of 10 animals after 500 and 1000 ppm.
There were increased incidences of retinal atrophy (females), lens degeneration/opacity (females), radiculoneuropathy (females), sciatic nerve degeneration (both sexes) and skeletal muscle atrophy (females). These age-associated findings were mainly seen at the top dose level or were sex specific but are consistent with the neurotoxic mode of action of the test material.

In mice, there were effects on male body weight and food intake. Leukocyte counts were increased in males and in females at some sampling points. Liver effects were seen in males and females and included increased weight, hypertrophy, fat storage, necrosis and degeneration. In females, increased adrenal weight was associated with hypertrophy and vacuolisation of the cortical X-zone in females. The liver effects and the concomitant hormonal changes may have caused these adrenal changes by affecting the development of the hormone-dependent X-zone. The incidence of eosinophilic luteinised cells in the ovarian stroma or the surrounding adipose tissue and ovarian luteomas were increased. The report considered these effects to be secondary to the known liver enzyme induction and the subsequent hormone imbalance.

Table 5.5-1: Summary of long-term studies

Study	Sex	NO(A)EL mg/kg bw/day	LO(A)EL mg/kg bw/day	Main findings seen at LO(A)EL	Reference
Rat 2-year, oral (diet) *0-25-50-500-1000 ppm*	M F	1.2 *(25 ppm)* 3.3 *(50 ppm)*	2.5 *(50 ppm)* 33.5 *(500 ppm)*	50 ppm (M), 500 ppm (F): liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy, cytoplasmatic changes, ↑ incidences of mixed eosinophilic/ clear cell foci, thyroid follicular cell hypertrophy, colloid alteration, ↑ incidences of thyroid follicular cell adenoma (M) ↑ incidences of uterine adenocarcinoma & ovarial cysts, 500 ppm (M&F): ↓ bw, slightly ↓ food consumption, ↑ incidences of skeletal muscle atrophy (F)	[redacted] & [redacted], 1998 M-003817-02-1
additional uterus histo-pathology	***F***	***3.3*** *(50 ppm)*	***33.5*** *(500 ppm)*	***slight increase of uterine glandular hyperplasia after 1 year of treatment***	[redacted]***, 2007*** ***M-003817-02-1 (amendment)***
Mouse 2-year, oral (diet) *0-30-1250-2500 ppm*	M F	5.7 10. *(30 ppm)*	234.1 475.3 *(1250 ppm)*	↑ liver weight, hepatocellular hypertrophy, fat storage (M) & degeneration (M), ↑ adrenal weights, hypertrophy & vacuolisation of the adrenal cortical X-zone (F) ↑ incidence of eosinophilic, luteinized cells in the ovarian stroma / surrounding adipose tissue & luteomas (F)	[redacted] & [redacted], 1998 M-003819-02-1

M: male F: female ↓: decrease(d) ↑: increase(d)

Report: [redacted]d; [redacted]; [redacted]; [redacted]; [redacted]; 2007; M-003817-02-1
Title: YRC 2894 - Combined chronic toxicity/carcinogenicity study in Wistar rats (dietary administration over 2 years)
Report No.: 27480
Document No.: M-003817-02-1
Guidelines: OECD 453; Directive 67/548/EEC; US-EPA (FIFRA) series 83.5; JMAFF Guidance on Toxicology Study Data for Application of Agricultural Chemical Registration; US EPA OPPTS 870.4300; The determination of TSH was not according to GLP. This deviation does not limit the assessment of the results.
GLP: yes

Report: KCA 5.5/03 , [redacted], U., 1998;M-003817-02; Amended: 2007-03-02
Title: YRC 2894 – Combined chronic toxicity / carcinogenicity study in Wistar rats – dietary administration over 2 years
Amendment I
Report No.: 27480A
Document No.: M-003817-02-1
Guidelines: No applicable guideline
Deviation(s): not applicable

GLP: yes

I. Materials and methods

A. Study design and methods

This report is an amendment to the chronic and cancerogenicity feeding study in rats over 2 years, interim sacrifice after 1 year (doses: 0, 25, 50, 500, 1000 ppm), ([redacted] & [redacted], 1998; M-003817-02-1; report no. 27480; DAR B.6.5.1)

The reason for this amendment was an increased incidence of adenocarcinomas in the 2-year cancerogenicity study at 500 ppm and above. Therefore the uterus was investigated throughout all groups (previously only controls, high dose animals and gross pathological findings). This was done in order to obtain a comprehensive general view of the functional state of the uterus mucosa after one year of treatment.

The results of the presented additional histopathological investigations of uteri in all groups do not change the toxicological interpretation of report No. 27480.

II. Results and discussion

A. Gross pathological findings in the uterus

There was no evidence of any gross finding in the uterus related to dosing with thiacloprid.

Table 5.5/03-1: Gross pathological uterus findings

Thiacloprid dose (ppm) Uterus finding	0	25	50	500	1000
Dilation(s)	1	0	1	3	2
Change in contents	0	0	0	1	0
Fluid	0	0	0	2	0
Nodule(s)	0	0	1	2	0

B. Histopathological findings in the uterus

Endometritis or endometrial polyp occurred unrelated to dosing with the test compound and are frequent spontaneous findings in rats of this age. Other findings observed sporadically among the animals were fibrosis, stromal hyperplasia of the cervix, squamous metaplasia in an inflamed uterus, uterine dilation and increased mucification of the cervix. In one female dosed at 50 ppm, a granular cell tumour was seen in the adnexa of the uterine cervix which is considered as a spontaneous finding.

Glandular hyperplasia of the uterine mucosa was encountered in one control female and in some females of the treatment groups. The grade was generally minimal or slight except in one female dosed at 1000 ppm and in one female at 500 ppm, in which the finding was moderate. The incidence of this finding was 1 - 0 - 2 - 4 - 4 suggesting an increase in the 500 and 1000 ppm dose groups. However, glandular hyperplasia occurred as a reactive change in some animals up to and including 500 ppm due to

- endometritis in control female no. 342 and 500 ppm female no. 501,
- stromal polyp in 50 ppm female no. 438
- or both conditions in female no. 521 of the 500 ppm group.

Table 5.5/03-2: Histopathological uterus findings

Thiacloprid dose (ppm) **Uterus finding** *Number of animals*	**0** *10*	**25** *10*	**50** *10*	**500** *10*	**1000** *10*
Endometritis	1	0	0	3	0
Stromal polyp	0	0	1	1	0
Glandular hyperplasia of the mucosa	1	0	2	4	4
Glandular hyperplasia of the mucosa: non-reactive changes*	0	0	1	2	4

*: not secondary to endometritis and / or a stromal polyp; possibly due to thiacloprid related hormonal imbalance

III. Conclusion

The additional histopathological evaluation revealed a slight increase of uterine glandular hyperplasia in animals of the higher dose levels (500 and 1000 ppm). This lesion is considered to be a spontaneous finding that develops during the physiological involution process and can occur as a reactive change to other conditions of the uterus.
However, a slight treatment-related effect due to hormonal imbalance is also a possible reason for this finding.

CA 5.6 Reproductive toxicity

Summary of reproductive and developmental toxicity studies

In a rat two generation study, decreased food consumption and bodyweight gain were seen at the top dose level of 600 ppm. Clinical signs of toxicity were noted in dams at 300 and 600 ppm, the incidence of dystocia was also increased at these dose levels. Litter size and pup survival were significantly decreased at the top dose level, pup growth was significantly decreased at 300 ppm and 600 ppm in both generations. Increased thyroid, liver and gonad weights were seen in adults of both generations at ≥ 300 ppm. Histological correlates of hepatocyte and thyroid follicular hypertrophy were also reported.

In the rat developmental study, decreased bodyweights and food consumption were noted at the top dose level of 50 mg/kg bw/day. Effects on urine and faecal production were also seen. Forelimb malformations (bone dysplasia) were also seen in the presence of marked maternal toxicity and the incidence was within the historical control range. Post-implantation loss was increased in this group as a result of late resorption. The incidences of placental border necrosis and foetal renal pelvic dilatation were increased in treated groups, however values were within or close to the historical control ranges. Numerous skeletal findings indicative of delayed or reduced ossification were noted at the top dose level.

In the rabbit developmental study, increased abortion, decreased food consumption and bodyweight gain were seen at 10 mg/kg bw/day. Foetal skeletal effects indicative of reduced or delayed ossification were noted in the top dose group. A marginal increase in the incidence of supernumerary 13th ribs was also noted in this group. An increase in the number of foetal malformations in this group is largely attributable to the incidence of forelimb arthrogryposis. This effect is a common spontaneous malformation ***(nowadays termed "malposition of forelimb(s)" - ventral flexure in the region of the wrist)***[3] in this strain of rabbit, the incidence is within the historical control range and there is no clear dose-response relationship.

Although forelimb malformations were seen in the rat and rabbit, these findings are not directly comparable. Both the bone dysplasia and arthrogryposis are common spontaneous findings and the incidences of these effects are within the relevant historical control data.

[3] ***Malposition of forelimb(s): This finding is the most common spontaneous malformation in the strain of rabbits used and some companies regard this finding as variation only. It is most likely the consequence of restriction of fetal movements in the uterus, which results in a caudal flexure of the forelimb(s) in the region of the wrist (carpal joint). Except from the flexion of the limb, there are no further morphological changes involved in this joint region, otherwise it would not be regarded as a "common finding". Furthermore, arthrogryposis in rabbits is considered to be reversible after birth, since such a finding was never observed in the young by the breeder. Therefore, this finding in rabbits is completely different from the dysplasia of limb bones seen in the rat, where the bones are changed morphologically.***

Table 5.6-1: Summary of reproductive and developmental toxicity studies

Study *Doses tested*	Sex	NO(A)EL (mg/kg bw/d)	LO(A)EL	Main effects seen at LOAEL	Reference
Rat 1-generation dietary (dose range finder) *0-100-400-1600 ppm*	M F	100 ppm	400 ppm	Liver: hepatocellular hypertrophy (1/7 P females) Thyroid: elongated follicular cells (1/7 P males)	[redacted], [redacted], [redacted], 1995 M-000911-01-1
Rat 2-generation dietary *0-50-300-600 ppm*	M F	2.6/2.7 (M/F) *(50 ppm)*	21/26 (M/F) *(300 ppm)*	Reproductive: dystochia (4/30 P females) Parental: ↑ liver weight, hepatocyto-megaly, necrosis (F with dystocia only), ↑ thyroid weight, thyroid follicular cell hypertrophy Offspring: ↓ pup weights	[redacted] & [redacted], 1997 M-001304-01-1
Rat oral (gavage) developmental *0-2-10-50 mg/kg bw/day*	Dam Fetal	10 10	50 50	Maternal: markedly ↓ food & water consumption, ↓ feces, transient bw loss during the first treatment days, later: ↑ water consumption & urine excretion, overall: ↓ bw gain Fetal: ↑ incidences of late & total resorptions, ↓ number of viable fetuses, ↓ fetal weight, ↑ incidences of extremital bone dysplasia, one fetus with multiple malformations, ↑ incidences of skeletal retardations (impaired ossification) & variations (wavy ribs, asymmetrical sternebrae)	[redacted], 1997 M-000832-01-1
Rat oral (gavage) developmental, historical control data	Fetal	10	50	Fetal: fetal & litter incidences of dysplasia of limb bones in the 50 mg/kg group lay within the historical control range of the used rat strain	[redacted], 2000 M-031344-01-1
Rabbit oral (gavage) developmental *0-2-10-45 mg/kg bw/day*	Dam Fetal	2 2	10 10	Maternal: ↓ food consumption & bw loss during the first treatment week, ↓ bw gain, Fetal: marginally ↓ fetal weights	[redacted], 1996 M-000780-01-1

M: male F: female ↓: decrease(d) ↑: increase(d)
D: dam
bw: body weight

CA 5.6.1 Generational studies

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of thiacloprid. (M-000911-01-1; M-001304-01-1)

CA 5.6.2 Developmental toxicity studies

All necessary studies were presented and evaluated during the EU process for Annex I listing. Please refer to the Monograph and the baseline dossier of thiacloprid. (M-000832-01-1; M-031344-01-1; M-000780-01-1)

CA 5.7 Neurotoxicity studies

Summary of neurotoxicity studies

As thiacloprid does not belong to the class of the organophosphates and has a different mode of action, i.e. action at the nicotinic receptor, testing for delayed neurotoxicity was not necessary. However, thiacloprid was tested in acute and sub-chronic neurotoxicity studies, as well as in a developmental neurotoxicity study.

Administration of single oral doses of thiacloprid to rats by gavage produced only transient clinical signs of toxicity. The overt signs included tremors, decreased activity, ataxia, repetitive chewing movements, dilated pupils, eyelid ptosis, and clear lacrimation, oral and nasal staining and reduced body temperature. Brain weight was not affected by treatment. Histopathology did not reveal any lesions in the nervous system, eyes or skeletal muscle. The only treatment-related effects in the 13 week feeding study were reduced body weight and food consumption. The large differences between the NOELs determined for neurotoxicity in the acute and short-term feeding study may be due to bolus dosing or possibly adaptation.
For the registration of thiacloprid in the United States, a developmental neurotoxicity study was conducted. The study was already submitted for Annex I inclusion, but not discussed in the Monograph. In this study dietary exposure to thiacloprid did not cause any neurotoxic effects in parental as well as offspring animals. Treatment-related findings consisted of reduced maternal body weights and body weight gain during gestation and lactation, as well as reduced food consumption during gestation in the mid and high dose. Body weights as well as body weight gain were also reduced in mid- and high dose F1 males and females and absolute food consumption was also reduced in mid and high dose F1 males. Relative food consumption was increased in mid and high dose F1 rats of both sexes due to the reduced body weights. Terminal body weights were also decreased in mid and high dose males and high dose females of the F1 generation. F1 offspring of the mid- and high-dose groups exhibited also a delay in development (preputial separation in mid and high dose males and vaginal patency in high dose females) which is considered to be secondary to body weight changes.

A publication on histopathological alterations in chicken after oral subacute treatment with thiacloprid was reviewed for its possible relevance for delayed neurotoxicity. However, the focus of the study was on general toxicity in hens and investigations regarding delayed neurotoxicity were not included. Therefore, the publication is not relevant for the endpoint delayed neurotoxicity.

Table 5.7-1: Summary of neurotoxicity studies on the active substance

Study *Doses tested*	NO(A)EL (mg/kg bw/d)	LO(A)EL (mg/kg bw/d)	Main effects seen at LOAEL	Reference
Rat acute oral neurotoxicity *0-20-50-100 mg/kg bw/d*	< 20	20	Eyelid ptosis, slight tremors (M, FOB), impaired aerial righting response (F, FOB), ↓ motor- and locomotor activity (F, figure – eight maze)	[redacted], [redacted] & [redacted], 1997 M-000894-03-1
Rat acute oral neurotoxicity *0-3.1-11 mg/kg bw/d*	11 (M) 3.1 (F)	> 11 11	↓ motor- and locomotor activity (F only, figure-eight maze)	[redacted], 1998 M-000894-03-1
Rat, 13-week, diet, neuro-toxicity *0-50-400-1600 ppm*	101 (M) 115 (F) *(1600 ppm in M/F)*	> 101 (M) > 115 (F) *(> 1600 ppm in M/F)*	↓ body weight & food consumption, no neurotoxic effects	[redacted] & [redacted], 1997 M-003815-01-1
Rat, developmental neurotoxicity *0-50-300-500 ppm*	4.4 (Mat & Dev) *(50 ppm)* Neurotox: 40.8 *(500 ppm)*	25.6 (Mat & Dev) *(300 ppm)* Neurotox: > 40.8 *(> 500 ppm)*	Maternal: ↓ body weight/body weight gain & food consumption Developmental offspring: ↓ body weight/ body weight gain (M/F), ↓ absolute food consumption (M), ↑ relative feed consumption (M/F), delayed sexual maturation (preputial separation), ↓ terminal body weight (DP 12 M)	[redacted], 2001 M-088059-01-1

M: male F: female ↓: decrease ↑: increase
D: dam Mat: maternal Dev: developmental
gest: gestation lact: lactation

CA 5.7.1 Neurotoxicity studies in rodents

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

CA 5.7.2 Delayed polyneuropathy studies

Not necessary, since thiacloprid is no organophosphate.

However, since chicken is the species used for delayed neurotoxicity studies, the following publication on histopathological alterations in chicken after oral subacute treatment with thiacloprid was reviewed for its relevance concerning delayed neurotoxicity.

Publication(s)

Report:	[redacted]6; [redacted]; [redacted]; [redacted]; 2010; M-437662-01-1
Title:	Histopathological alterations induced after oral sub-acute thiacloprid toxicity in *Gallus domesticus*
Reference:	Veterinarski Arhiv 80 (5), 673-682, 2010
Document No.:	M-437662-01-1
Guidelines:	None Deviation(s): not applicable
GLP:	no

I. Materials and methods

A. Materials

1. Test material: Alanto 240 SC

Description:	not reported
Lot/Batch no:	not reported
Purity:	21.7%
Stability of test compound:	not reported, but should be given, since the formulation is a marketed product of thiacloprid

2. Vehicle and positive controls: vehicle: distilled water

3. Test animals:

Species:	Gallus domesticus
Strain:	not reported
Age:	1 ½ years
Sex:	females
Weight at dosing:	not reported
Source:	not reported
Acclimatisation period:	10 days
Diet:	standard feed, *ad libitum*; mineral mixture, Vimeral, coccidiostat amprolium hydrochloride and anti-stress vitamins were provided to the hens before start of treatment
Water:	water, *ad libitum;*
Housing:	in pens at the layer house of a poultry farm

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0-10 mg/kg bw/day thiacloprid
Application route:	oral gavage
Duration:	7, 14, 21 and 28 days
Group size:	control groups (I, II, III): 4 thiacloprid groups (IV, V, VI, VII): 6

Application volume:	not exactly reported, administration with a 2 mL syringe
Termination:	control groups I, II, III: days 0, 14 and 28, respectively thiacloprid groups IV, V, VI, VII: days 7, 14, 21, 28, respectively
Observations:	gross pathology, histopathology (liver, heart, kidney, brain, lung, intestine and ovaries)

II. Results and discussion

A. Gross pathological findings

Repeated oral administration of 10 mg/kg/day thiacloprid for 28 consecutive days in *Gallus domesticus* resulted in significant changes in the gross morphology of liver, lungs and intestine but no alterations in the kidneys, brain, heart and ovaries. The severity of liver findings depended on the number of treatment days.

B. Histopathological findings

Histopathologically significant alterations in the liver were observed, such as mild fatty changes, congestion and degeneration of hepatocytes. Alterations in the histoarchitecture of the kidneys included marked congestion, tubular cell degeneration and sloughing of epithelial cells. The cerebral hemisphere revealed changes comprising of mild neuronal degeneration with surrounding glial cells, satellitosis and vacuolation. Mild congestion and haemorrhage was observed in the lungs and myocardial tissues following oral administration of thiacloprid. No adverse effect on the ovarian histoarchitecture and thus the reproductive performance of *Gallus domesticus* was seen.

III. Conclusion

The authors concluded that thiacloprid is of moderate risk in Gallus domesticus.

BCS conclusion: With regard to delayed neurotoxicity in hens this oral subacute study revealed only supplemental information because the focus of the study was general toxicity and not neurotoxicity. There was no assessment of neurotoxic signs (motor activity, etc) and no details on the grade of a possible neuronal degeneration were provided (no histopathological investigation of the spinal cord or of peripheral nerves for axonal degeneration and demyelination were conducted). The results do not change existing endpoints.

The reliability evaluation is given below.

Klimisch evaluation

Reliability of study:	Reliable with restrictions (Klimisch code 2)
Comment on reliability:	- only one dose tested - no signs of toxicity were reported - no assessment of neurotoxic signs (motor activity, etc), no details on the grade of neuronal degeneration were provided (no histopathological investigation of the spinal cord or of

	peripheral nerves for axonal degeneration and demyelination).
Relevance of study:	**Not relevant for the endpoint delayed neurotoxicity, since the investigations needed for the assessment of delayed neurotoxicity were not investigated in this study.**

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

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

Summary of studies with metabolites

During the previous EU review, the toxicological properties of several plant and/or soil/groundwater metabolites (YRC 2894-amide (M02), YRC 2894-sulfonic acid (M30), and YRC2894-sulfonic acid amide (M34)) had already been evaluated based on studies on acute oral toxicity in rats, genotoxicity and liver enzyme induction in rats.

In addition, new studies on 6-chloronicotinic acid (M03), YRC 2894-sulfonic acid (M30), sulfonic acid amide (M34) and thiacloprid-thiadiazine (Z5) on acute oral toxicity, genotoxicity, steroidogenesis in vitro or liver enzyme induction in rats are now available or were conducted, respectively. With regard to the in vitro steroidogenesis assays on the metabolites thiacloprid was tested in parallel again in order to be able to compare the results obtained with the metabolites with those of the parent compound.
The results of all available studies on the above mentioned metabolites are provided in the following paragraphs.

Summary of studies with thiacloprid-amide (M02)

For the plant metabolite thiacloprid-amide (M02), which is also a postulated intermediate in rat metabolism, an acute oral toxicity study in rat and an Ames test have been conducted.
The acute oral toxicity study revealed an LD_{50} > 2000 mg/kg bw showing that M02 is of less acute toxicity than thiacloprid. There was no indication for point mutations in the bacterial reverse mutation test.

Table 5.8.1-1: Summary of studies with thiacloprid-amide (M02 / KKO 2254)

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral (fasted)	500-2000 mg/kg bw/day	LD_{50} > 2000 mg/kg bw	[redacted], 1995 M-000765-01-1
Bacterial reverse mutation assay (*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102)	16-5000 µg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted], 1995 M-000733-01-1

bw: body weight

Summary of studies with YRC 2894-sulfonic acid / sulfonic acid Na-salt (M30)

The groundwater metabolite YRC 2894-sulfonic acid, which is also a postulated intermediate in rat metabolism, has been characterized toxicologically in an acute oral toxicity study in rat, a set of 3 *in vitro* genotoxicity assays and a 7-day dietary study on liver enzyme induction in rats. ***In addition, a new in vitro test on steroidogenesis has been conducted.***

The metabolite has an acute oral LD_{50} > 2000 mg/kg bw and is therefore less acutely toxic than thiacloprid. YRC 2894-sulfonic acid did not induce mutations in bacteria and mammalian cells and displayed no clastogenic potential in mammalian cells *in vitro*. Thus, YRC 2894-sulfonic acid is considered to be non-genotoxic.

Furthermore, YRC 2894-sulfonic acid did not induce liver enzymes in female rats after seven days of dietary exposure with 1000 ppm. **An *in vitro* H295R steroidogenesis assay did not give any indication for an effect on estradiol and testosterone secretion and, hence, on steroidogenesis.**

Table 5.8.1-2: Summary of studies with YRC 2894-sulfonic acid / sulfonic acid Na-salt (M30)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral (fasted)	2000 mg/kg bw/day	LD_{50} > 2000 mg/kg bw	[redacted], 1996 M-000811-01-1
Bacterial reverse mutation assay (*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102)	10-5000 µg/plate (+/- S9 mix)	Negative (+/- S9 mix)	[redacted], 1995 M-000777-01-1
Mammalian cell gene mutation test (Chinese hamster V79 cells)	12.5-3200 µg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted], 2003 M-110485-01-1
Mammalian chromosome aberration test (Chinese hamster V79 cells)	800-1600-3200 µg/mL (+/- S9 mix)	Negative (+/- S9 mix)	[redacted], 2003 M-110494-01-1
Rat, 7-day oral (diet), assessment of liver enzyme induction	1000 ppm	no liver enzyme induction	[redacted], 2003 M-103210-01-1
In vitro H295R steroidogenesis assay	***10^{-4} - 10^{-10} µM***	***no effect on steroidogenesis***	[redacted], ***2014*** ***M-490179-01-1***

*: New studies, i.e. studies that were not previously submitted, are written in bold and italic
bw: body weight

Summary of studies with YRC 2894-sulfonic acid amide (M34)

The toxicological properties of the groundwater metabolite YRC 2894-sulfonic acid amide have been investigated in an acute oral toxicity study, a set of 3 *in vitro* tests on genotoxicity and a 7-day dietary study in rats on liver enzyme induction. ***In addition, a new in vitro steroidogenesis assay has been conducted.***

With a LD_{50} > 2000 mg/kg bw YRC 2894-sulfonic acid amide is of less acute toxicity than the parent compound thiacloprid.

In a set of genotoxicity tests M34 did not induce point mutations in bacteria and mammalian cells. Furthermore, there was no evidence of a clastogenic potential in mammalian cells *in vitro*. Thus, YRC 2894-sulfonic acid amide (M34) is considered to be non-genotoxic.

Furthermore, YRC 2894-sulfonic acid amide showed no liver enzyme induction after dietary exposure with 1000 ppm for 7 days in female Wistar rats. ***An in vitro H295R steroidogenesis assay did not give any indication for an effect on steroidogenesis.***

Table 5.8.1-3: Summary of studies with YRC 2894-sulfonic acid amide (M34)*

Study	Dose levels / concentrations tested	Result	Reference
Rat Acute oral	2000 mg/kg bw/day	LD_{50} > 2000 mg/kg bw	[redacted], 2003 M-110389-01-1
Bacterial reverse mutation assay (*S. typhimurium* TA1535, TA100, TA1537, TA98, TA102)	16 - 5000 µg/plate (+/- S9 mix)	negative (+/- S9 mix)	[redacted], 2003 M-110534-01-1
Mammalian cell gene mutation test (Chinese hamster V79 cells)	125 - 4000 µg/mL (+/- S9 mix)	negative (+/- S9 mix)	[redacted], 2003 M-110532-01-1
Mammalian chromosome aberration test (Chinese hamster V79 cells)	250-500-2000-4000 µg/mL (+/- S9 mix)	negative (+/- S9 mix)	[redacted], 2003 M-110518-01-1
Rat, 7-day oral (diet), assessment of liver ezyme induction	1000 ppm	no liver enzyme induction	[redacted], 2003 M-103210-01-1
In vitro H295R steroidogenesis assay	***10^{-5} - 10^{-10} µM***	***no effects on steroidogenesis***	[redacted], ***2014*** ***M-490176-01-1***

*: New studies, i.e. studies that were not previously submitted, are written in bold and italic
bw: body weight

Summary of studies with 6-chloronicotinic acid (M03)

An acute oral toxicity study and an Ames test are available on the rat, plant and soil metabolite 6-chloronicotinic acid.

An acute oral toxicity study in rats with an LD_{50} > 5000 mg/kg bw revealed that 6-chloronicotininc acid has a lower acute oral toxicity than the parent compound thiacloprid. Furthermore, 6-chloronicotininc acid was negative for point mutations in the bacterial reverse mutation test.

Table 5.8.1-4: Summary of studies with 6-chloronicotinic acid*

Study	Dose levels / concentrations tested	Result	Reference
Rat ***Acute oral***	***2000, 5000 mg/kg bw***	***LD_{50} > 5000 mg/kg bw***	[redacted] ***&*** [redacted]***, 1997*** ***M-195930-01-1***
Bacterial reverse mutation assay ***S. typhimurium TA1535, TA100, TA1537, TA98*** ***E. coli WP2 uvrA***	***313 - 5000 µg/plate*** ***(+/- S9 mix)***	***negative*** ***(+/- S9 mix)***	[redacted] ***&*** [redacted]***, 1997*** ***M-195932-01-1***

*: New studies, i.e. studies that were not previously submitted, are written in bold and italic
bw: body weight

Summary of studies with thiacloprid-thiadiazine (Z5)

The toxicological properties of the groundwater metabolite thiacloprid-thiadiazine were assessed in an acute oral toxicity study, three in vitro genotoxicity studies as well as in an in vitro steroidogenesis assay and a study on liver enzyme induction in male rats.
Thiacloprid-thiadiazine has a low acute oral toxicity with a LD_{50} > 2000 mg/kg bw. No mortalities or any signs of toxicity were observed in this study.
There was no evidence for point mutations in the bacterial reverse mutation assay as well as in the mammalian cell gene mutation test. The micronucleus test in human lymphocytes gave no indication for a clastogenic potential. Thus, thiacloprid-thiadiazine is considered to be non-mutagenic or non-clastogenic, respectively.
Thiacloprid-thiadiazine also has no effect on steroidogenesis in the in vitro H295R steroidogenesis assay and does not lead to liver enzyme induction after dietary administration of 1000 ppm for 7 days in male rats.

Table 5.8.1-5: Summary of studies with thiacloprid-thiadiazine*

Study	Dose levels	Result	Reference
Rat Acute oral (up-and-down method)	***175-550-2000 mg/kg bw/day***	***LD_{50} > 2000 mg/kg bw***	***[redacted], 2014 M-485201-01-1***
Bacterial reverse mutation assay (S. typhimurium TA1535, TA100, TA1537, TA98, TA102)	***3-5000 µg/plate (plate incorporation) 33-5000 µg/plate (pre-incubation) (+/- S9 mix)***	***negative (+/- S9 mix)***	***[redacted], 2014 M-478073-01-1***
Mammalian cell gene mutation test (HPRT / Chinese hamster V79 cells)	***175-2800 µg/mL (+/- S9 mix)***	***negative (+/- S9 mix)***	***[redacted], 2014 M-484705-01-1***
In vitro micronucleus test (human lymphocytes)	***294.3-2760 µg/mL (+ S9 mix) 901.2-2760 µg/mL (- S9 mix)***	***negative (+/- S9 mix)***	***[redacted], 2014 M-486183-01-1***
7-day dietary study on liver enzyme induction	***1000 ppm in the diet (87.4 mg/kg bw/day)***	***no liver enzyme induction***	***[redacted], 2014 M-495981-01-1***
In vitro H295R steroidogenesis assay	***10^{-4} - 10^{-10} µM***	***no effects on steroidogenesis***	***[redacted], 2014 M-490181-01-1***

*: New studies, i.e. studies that were not previously submitted, are written in bold and italics
bw: body weight

Additional H295R steroidogenesis assay with thiacloprid

For the evaluation of possible effects of the metabolites on steroidogenesis in comparison with the parent compound, an additional H295R steroidogenesis assay was conducted in parallel with thiacloprid.

Thiacloprid caused a slight, but statistically significant treatment-related reduction in both testosterone and estradiol secretion in the H295R steroidogenesis assay at the highest concentration tested (10^{-4} M, equivalent to 100 µM).

Table 5.8.1-6: Summary of studies with thiacloprid parent compound*

Study	Dose levels	Result	Reference
In vitro H295R steroidogenesis assay	***10^{-4} - 10^{-10} µM***	***Slight statistically significant reduction of testosterone and estradiol secretion at the highest concentration (10^{-4} M) tested.***	[redacted], ***2014 M-490186-01-1***

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

YRC 2894-sulfonic acid Na-salt

Report:	KCA 5.8.1/14[redacted]; 2014; M-490179-01-1
Title:	Evaluation of YRC 2894-sulfonic acid Na-salt in the H295R steroidogenesis assay
Report No.:	SA 13332
Document No.	M-490179-01-1
Guidelines:	US-EPA, OPPTS Series 890, Endocrine Disruptor Screening Program test guidelines, No. 890.1550: Steroidogenesis (Human Cell Line – H295R) (October 2009) Deviation(s): none
GLP:	yes

I. Materials and methods

A. Materials

1. Test material: YRC2894-sulfonic acid Na-salt

Description:	white solid
Lot/Batch no:	GSB 61235-2-2
Purity:	94.6%
Stability of test compound:	guaranteed for study duration; expiry date: 2015-02-25

2. Vehicle and positive controls: vehicle: 0.1% dimethylsulfoxid (DMSO)
positive controls:
Forskolin – for sex steroid hormone biosynthesis stimulation
Prochloraz – for sex steroid hormone biosynthesis inhibition

3. Test organism / cells:

Species:	human
Cell line	adrenal carcinoma immortal cell line H295R

Source: [redacted] (Batch No. 58660579)

4. Culture maintenance:

Medium: DMEM:F12, supplemented with ITS+ premix and 2.5% Nu-Serum I and 0.1 % Penicillin-Streptomycin

Conditions: 37°C ± 1°C and 5% CO_2

B. Study design and methods

1. Test conditions:

Cell isolation: H295R cells isolated from flasks of ≥ 75 % confluency

Medium: DMEM: F12, supplemented with ITS + premix and 2.5 % Nu-serum I and 0.1 % Penicillin-Streptomycin

Test substance concentrations: YRC2894-sulfonic acid Na-salt: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M;
100 µM and 250 µM for cytotoxicity assessment
Forskolin: 0.03, 0.1, 0.3, 1, 3, 10 µM;
Prochloraz: 0.01, 0.03, 0.1, 0,3, 1, 3 µM

Cell density: H295R cells seeded into 24-well plates at a density of 0.3×10^6 cells/mL and a final volume of 1 mL/well and cultured for approx. 24 h prior treatment

Group size: 3 wells per compound/vehicle control, concentration and treatment period

Incubation time: 48 h

Incubation conditions: 37 ± 1°C and 5% CO_2

2. Sample collection and analysis:

Sampling: four aliquots of culture medium / well

Analyses: using specific enzyme-immunoassay kits (Enzo Life Science Inc., USA), method detection limits: 5.67 pg/mL for testosterone, 14 pg/mL for estradiol

II. Results and discussion

A. Interference evaluation

YRC2894-sulfonic acid Na-salt did not interfere with the EIA kit for testosterone or estradiol at any concentration evaluated.

B. Cytotoxicity

Cytotoxicity was not observed following treatment of the H295R cells at 250 µM YRC2894-sulfonic acid Na-salt when evaluated using the XTT method. In addition, staining of the cells with trypan blue at the end of the evaluation demonstrated the viability of the cells following treatment with 10^{-4} M to 10^{-10} M YRC 2894-sulfonic acid Na-salt.

C. Hormone evaluations

Testosterone concentrations

The variability (CV) between the runs for the solvent controls was slightly outside the guideline recommendation (~32% compared to the recommended guideline of 30%).

YRC2894-sulfonic acid Na-salt was considered not to interfere with testosterone secretion in the H295R steroidogenesis assay as no concentration-related effects could be established in any of the three evaluations of the test item. The slight changes recorded for testosterone secretion ranged from –21.5% at 10^{-8} M in the first evaluation to +8.6% at 10^{-4} M in the third evaluation. These changes were considered to be within the normal variability of the assay.

Estradiol concentrations

The variability (CV) between the runs for the solvent controls was within the guideline recommendation (~14 % compared to the recommended guideline of 30%).

YRC2894-sulfonic acid amide was considered not to interfere with estradiol secretion in the H295R steroidogenesis assay. The slight changes recorded for estradiol secretion ranged from –23.3% at 10^{-8} M to +12.5% at 10^{-4} M in the same evaluation. These changes were considered to be within the normal variability of the assay.

Table 5.8.1/14-1: Mean hormone concentrations, standard deviation and % change in comparison to controls after incubation of H295R cells with YRC2894-sulfonic acid Na-salt for 24 h (mean of three evaluations)

YRC2894-sulfonic acid Na-salt conc.	Testosterone (pg/mL)			Estradiol (pg/mL)		
	Mean	SD	% change	Mean	SD	% change
DMSO	8880.9	2826.06	--	201.2	28.61	--
10^{-10} M	7921.1	1999.44	-10.8%	171.3	25.18	-14.9%
10^{-9} M	8413.9	2420.90	-5.3%	190.1	24.82	-5.5%
10^{-8} M	7518.3	1867.02	-15.3%	170.8	25.88	-15.1%
10^{-7} M	8699.7	2185.93	-2.0%	195.9	19.93	-2.6%
10^{-6} M	8559.0	2218.32	-3.6%	204.0	18.84	+1.4%
10^{-5} M	9040.4	2471.33	+1.8%	189.9	26.36	-5.6%
10^{-4} M	9429.6	2796.10	+6.2%	214.2	22.65	+6.5%

Statistical evaluation conducted on overall data only. Data have been rounded up.
conc.: concentration
SD: standard deviation

Table 5.8.1/14-2: Mean fold change and standard deviation of hormone concentrations relative to DMSO controls after incubation of H295R cells with YRC2894-sulfonic acid Na-salt for 24 h (mean of three evaluations)

YRC2894-sulfonic acid Na-salt concentration	Testosterone		Estradiol	
	Mean fold change	SD	Mean fold change	SD
10^{-10} M	0.89	0.23	0.85	0.13
10^{-9} M	0.95	0.27	0.94	0.12
10^{-8} M	0.85	0.21	0.85	0.13
10^{-7} M	0.98	0.25	0.97	0.10
10^{-6} M	0.96	0.25	1.01	0.09
10^{-5} M	1.02	0.28	0.94	0.13
10^{-4} M	1.06	0.31	1.06	0.11

SD: standard deviation
Data have been rounded up.

Positive controls

The comparison of the data generated in the concurrent positive control study (M-490174-01-1) with the guideline criteria are given in the table below. These data indicate that, with the exception of the testosterone increases induced by forskolin in the first and fourth evaluations, which were < 2-times the solvent control, all other criteria were met. It should be borne in mind that steroidogenesis in the H295R assay is dynamic and the limited increase in testosterone secretion following forskolin treatment could, therefore, be a reflection of the marked increase in estradiol secretion (~23- to ~44-fold increase compared to 7.5-fold increase proposed in the guideline).

Table 5.8.1/14-3: Mean hormone concentrations and standard deviation of concurrent positive controls (mean of four evaluations)

		Testosterone		Estradiol	
		Mean	SD	Mean	SD
Minimum Basal Production (pg/mL) (Testosterone: 500 pg/mL; Estradiol: 40 pg/mL)	1st evaluation				
	2nd evaluation				
	3rd evaluation				
	4th evaluation				
	Overall				

		Testosterone	Estradiol
Basal Production (Testosterone :≥ 5-times MDL; Estradiol: ≥ 2.5-times MDL)	1st evaluation	2133-times MDL	19-times MDL
	2nd evaluation	1343-times MDL	18-times MDL
	3rd evaluation	1218-times MDL	21-times MDL
	4thevaluation	1595-times MDL	30-times MDL
	Overall	1572-times MDL	22-times MDL
Induction (10 µM Forskolin) (Testosterone: ≥ 2-times SC; Estradiol: ≥ 7.5-times SC)	1st evaluation	1.8-times SC	41.4-times SC
	2nd evaluation	2.1-times SC	44.3-times SC
	3rd evaluation	2.0-times SC	28.1-times SC
	4thevaluation	1.9-times SC	23.4-times SC
	Overall	1.9-times SC	32.9-times SC
Inhibition (1 µM Prochloraz) (Testosterone: ≤ 0.5-times SC; Estradiol: ≤ 0.5-times SC)	1st evaluation	0.04-times SC	Complete inhibition
	2nd evaluation	0.05-times SC	Complete inhibition
	3rd evaluation	0.05-times SC	Complete inhibition
	4thevaluation	0.06-times SC	Complete inhibition
	Overall	0.05-times SC	Complete inhibition

MDL: minimum detection limit (5.67 pg/mL for testosterone, 14 pg/mL for estradiol)
SC: solvent control
Note: Four positive control evaluations were conducted to cover the assessment of several test items. Evaluations 1 - 3 were conducted concurrently with the three evaluations of YRC2894-sulfonic acid Na-salt.

III. Conclusion

Overall, YRC2894-sulfonic acid Na-salt, tested at concentrations between 10^{-10} M and 10^{-4} M, was considered not to interfere with testosterone or estradiol secretion in the H295R steroidogenesis assay.

YRC 2894-sulfonic acid amide

Report: [redacted], 2014; M-490176-01-1
Title: Evaluation of YRC 2894-sulfonic acid amide in the H295R steroidogenesis assay
Report No.: SA 13331
Document No.: M-490176-01-1
Guidelines: US-EPA, OPPTS Series 890, Endocrine Disruptor Screening Program test guidelines, No. 890.1550: Steroidogenesis (Human Cell Line – H295R) (October 2009)
Deviation(s): none
GLP: yes

I. Materials and methods

A. Materials

1. Test material: YRC2894-sulfonic acid amide
Description: white solid

Lot/Batch no:	SES 12253-5-4
Purity:	97.7%
Stability of test compound:	guaranteed for study duration; expiry date: 2014-04-11
2. Vehicle and positive controls:	vehicle: 0.1% dimethylsulfoxid (DMSO) positive controls: Forskolin – for sex steroid hormone biosynthesis stimulation Prochloraz – for sex steroid hormone biosynthesis inhibition
3. Test organism / cells:	
Species:	human
Cell line	adrenal carcinoma immortal cell line H295R
Source:	[redacted] (Batch No. 58660579)
4. Culture maintenance:	
Medium:	DMEM:F12, supplemented with ITS+ premix and 2.5% Nu-Serum I and 0.1 % Penicillin-Streptomycin
Conditions:	37°C ± 1°C and 5% CO_2

B. Study design and methods

1. Test conditions:	
Cell isolation:	H295R cells isolated from flasks of ≥ 75 % confluency
Medium:	DMEM: F12, supplemented with ITS + premix and 2.5 % Nu-serum I and 0.1 % Penicillin-Streptomycin
Test substance concentrations:	YRC2894-sulfonic acid amide: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M (equivalent to 100 - 0.0001 µM) 100 µM and 250 µM for cytotoxicity assessment Forskolin: 0.03, 0.1, 0.3, 1, 3, 10 µM; Prochloraz: 0.01, 0.03, 0.1, 0,3, 1, 3 µM
Cell density:	H295R cells seeded into 24-well plates at a density of 0.3×10^6 cells/mL and a final volume of 1 mL/well and cultured for approx. 24 h prior treatment
Group size:	3 wells per compound/vehicle control, concentration and treatment period
Incubation time:	48 h
Incubation conditions:	37°C ± 1°C and 5% CO_2
2. Sample collection and analysis:	
Sampling:	four aliquots of culture medium / well
Analyses:	using specific enzyme-immunoassay kits (Enzo Life Science Inc., USA)., method detection limits: 5.67 pg/mL for testosterone, 14 pg/mL for estradiol

Replicates: 3 per dose level

II. Results and discussion

A. Interference evaluation

YRC2894-sulfonic acid amide did not interfere with the EIA kit for testosterone or estradiol at any concentration evaluated.

B. Cytotoxicity

Cytotoxicity was not observed following treatment of the H295R cells at 100 and 250 µM YRC2894-sulfonic acid amide when evaluated using the XTT method. In addition, staining of the cells with trypan blue at the end of the evaluation demonstrated the viability of the cells following treatment with 10^{-4} M to 10^{-10} M YRC 2894-sulfonic acid amide.

C. Hormone evaluations

<u>Testosterone concentrations</u>

The variability (CV) between the runs for the solvent controls was within the guideline recommendation (~22% compared to the recommended guideline of 30%).

The effect of YRC2894-sulfonic acid on testosterone secretion ranged between +6.6% at 10^{-10} M in the first evaluation and -18.2% at 10^{-7} M in the second evaluation. In the absence of any concentration-related responses (either for each individual evaluation or overall), YRC2894-sulfonic acid amide was considered not to interfere with testosterone secretion in the H295R steroidogenesis assay.

<u>Estradiol concentrations</u>

The variability (CV) between the runs for the solvent controls was within the guideline recommendation (~5.8 % compared to the recommended guideline of 30%).

No concentration-related effects could be established for YRC2894-sulfonic acid amide in any of the three evaluations. The marginal though statistically significant reduction in estradiol secretion at the two lowest concentrations tested (10^{-10} M and 10^{-9} M) were considered non-relevant due to the variability observed among the three evaluations for each concentration (between 0.98 and 0.75-fold for 10^{-10} M and between 0.96 and 0.82-fold for 10^{-9} M). YRC2894-sulfonic acid amide was therefore considered not to interfere with estradiol secretion in the H295R steroidogenesis assay.

Table 5.8.1/15-1: Mean hormone concentrations, standard deviation and % change in comparison to controls after incubation of H295R cells with YRC2894-sulfonic acid amide for 24 h (mean of three evaluations)

YRC2894-sulfonic acid amide conc.	Testosterone (pg/mL)			Estradiol (pg/mL)		
	Mean	SD	% change	Mean	SD	% change
DMSO	9173.1	2029.28	--	228.3	13.35	--
10^{-10} M	8963.4	2754.66	-2.3%	202.7*	21.86*	-11.2%
10^{-9} M	8350.0	2261.35	-9.0%	200.1*	23.69*	-12.4%
10^{-8} M	7989.8	1732.03	-12.9%	212.7	15.54	-6.8%
10^{-7} M	7810.1	1738.18	-14.9%	210.3	13.67	-7.9%
10^{-6} M	8270.6	1845.21	-9.8%	209.2	24.32	-8.4%
10^{-5} M	8576.4	2031.23	-6.5%	228.0	19.51	nc
10^{-4} M	8181.8	1864.94	-10.8%	216.4	23.22	-5.2%

Statistical evaluation conducted on overall data only. Data have been rounded up.
conc.: concentration
SD: standard deviation
*: significantly diferent from controls; $p \leq 0.05$
nc: no change compared to controls

Table 5.8.1/15-2: Mean fold change and standard deviation of hormone concentrations relative to DMSO controls after incubation of H295R cells with YRC2894-sulfonic acid amide for 24 h (mean of three evaluations)

YRC2894-sulfonic acid amide concentration	Testosterone		Estradiol	
	Mean fold change	SD	Mean fold change	SD
10^{-10} M	0.98	0.30	0.89	0.10
10^{-9} M	0.91	0.25	0.88	0.10
10^{-8} M	0.87	0.19	0.93	0.07
10^{-7} M	0.85	0.19	0.92	0.06
10^{-6} M	0.90	0.20	0.92	0.11
10^{-5} M	0.93	0.22	1.00	0.09
10^{-4} M	0.89	0.20	0.95	0.10

SD: standard deviation
Data have been rounded up.

Positive controls

The comparison of the data generated in the concurrent positive control study (M-490174-01-1) with the guideline criteria are given in the table below. These data indicate that, with the exception of the testosterone increases induced by forskolin in the first and fourth evaluations, which were 2-times the solvent control, all other criteria were met. It should be borne in mind that steroidogenesis in the H295R assay is dynamic and the limited increase in testosterone secretion following forskolin treatment could, therefore, be a reflection of the marked increase in estradiol secretion (~23- to ~44-fold increase compared to 7.5-fold increase proposed in the guideline).

Table 5.8.1/15-3: Mean hormone concentrations and standard deviation of concurrent positive controls (mean of four evaluations)

		Testosterone		Estradiol	
		Mean	SD	Mean	SD
Minimum Basal Production (pg/mL) (Testosterone: 500 pg/mL; Estradiol: 40 pg/mL)	1st evaluation	12093	1224.5	272	22.4
	2nd evaluation	7616	458.1	250	13.8
	3rd evaluation	6905	398.7	298	16.4
	4thevaluation	9041	891.2	421	29.8
	Overall	8914	2170.8	310	70.4
		Testosterone		Estradiol	
Basal Production (Testosterone :≥ 5-times MDL; Estradiol: ≥ 2.5-times MDL)	1st evaluation	2133-times MDL		19-times MDL	
	2nd evaluation	1343-times MDL		18-times MDL	
	3rd evaluation	1218-times MDL		21-times MDL	
	4thevaluation	1595-times MDL		30-times MDL	
	Overall	1372-times MDL		22-times MDL	
Induction (10 µM Forskolin) (Testosterone: ≥ 2-times SC; Estradiol: ≥ 7.5-times SC)	1st evaluation	1.8-times SC		41.4-times SC	
	2nd evaluation	2.1-times SC		44.3-times SC	
	3rd evaluation	2.0-times SC		28.1-times SC	
	4thevaluation	1.9-times SC		23.4-times SC	
	Overall	1.9-times SC		32.9-times SC	
Inhibition (1 µM Prochloraz) (Testosterone: ≤ 0.5-times SC; Estradiol: ≤ 0.5-times SC)	1st evaluation	0.04-times SC		Complete inhibition	
	2nd evaluation	0.05-times SC		Complete inhibition	
	3rd evaluation	0.05-times SC		Complete inhibition	
	4thevaluation	0.06-times SC		Complete inhibition	
	Overall	0.05-times SC		Complete inhibition	

MDL: minimum detection limit (5.67 pg/mL for testosterone, 14 pg/mL for estradiol)
SC: solvent control
Note: Four positive control evaluations were conducted to cover the assessment of several test items. Evaluations 1 - 3 were conducted concurrently with the three evaluations of YRC2894-sulfonic acid amide.

III. Conclusion

Overall, YRC2894-sulfonic acide amide, tested at concentrations between 10^{-10} M and 10^{-4} M, was considered not to interfere with testosterone or estradiol secretion in the H295R steroidogenesis assay.

6-Chloronicotinic acid

Report:	[redacted]ö; [redacted]; [redacted]; 1997; M-195930-01-1
Title:	IC-0- Acute oral toxicity study in rats
Report No:	C007439
Document No:	M-195930-01-1
Guidelines:	OECD 401; US-EPA Guideline 81-1; JMAFF Guideline 59 NohSan No. 4200 Deviations: none
GLP:	yes

I. Materials and methods

A. Materials

1. Test material:	6-chloronicotinic acid
Synonym:	IC-0
Description:	Crystal appearance
Lot/Batch no:	Batch No. 5
Purity:	99.4% (analysis: 1993-08-26), 98.4% (analysis: 1994-10-21)
Stability of test compound:	guaranteed for study duration; expiry date: 1995-11-21
2. Vehicle:	Tween 80 / ion exchange water (IC-0 was ground into a powder by a mortar and was suspended in Tween 80 - ion exchange water.)
3. Test animals:	
Species:	rat
Strain:	Crj:CD (SD), SPF
Age:	7 weeks
Sex:	males and females
Weight at dosing:	males: 203.2 – 223.7 g females: 144.1 g – 154.1 g
Source:	[redacted].
Acclimatisation period:	7 days
Diet:	pelleted diet "MF" (Oriental Yeat Co., Ltd.), *ad libitum*
Water:	tap water, *ad libitum*
Housing:	5 animals per cage in stainless steel mesh cages for rats (D 40.0 x W 23.0 x H 18.5 cm)

B. Study design and methods

1. Animal assignment and treatment:	
Dose:	2000-5000 mg/kg bw
Application route:	Oral (gavage)
Application volume:	10 mL/kg bw
Fasting time:	before administration: overnight after administration: 3 hours
Group size:	5 / sex / dose
Post-treatment observation period:	14 days
Observations:	mortality, clinical signs, body weight, gross necropsy

II. Results and discussion

A. Mortality

Table 5.8.1/16-1: Doses, mortality / animals treated

Dose (mg/kg bw)	Toxicological result*			Occurrence of signs	Time of death	Mortality (%)
Male rats						
2000	0	0	5	--	--	0
5000	0	0	5	--		0
Female rats						
2000	0	0	5		--	0
5000	0	0	5	--	--	0
LD_{50}: > 5000 mg/kg bw						

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

6-Chloronicotinic acid did not cause mortality at dose levels of 2000 or 5000 mg/kg bw in both sexes.

B. Clinical observations

There were no clinical signs observed in any animal at any dose level.

C. Body weight

No unusual changes in body weight were observed in 2000 mg/kg in males. Body weights in 5000 mg/kg males decreased on day 1 to day 2 after the administration, and recovered from day 3. Body weights in 2000 mg/kg females decreased on day 1 after administration. Body weights in 5000 mg/kg females decreased on days 1 to 3 after administration and recovered thereafter.

D. Necropsy

There were no macroscopic observations at the dose levels of 2000 or 5000 mg/kg bw at necropsy.

III. Conclusion

6-Chloronicotinic acid is non-toxic after acute oral administration with an LD_{50} above 5000 mg/kg bw in male and female rats.

Report: [redacted]t; [redacted]; [redacted]; 1997; M-195932-01-1
Title: IC-0 - Reverse mutation study on bacteria
Report No: C007440
Document No: M-195932-01-1
Guidelines: OECD 471 (1983); US-FIFRA guideline 84-2 8 (1984); JMAFF Guideline 59 NohSan No. 4200 (1995)
Deviations: none
GLP: yes

I. Materials and methods

A. Materials

1. Test material:	6-chloronicotinic acid
Synonym:	IC-0
Description:	crystal appearance
Lot/Batch no:	Batch Nr. 5
Purity:	99.4% (analysis: 1993-08-26), 98.4% (analysis: 1994-10-21)
Stability of test compound:	guaranteed for study duration; expiry date: 1995-11-21
2. Vehicle and/or positive control:	Vehicle: DMSO positive controls: N-ethyl-N´-nitro-nitrososguanidine (ENNG) 2-nitrofluorene (2-NF) 9-aminoacridine hydrochloride (9-AA) 2-aminoanthracene (2-AA)
3. Test system:	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100, *E. coli* WP2 uvrA
Metabolic activation:	S9 mix prepared from the liver of male Sprague Dawley rats treated with 5,6-benzoflavone and phenobarbital

B. Study design and methods

1. Treatment:	
Dose:	0-313-625-1250-2500-5000 µg/plate positive controls: ENNG: 2, 3, 5 µg/plate 2-NF: 0.2 µg/plate 9-AA: 80 µg/plate 2-AA: 0.5, 2, 10 µg/plate
Application volume:	0.1 mL/plate
Pre-incubation conditions:	20 min, 37°C
Incubation time:	not reported
Incubation temperature:	37°C
Replicates:	3 per concentration and strain, including controls

II. Results and discussion

First experiment:

There was no increase in the number of reverse mutant colonies observed in any strain with and without metabolic activation. In TA98 growth inhibition but no precipitation was observed at 5000 µg/plate without metabolic activation. Growth inhibition and precipitation was observed in all Salmonella strains at 5000 µg/plate with metabolic activation.

Second experiment:

There was no increase in the number of reverse mutant colonies observed in any strain with and without metabolic activation. In TA98 growth inhibition but no precipitation was observed at 5000 µg/plate without metabolic activation.

Table 5.8.1/17-1: Experiment I: Revertant counts (mean ±SD)

Dose (µg/plate)	S9 mix	*S. typhimurium* strain				*E. coli*
		TA 1535	TA 1537	TA 98	TA 100	WP2 *uvrA*
Vehicle control	–	10 ± 7.23	11 ± 2.52	24 ± 4.36	107 ± 2.65	19 ± 2.65
Test item 313	–	8 ± 2.65	14 ± 3.21	26 ± 2.31	116 ± 4.62	17 ± 3.61
625	–	6 ± 1.15	14 ± 5.03	26 ± 3.61	109 ± 9.29	22 ± 4.62
1250	–	8 ± 1.00	19 ± 4.58	25 ± 2.31	135 ± 4.91	20 ± 5.77
2500	–	8 ± 2.52	12 ± 4.04	30 ± 2.89	122 ± 11.37	24 ± 2.65
5000	–	4 ± 1.53	10 ± 3.51	10 ± 5.00*	134 ± 8.72	18 ± 3.21
ENNG 2	–					445 ± 37.87
ENNG 3	–				557 ± 25.01	
ENNG 5	–	496 ± 65.77				
2-NF 0.2	–			88 ± 4.58		
9-AA 80	–		548 ± 26.76			
Vehicle control	+	14 ± 0.58	20 ± 2.00	46 ± 6.66	127 ± 4.51	32 ± 12.06
Test item 313	+	10 ± 4.16	15 ± 2.65	47 ± 2.31	121 ± 7.00	36 ± 3.79
625	+	14 ± 2.00	14 ± 3.61	42 ± 1.53	123 ± 9.87	28 ± 6.43
1250	+	11 ± 5.51	13 ± 1.53	38 ± 2.00	129 ± 14.00	31 ± 11.27
2500	+	22 ± 5.86	20 ± 2.00	34 ± 7.00	133 ± 9.24	22 ± 4.73
5000	+	9 ± 2.00*#	10 ± 4.04*#	13 ± 5.51*#	90 ± 21.55*#	28 ± 10.44#
2-AA 0.5	+			154 ± 4.36		
2-AA 1	+				592 ± 23.39	
2-AA 2	+	184 ± 9.17	164 ± 8.39			
2-AA 10	+					663 ± 13.75

ENNG: N-ethyl-N´-nitro-N-nitrosoguanidine 2-AA: 2-aminoanthracene
9-AA: 9-aminoacridine hydrochloride 2-NF: 2-nitrofluorene
*: bacterial growth inhibition #: precipitation

Table 5.8.1/17-2: Experiment II: Revertant counts (mean ±SD)

Dose (µg/plate)	S9 mix	*S. typhimurium* strain				*E. coli*
		TA 1535	TA 1537	TA 98	TA 100	WP2 *uvrA*
Vehicle control	–	12 ± 3.06	14 ± 3.21	24 ± 4.04	127 ± 2.52	24 ± 2.65
Test item 313	–	9 ± 2.52	11 ± 2.00	27 ± 4.04	125 ± 16.04	22 ± 7.02
625	–	11 ± 2.31	10 ± 3.61	35 ± 2.00	132 ± 4.93	27 ± 6.35
1250	–	9 ± 6.08	13 ± 5.00	25 ± 1.15	138 ± 5.00	26 ± 6.11
2500	–	15 ± 3.51	14 ± 4.93	31 ± 8.72	134 ± 4.36	20 ± 4.04
5000	–	9 ± 2.52	12 ± 0.73	18 ± 7.21*	134 ± 8.72	23 ± 3.06
ENNG 2	–					436 ± 9.07
ENNG 3	–				657 ± 25.00	
ENNG 5	–	516 ± 24.99				
2-NF 0.2	–			91 ± 9.17		
9-AA 80	–		574 ± 40.04			
Vehicle control	+	15 ± 2.08	12 ± 4.62	35 ± 2.52	120 ± 6.08	27 ± 5.20
Test item 313	+	17 ± 4.51	19 ± 2.31	43 ± 8.62	124 ± 15.50	24 ± 1.73
625	+	16 ± 5.20	14 ± 3.79	45 ± 17.04	107 ± 6.43	21 ± 4.04
1250	+	11 ± 2.52	14 ± 4.16	39 ± 8.74	130 ± 9.61	28 ± 6.00
2500	+	15 ± 3.21	17 ± 2.52	39 ± 3.21	114 ± 3.79	23 ± 5.13
5000	+	22 ± 2.08*#	18 ± 4.04*#	18 ± 1.15*#	113 ± 11.02*#	28 ± 4.58#
2-AA 0.5	+			425 ± 14.57		
2-AA 1	+				652 ± 20.03	
2-AA 2	+	201 ± 17.67	148 ± 8.72			
2-AA 10	+					682 ± 15.04

ENNG: N-ethyl-N´-nitro-N-nitrosoguanidine 2-AA: 2-aminoanthracene
9-AA: 9-aminoacridine hydrochloride 2-NF: 2-nitrofluorene
*: bacterial growth inhibition #: precipitation

III. Conclusion

6-Chloronicotinic acid is non-mutagenic in this bacterial reverse mutation assay.

Thiacloprid-Thiadiazine

Report: [redacted]x; [redacted]; 2014; M-485201-01-1
Title: Thiacloprid-thiadiazine - Acute oral toxicity study in rat (Up and Down Procedure)
Report No: 13/357-001P
Document No: M-485201-01-1
Guidelines: OECD 425; Commission Regulation (EC) No 440/2008; B.1.TRIS; US-EPA 712-C-02-190 , OPPTS 870.1100;
Deviations: none
GLP: yes

I. Materials and methods

A. Materials

1. Test material:	thiacloprid-thiadiazine
Description:	light brown solid
Lot/Batch no:	SES 11969-11-8
Purity:	99.1%
Stability of test compound:	guaranteed for study duration; expiry date 2014-03-18
2. Vehicle:	0.5 % aqueous carboxymethyl cellulose
3. Test animals:	
Species:	rat
Strain:	Wistar rat, RccHan:(WIST)
Age:	10 - 12 weeks
Sex:	female
Weight at dosing:	191 g - 206 g
Source:	[redacted], Italy
Acclimatisation period:	at least 20 days
Diet:	[redacted] SM R/M "Autoclavable complete diet for rats and mice - breeding and maintenance" ([redacted], Germany) *ad libitum*
Water:	tap water, *ad libitum*
Housing:	individually in Type II polypropylene/polycarbonate cages; Lignocel bedding for laboratory animals and Cotton rolls (Supplement to Ani-Pet Bedding) were available.

B. Study design and methods

1. Animal assignment and treatment:	
Dose:	175-550-2000 mg/kg bw
Application route:	Oral (gavage)
Application volume:	10 mL/kg bw
Fasting time:	before administration: overnight after administration: 3 hours
Group size:	3 females
Post-treatment observation period:	14 days
Observations:	mortality, clinical signs, body weight, gross necropsy

II. Results and discussion

A. Mortality

Table 5.8.1/18-1: Doses, mortality / animals treated

Dose (mg/kg bw)	Toxicological result*			Occurrence of signs	Time of death	Mortality (%)
Female rats						
175	0	0	1	--		0
550	0	0	1	--	--	0
2000	0	0	3		--	0
LD_{50}: > 2000 mg/kg bw						

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

Thiacloprid-thiadiazine did not cause mortality after single oral administration of 175, 550 or 2000 mg/kg bw to rats.

B. Clinical observations

There were no clinical signs in any animal at any dose level.

C. Body weight

Body weight and body weight gain of thiacloprid-thiadiazine treated animals showed no indication of a treatment-related effect.

D. Necropsy

There were no macroscopic observations at the dose levels of 175, 550 or 2000 mg/kg bw at necropsy.

III. Conclusion

Thiacloprid-thiadiazine is non-toxic after acute oral administration to female rats with an LD_{50} value above 2000 mg/kg bw.

[redacted]; 2014; M-478073-01-1

Report:
Title: Thiacloprid-thiadiazine: *Salmonella typhimurium reverse* mutation assay
Report No: 1597201
Document No: M-478073-01-1
Guidelines: OECD 471 (1997); Commission Regulation (EC) No. 440/2008, Method B.13/14; US-EPA 712-C-98-247, OPPTS 870.5100 (1998);
Deviations: none
GLP: yes

I. Materials and methods

A. Materials

1. Test material: thiacloprid-thiadiazine
Synonym: BCS-CJ16425
Description: light brown solid
Lot/Batch no: SES 11969-11-8

Purity:	99.1%
Stability of test compound:	guaranteed for study duration; expiry date: 2014-03-18
2. Vehicle and/or positive control:	vehicle: dimethylsulfoxide (DMSO) for thiacloprid-thiadiazine and positive controls 4-NOPD and 2-AA deionised water for positive controls NaN3 and MMS positive controls: sodium azide (NaN_3) 4-nitro-o-phenylene-diamine (4-NOPD methyl methane sulfonate (MMS) 2-aminoanthracene (2-AA)
3. Test system:	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100, TA102
metabolic activation:	S9 mix, prepared from phenobarbital/ß-naphthoflavone induced rat liver at Harlan CCR, Lot. No.: 101013, protein concentration: 37.8 mg/mL

B. Study design and methods

1. Treatment:

Dose:	plate incorporation: 0-3-10-33-100-333-1000-2500-5000 µg/plate pre-incubation: 0-33-100-333-1000-2500-5000 µg/plate positive controls: NaN_3: 10 µg/plate 4-NOPD: 10, 50 µg/plate MMS: 2.0 µL/plate 2-AA: 2.5, 10 µg/plate
Application volume:	0.1 mL/plate
Pre-incubation conditions:	60 minutes, 37°C
Incubation time:	48 hours, 37°C
Replicates:	3 per concentration and strain, including controls

II. Results and discussion

No precipitation of the test item occurred up to the highest investigated dose. The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without metabolic activation in both independent experiments.

No toxic effects, evident as a reduction in the number of revertants (below the indication factor of 0.5) occurred in the test groups with and without metabolic activation.

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

In both experiments, the data in the negative control of strain TA 102 without S9 mix were slightly above the historical control range. Since this deviation is rather small, this effect is considered to be based upon biologically irrelevant fluctuations in the number of colonies.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.
In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, the test item did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used.

Table 5.8.1/19-1: Experiment I: Revertant counts (mean ±SD)

Dose (µg/plate)	S9 mix	*Salmonella typhimurium* strain				
		TA 1535	TA 1537	TA 98	TA 100	TA 102
Vehicle control	–	18 ± 6	9 ± 1	28 ± 1	94 ± 3	459 ± 7
Untreated	–	17 ± 3	8 ± 2	32 ± 10	106 ± 12	504 ± 46
Test item 3	–	18 ± 3	8 ± 2	23 ± 4	92 ± 1	514 ± 7
10	–	16 ± 6	8 ± 2	24 ± 2	99 ± 15	499 ± 14
33	–	19 ± 3	8 ± 1	28 ± 5	93 ± 14	518 ± 26
100	–	21 ± 7	8 ± 2	26 ± 2	96 ± 7	522 ± 42
333	–	16 ± 6	8 ± 2	27 ± 3	90 ± 5	507 ± 36
1000	–	14 ± 2	10 ± 2	30 ± 8	89 ± 7	496 ± 14
2500	–	13 ± 6	9 ± 4	25 ± 5	99 ± 5	467 ± 12
5000	–	14 ± 4	9 ± 4	22 ± 5	101 ± 11	496 ± 28
NaN_3 10	–	2854 ± 98			2148 ± 33	
4-NOPD 10	–			277 ± 20		
4-NOPD 50	–			762 ± 3		
MMS 2.0 µL	–					5244 ± 858
Vehicle control	+	15 ± 4	15 ± 6	41 ± 2	101 ± 4	585 ± 49
Untreated	+	12 ± 6	19 ± 3	34 ± 5	115 ± 6	575 ± 8
Test item 3	+	16 ± 4	19 ± 5	36 ± 5	94 ± 19	584 ± 43
10	+	14 ± 4	16 ± 4	40 ± 6	115 ± 10	575 ± 32
33	+	10 ± 2	17 ± 2	38 ± 8	97 ± 15	635 ± 31
100	+	21 ± 2	13 ± 4	33 ± 4	110 ± 5	591 ± 41
333	+	15 ± 4	14 ± 5	43 ± 8	101 ± 6	606 ± 25
1000	+	14 ± 3	17 ± 0	38 ± 12	107 ± 11	632 ± 34
2500	+	25 ± 2	15 ± 5	45 ± 3	117 ± 7	583 ± 13
5000	+	31 ± 10	12 ± 2	32 ± 3	128 ± 9	611 ± 58
2-AA 2.5	+	415 ± 22	344 ± 10	2842 ± 406	3555 ± 95	
2-AA 10	+					1981 ± 294

NaN_3: sodium azide
MMS: methyl methane sulfonate
2-AA: 2-aminoanthracene
4-NOPD: 4-nitro-o-phenylene-diamine

Table 5.8.1/19-2: Experiment II: Revertant counts (mean ±SD)

Dose (µg/plate)	S9 mix	*Salmonella typhimurium* strain				
		TA 1535	TA 1537	TA 98	TA 100	TA 102
Vehicle control	–	15 ± 3	9 ± 4	24 ± 5	87 ± 11	501 ± 24
Untreated	–	17 ± 3	8 ± 3	24 ± 5	99 ± 7	509 ± 2
Test item 33	–	15 ± 4	10 ± 0	19 ± 5	75 ± 4	522 ± 11
100	–	16 ± 1	10 ± 2	20 ± 5	84 ± 12	511 ± 16
333	–	16 ± 4	8 ± 2	25 ± 10	91 ± 10	542 ± 4
1000	–	17 ± 3	8 ± 1	21 ± 6	94 ± 17	515 ± 11
2500	–	17 ± 4	12 ± 5	15 ± 5	79 ± 9	475 ± 18
5000	–	14 ± 5	9 ± 3	22 ± 5	74 ± 12	536 ± 28
NaN_3 10	–	2836 ± 31			2219 ± 17	
4-NOPD 10	–			326 ± 14		
4-NOPD 50	–		66 ± 6			
MMS 2.0 µL	–					4723 ± 37
Vehicle control	+	14 ± 2	18 ± 2	36 ± 7	101 ± 8	558 ± 53
Untreated	+	15 ± 1	16 ± 3	37 ± 2	114 ± 20	584 ± 9
Test item 33	+	15 ± 2	14 ± 5	30 ± 7	87 ± 7	611 ± 30
100	+	18 ± 3	18 ± 2	40 ± 8	104 ± 8	580 ± 45
333	+	18 ± 4	19 ± 5	31 ± 9	95 ± 4	600 ± 103
1000	+	20 ± 7	23 ± 3	41 ± 7	91 ± 3	581 ± 45
2500	+	28 ± 7	17 ± 3	38 ± 3	98 ± 17	536 ± 28
5000	+	28 ± 3	19 ± 5	32 ± 7	106 ± 13	518 ± 17
2-AA 2.5	+	459 ± 6	321 ± 15	2524 ± 217	2867 ± 61	
2-AA 10	+					1433 ± 40

NaN_3: sodium azide 2-AA: 2-aminoanthracene
MMS: methyl methane sulfonate 4-NOPD: 4-nitro-o-phenylene-diamine

III. Conclusion

Thiacloprid-thiadiazine is non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

Report: [redacted]; 2014; M-484705-01-1
Title: Thiacloprid-thiadiazine - Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT)
Report No: 1597203
Document No: M-484705-01-1
Guidelines: OECD 476; Commission Regulation (EC) No. 440/2008, B.17; US-EPA 712-C-98-221, OPPTS 870.5300 (1998), JMAFF No 12 Nousan-8147 (2000)
Deviation(s): none
GLP: yes

I. Materials and methods

A. Materials

1. Test material:

Name:	thiacloprid-thiadiazine
Article no:	not specified
Description:	light brown solid
Lot/Batch no:	SES 11969-11-8
Purity:	99.1% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2014-09-11

2. Vehicle and or positive control: vehicle: dimethylsulfoxid (DMSO) for thiacloprid-thiadiazine and positive control DMBA
nutrient medium for positive control EMS

positive controls:
ethylmethane sulfonate (EMS)
7,12-dimethylbenz(a)anthracene (DMBA)

3. Test system: Chinese hamster V79 cells

Medium: MEM (minimal essential medium) containing Hank's salts, 10% foetal bovine serum (FBS) (except during 4 h treatment), neomycin (5 µg/mL) and amphotericin B (1 %). For the selection of mutant cells the complete medium was supplemented with 11 µg/mL 6-thioguanine.

Metabolic activation: mammalian microsomal fraction S9 mix prepared from livers of phenobarbital and ß-naphthoflavone induced rats at Harlan CCR, Lot No. 260913, protein concentration: 31.4 mg/mL.

B. Study design and methods

1. Treatment:

Dose:

Exposure period	S9 mix	Test item concentrations [µg/mL]
Experiment I		
4 h	−	87.5, **175.0**, **350.0**, **700.0**, **1400.0**[P], **2800.0** [P]
4 h	+	87.5, **175.0**, **350.0**, **700.0**, **1400.0** [P], **2800.0** [P]
Experiment II		
24 h	−	87.5, **175.0**, **350.0**, **700.0**, **1400.0**, **2800.0** [P]
4 h	+	87.5, **175.0**, **350.0**, **700.0**, **1400.0**, **2800.0** [P]

[P]: Precipitation visible to the unaided eye

Concentrations given in bold letters were chosen for the mutation rate analysis, all concentrations were used for toxicity assessment. The high concentration of 2800 µg/mL test item is equal to a molar concentration of approximately 10 mM.

For each test solution or control two parallel cultures were used.

Incubation conditions: 4 or 24 hours, at 37°C in a humidified atmosphere with 1.5% CO_2

2. Statistical analysis:

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

Exposure period	S9 mix	p-value Culture I	Culture II
Experiment I			
4 h	−	0.533	0.071
4 h	+	0.252	0.814
Experiment II			
24 h	−	0.478	0.003[s]
4 h	+	0.000[s]	0.198

[s]: significant trend

II. Results and discussion

Precipitation at the end of treatment was noted in experiment I at 1400 µg/mL and above with and without metabolic activation. In experiment II precipitation occurred at 2800 µg/mL with and without metabolic activation.

No relevant toxic effects indicated by a relative cloning efficiency I[4] or a relative cell density below 50% was noted up to the maximum concentration with and without metabolic activation.

No relevant and reproducible increase in mutant colony numbers/10^6 cells was observed in the main experiments up to the maximum concentration. The mutation frequency did not exceed the historical range of solvent controls.

The threshold of three times the mutation frequency of the corresponding solvent control was reached or exceeded in the second culture of the first experiment at 1400 µg/mL without metabolic activation and in the second experiment, culture I, at 2800 µg/mL with and 175 µg/mL without metabolic activation. However, these effects were based on relatively low solvent controls as the absolute mutation frequency remained well within the historical range of solvent controls. These increases were judged as biologically irrelevant artifacts as none was reproduced in the parallel cultures under identical conditions.

[4]: cloning efficiency I (survival, relative): (mean number of colonies per flask divided by the mean number of colonies per flask of the corresponding control) x 100

A linear regression analysis (least squares) was performed to assess a possible dose dependent increase of mutant frequencies. A significant dose dependent trend of the mutation frequency indicated by a probability value of <0.05 was determined in the second experiment in the first culture with and the second culture without metabolic activation. Again, the trends were not reproduced in the parallel cultures and consequently judged as irrelevant fluctuation.
In both experiments of this study (with and without S9 mix) the range of the solvent controls was from 2.4 up to 13.4 mutants per 10^6 cells; the range of the groups treated with the test item was from 3.2 up to 25.2 mutants per 10^6 cells. The solvent control of culture II of the second experiment with metabolic activation fell short of the lower limit of the historical range (2.4 versus 3.4 colonies per 10^6 cells). The data are acceptable however, as the solvent control of the parallel culture remained well within the historical range.

The positive controls EMS (150 µg/mL) and DMBA (1.1 µg/mL) showed a distinct increase in induced mutant colonies, and therefore demonstrated the validity of the test system.

The results of experiment I and II are summarised in the following tables.

Table 5.8.1/20-1: Summary of results: Experiment I

	Concentration [µg/mL]	S9 mix	Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Experiment I / 4 h treatment			**Culture I**		**Culture II**	
DMSO		–	10.7	1.0	8.3	1.0
EMS	150.0	–	90.0	8.4	90.2	10.8
Thiacloprid-thiadiazine	87.5	–	Culture was not continued[#]			
	175.0	–	10.0	0.9	7.0	0.8
	350.0	–	21.2	2.0	9.8	1.2
	700.0	–	8.6	0.8	8.7	1.0
	1400.0[P]	–	15.0	1.4	25.2	3.0
	2800.0[P]	–	7.6	0.7	20.1	2.4
DMSO		+	8.7	1.0	2.4	1.0
DMBA	1.1	+	281.2	32.4	109.7	46.3
Thiacloprid-thiadiazine	87.5	+	Culture was not continued[#]			
	175.0	+	7.7	0.9	5.0	2.1
	350.0	+	10.5	1.2	4.6	2.0
	700.0	+	8.6	1.0	4.1	1.7
	1400.0[P]	+	11.5	1.3	3.2	1.4
	2800.0[P]	+	4.2	0.5	3.7	1.5

[#]: Culture was not continued since a minimum of only four analysable concentrations is required
[P]: Precipitation

Table 5.8.1/20-2: Summary of results: Experiment II

	Concentration [µg/mL]	S9 mix	Mutant colonies per 10^6 cells	Induction factor	Mutant colonies per 10^6 cells	Induction factor
Experiment II / 24 h treatment			**Culture I**		**Culture II**	
DMSO		−	6.0	1.0	5.9	1.0
EMS	150.0	−	444.3	73.6	403.5	68.0
Thiacloprid-thiadiazine	87.5	−	Culture was not continued[#]			
	175.0	−	18.3	3.0	6.7	1.1
	350.0	−	8.5	1.4	5.0	0.8
	700.0	−	5.6	0.9	7.9	1.3
	1400.0		4.8	0.8	9.1	1.5
	2800.0[P]	−	6.6	1.1	12.7	2.1
Experiment II / 4 h treatment			**Culture I**		**Culture II**	
DMSO		+	6.0	1.0	6.4	1.0
DMBA	1.1	+	337.3	56.0	325.2	24.3
Thiacloprid-thiadiazine	87.5	+	Culture was not continued[#]			
	175.0	+	6.9	1.1	7.4	0.6
	350.0	+	7.2	1.2	10.2	0.8
	700.0	+	6.9	1.2	17.9	1.3
	1400.0	+	13.2	2.2	12.5	0.9
	2800.0[P]	+	20.4	3.4	18.0	1.3

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

III. Conclusions

The test item thiacloprid-thiadiazine did not induce gene mutations at the HPRT locus in V79 cells under the experimental conditions reported. Therefore, thiacloprid-thiadiazine is considered to be non-mutagenic in this HPRT assay.

Report: [redacted]; 2014; M-486183-01-1
Title: Thiacloprid-thiadiazine: Micronucleus test in human lymphocytes in vitro
Report No: 1597202
Document No: M-486183-01-1
Guidelines: OECD 487 (2010); Commission Regulation (EU) 640/2012, B.49 (2012).
Deviation(s): none
GLP: yes

I. Materials and methods

A. Materials

1. Test material:

Name: thiacloprid-thiadiazine
Description: light brown solid

Lot/Batch no: SES 11969-11-8
Content: 99.1 %
Stability of test compound: guaranteed for study duration; expiry date: 2014-09-11

2. Vehicle / positive control: vehicle: dimethylsulfoxide (DMSO)
positive controls:
without metabolic activation
Mitomycin C (MMC): 2 µg/mL (pulse treatment)
Demecolcin: 0.1 µg/mL (continuous treatment)
with metabolic activation
Cyclophosphamide (CPA): 15.0 µg/mL

3. Test system: human peripheral blood lymphocytes from healthy non-smoking donors not receiving medication (experiment I: female donor, 29 years old; experiment IIA: male donor, 28 years old; experiment IIB: female donor, 40 years old)
After blood samples were drawn, human lymphocytes were stimulated for proliferation by the addition of phytohemeagglutinine (PHA) to the culture medium for a period of 48 hours. The cell harvest time point was approximately 2 to 2.5 x AGT (average generation time).

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

Metabolic activation: Mammalian microsomal fraction S9 mix prepared from livers of phenomarbital / ß-naphthoflavone induced rats at Harlan CCR. Protein concentration of the used S9 mix: 29.8 mg/mL (Lot No. 050913, used for experiment I) and 31.4 mg/mL (Lot No. 260913, used for experiment II).

B. Study design and methods

Dose:

Experiment	Exposure period	S9 mix	Concentrations in µg/mL
I	4 hrs	–	17.9, 31.4, 54.9, 96.1, 168.2, **294.3, 515.0, 901.2**[P], 1577.1[P], 2760.0[P]
IIA*	20 hrs	–	17.9, 31.4, 54.9, 96.1, 168.2, 294.3, 515.0, 901.2, 1577.1, 2760.0
IIB	20 hrs	–	17.9, 31.4, 54.9, 96.1, 168.2, 294.3, 515.0, **901.2, 1577.1, 2760.0**
I	4 hrs	+	17.9, 31.4, 54.9, 96.1, 168.2, 294.3, 515.0, **901.2, 1577.1, 2760.0**[P]

IIA	4 hrs	+	168.2, 294.3, 515.0, **901.2, 1577.1, 2760.0**

P: Precipitation was observed microscopically at the end of treatment

*: was replaced due to technical problems

Concentrations in bold letters: chosen for micronuclei analysis.

All concentrations: used for cytotoxicity assessment.

The highest treatment concentration in this study, 2760.0 µg/mL, was chosen since it is equivalent to approximately 10 mM, which is the highest dose for the *in vitro* mammalian cell micronucleus test according to OECD 487.

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

Recovery: 16 hours, after end of treatment for the experiments with 4 h treatment time; none for experiments with 20 h exposure

Cytochalasin B exposure: 20 hours

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

Total culture period: 88 h (exposure started 48 h after culture initiation)

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

Replicates: 2 parallel cultures / dose

Cytotoxicity assessment: To determine a cytotoxic effect the CBPI (cytokinesis-block proliferation index) was determined in 500 cells per culture and cytotoxicity is described as % cytostasis.

II. Results and discussion

A. Findings

Visible precipitation of the test item in the culture medium was observed microscopically in experiment I at 901.2 µg/mL and above in the absence of S9 mix and at 2760.0 µg/mL in the presence of S9 mix at the end of treatment. In all other experimental parts no precipitation was observed at the end of treatment up to the highest applied concentration.

No relevant influence on osmolarity was observed. The pH was adjusted to physiological values.

No relevant cytotoxicity, indicated by a reduced CBPI (cytokinesis-block proliferation index) and described as cytostasis, could be observed up to the highest applied concentration.

In the absence and presence of S9 mix, no biologically relevant increase in the number of cells carrying micronuclei was observed. The micronucleus rates of the cells after treatment with the test item (0.10 – 1.35 % micronucleated cells) exceeded the range of the solvent control values (0.20 – 0.90 % micronucleated cells), but were within the range of the laboratory historical control data (0.15 – 1.40 % (4 h treatment, pulse treatment) or 0.10 – 1.35% (20 h treatment, continuous treatment)).

Either Demecolcin (0.1 µg/mL), MMC (2.0 µg/mL) or CPA (15.0 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei.

Table 5.8.1/21-1: Summary of results of the *in vitro* micronucleus test in human lymphocytes with thiacloprid-thiadiazine without metabolic activation

Experi-ment	Preparation interval	Test item concentration [µg/mL]	Proliferation index CBPI	Cytostasis [%]*	Micronucleated cells [%]**
Exposure period 4 hrs without S9 mix					
IA	40 h	Solvent control[1]	1.59		0.55
		Positive control[2]	1.20	65.6	**8.00**[S]
		294.3	1.57	3.9	0.45
		515.0	1.67	n.c.	0.40
		901.2[P]	1.62	n.c.	0.45
Exposure period 20 hrs without S9 mix					
IIB	40 h	Solvent control[1]	1.95		0.20
		Positive control[3]	1.48	49.6	**2.75**[S]
		902.1	1.81	15.1	0.25
		1577.1	1.75	21.0	0.35
		2760.0	1.75	21.0	0.10

*: For positive control groups and test item treatment groups the values are related to the solvent controls
**: The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S: The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c.: not calculated as the CBPI is equal to or higher than the solvent control value
1: DMSO 0.5 % (v/v)
2: MMC 2.0 µg/mL
3: Demecolcin 0.1 µg/mL

Table 5.8.1/21-2: Summary of results of the *in vitro* micronucleus test in human lymphocytes with thiacloprid-thiadiazine with metabolic activation

Experi-ment	Preparation interval	Test item concentration [µg/mL]	Proliferation index CBPI	Cytostasis [%]*	Micronucleated cells [%]**
Exposure period 4 hrs with S9 mix					
I	40 h	Solvent control[1]	1.96		0.90
		Positive control[2]	1.58	39.6	**3.85**[S]
		901.2	1.89	7.3	1.35
		1577.1	1.86	10.8	0.80
		2760.0[P]	1.88	8.8	1.20
IIA	40 h	Solvent control[1]	1.80		0.55
		Positive control[2]	1.29	63.6	**7.15**[S]
		901.2	1.77	3.1	1.00
		1577.1	1.76	5.3	1.00
		2760.0	1.82	n.c.	0.25

*: For positive control groups and test item treatment groups the values are related to the solvent controls
**: The number of micronucleated cells was determined in a sample of 2000 binucleated cells
S: The number of micronucleated cells is statistically significantly higher than corresponding control values
n.c.: not calculated as the CBPI is equal to or higher than the solvent control value
1: DMSO 0.5 % (v/v)
2: CPA 15.0 µg/mL

III. Conclusion

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

Report:	[redacted]6; [redacted]; 2014; M-495981-01-1
Title:	Thiacloprid / Thiacloprid-thiadiazine - 7-day toxicity study in the rat by dietary administration
Report No.:	SA 13306
Document No.:	M-495981-01-1
Guidelines:	US EPA OCSPP 870.3100
GLP:	yes

I. Materials and methods

A. Materials

1. Test material:

	1) thiacloprid
	2) thiacloprid-thiadiazine
Description:	1) light grey solid
	2) light brown solid
Lot/Batch no:	1) EDFL021010
	2) SES 11969-11-8
Purity:	1) 98.8% (w/w)
	2) 99.1% (w/w)
Stability of test compound:	guaranteed for study duration (confirmed by analyses); expiry date: 1) 2015-01-11
	2) 2014-09-11

2. Vehicle: diet

3. Test animals:

Species:	rat
Strain:	Wistar Rj:WI (IOPS HAN)
Age:	approx. 7 weeks
Sex:	males
Weight at dosing:	249 – 280 g
Source:	[redacted] France.
Acclimatisation period:	at leat 6 days
Diet:	certified rodent powdered and irradiated diet A04CP1-10 from [redacted], France), *ad libitum*
Water:	Filtered and softened tap water, *ad libitum*
Housing:	individually in suspended, polycarbonate, wire-mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0 - 1000 ppm thiacloprid - 1000 ppm thiacloprid-thiadiazine
Application route:	oral (diet)
Group size:	10 per dose group
Observations:	mortality, clinical signs, detailed clinical examination, body weight, food consumption, gross necropsy, organ weights (brain, liver), liver histopathology, total cytochrome P 450 content and specific cytochrome P 450 isoenzyme profile (pentoxyresorufin (PROD), bezoxyresorufin (BROD), UDP-glucuronosyltransferase activity (UDPGT))

II. Results and discussion

A. Analyses of test substance formulations

Analyses of the test substance diets revealed that the dietary admixtures of the test substances were homogeneous. Homogeneity and concentration results ranged from 94 to 103% and from 96 to 99% of the nominal concentration for the diets containing thiacloprid and thiacloprid-thiadiazine, respectively. They were within the in-house target range of 85 to 115% of the nominal concentration. Both compounds proved to be stable at 1000 ppm in the rodent diet when stored frozen for a period of 7 days followed by 10 days at room temperature.

B. Mortality

There was no mortality in any group throughout the study.

C. Clinical observations

There were no treatment-related signs reported either at the daily clinical observation or at the weekly detailed physical examination. The few signs recorded (i.e. white area on the eye and skin scab on the neck) were observed in isolation and were therefore considered not to be treatment-related.

D. Body weight

After one week of treatment mean body weights and overall mean body weight gain in the thiacloprid dose group were decreased by 10% and 66% when compared to controls.

There were no effects on body weight parameters in the thiacloprid-thiadiazine treated group.

E. Food consumption and achieved dosages

In the thiacloprid-treated group, mean food consumption was 25% lower than in the control group while it was not affected in animals receiving thiacloprid-thiadiazine.

The mean achieved dietary intake of thiacloprid and thiacloprid-thiadiazine expressed in mg/kg/day received by the animals during the study are summarised in the following table.

Table 5.8.1/22-1: Achieved dose levels

Dietary concentration (ppm)	Achieved dosages – males (mg/kg bw/day)
1000 ppm thiacloprid	73.5
1000 ppm thiacloprid-thiadiazine	87.4

D. Terminal body weights and liver weights

Thiacloprid dose group

A significantly lower mean terminal body weight was observed in males treated with 1000 ppm thiacloprid (–11%) when compared with controls. Mean absolute and relative liver weights of the 1000 ppm thiacloprid group were significantly higher than in controls (+19 or +33%, resp.).

Table 5.8.1/22-2: Mean terminal body weights and liver weights after thiacloprid treatment

Thiacloprid dose (ppm)	Mean terminal body weight or liver weight ± standard deviation (% change when compared with controls)	
	0	1000
Mean terminal body weight (g)	285.92 ± 11.385	255.91** ± 12.989
Mean absolute liver weight (g)	8.103 ± 0.5486	9.642** ± 0.9499 (+19%)
Mean relative liver weight (%)	2.832 ± 0.1227	3.778** ± 0.2735 (+33%)
Mean absolute brain weight (g)	1.879 ± 0.0625	1.828 ± 0.0899
Mean liver to brain weight ratio (%)	431.157 ± 22.8204	529.281** ± 63.2554 (+23%)

**: significantly different to control $p \leq 0.001$

Thiacloprid-thiadiazine dose group

No test substance-related effects on terminal body weight and liver weights were observed in thiacloprid-thiadiazine treated rats.

D. Necropsy

Thiacloprid dose group

The only treatment-related finding was an enlarged liver observed in 4 out of 10 males treated with 1000 ppm thiacloprid.

Table 5.8.1/22-3: Macroscopic findings observed in the liver of male rats at scheduled necropsy

Thiacloprid dose (ppm)	0	1000
Enlarged liver	0/10	4/10

(x/y): number of animals affected / total number of animals per group

Thiacloprid-thiadiazine dose group

No test substance-related macroscopic findings were observed at necropsy.

D. Histopathology

Thiacloprid dose group

Slight centrilobular to panlobular hepatocellular hypertrophy was noted in all males treated with 1000 ppm thiacloprid. This finding was considered to be treatment-related and correlated with higher liver weights and enlarged liver at necropsy.

Table 5.8.1/22-4: Histopathological liver findings at scheduled necropsy - male rats

Thiacloprid dose (ppm)	0	1000
Hepatocellular hypertrophy: centrilobular to panlobular		
Slight	0/10	10/10
Total	**0/10**	**10/10**

(x/y): number of animals affected / total number of animals per group

Thiacloprid-thiadiazine dose group

There were no test substance-related histopathological findings.

D. Liver enzyme induction

Thiacloprid dose group

Total P-450, BROD, PROD and UDPGT activities were significantly increased by 1.8-, 31-, 17- and 2.5-fold, respectively compared to control.

Table 5.8.1/22-5: Mean liver enzyme activities and standard deviation in thiacloprid treated male rats

Thiacloprid dose (ppm)	0	1000
Total P450	1.460 ± 0.2338	2.602** ± 0.3395
BROD	27.312 ± 6.0507	841.008** ± 226.0059
PROD	11.294 ± 3.4767	190.313** ± 49.4472
UDPGT	19.277 ± 4.1485	48.444** ± 3.8686

**: significantly different to control, $p \leq 0.001$

Thiacloprid-thiadiazine dose group

There were no test-substance-related changes on liver enzyme induction observed.

Table 5.8.1/22-6: Mean liver enzyme activities and standard deviation in thiacloprid-thiadiazine treated male rats

Thiacloprid-thiadiazine dose level (ppm)	0	1000
Total P450	1.460 ± 0.2338	1.422 ± 0.1367
BROD	27.312 ± 6.0507	31.370 ± 6.8704
PROD	11.294 ± 3.4767	10.070 ± 3.1779
UDPGT	19.277 ± 4.1485	22.575 ± 4.2410

**: significantly different to control, $p \leq 0.001$

III. Conclusion

Dietary administration of 1000 ppm thiacloprid to male rats led to decreased body weight parameters and food consumption as well as pronounced liver enzyme induction indicated by increased liver weight, enlarged livers, hepatocellular hypertrophy and markedly increased levels of total P-450, BROD, PROD and UDPGT.
In contrast, no treatment-related effects and no indication for liver enzyme induction were observed in any animal of the 1000 ppm thiacloprid-thiadiazine group.

Analytical methods
A method for the determination of thiacloprid-thiadiazine by HPLC analysis in rodent diet was developed. The reference of the study report is presented in the following.

Report:	[redacted]; 2014; M-481765-01-1
Title:	Thiacloprid-thiadiazine - Determination by high performance liquid chromatography analysis in ground rodent diet
Report No.:	SA 14011
Document No.:	M-481765-01-1
Guidelines:	Good Laboratory Practice (Bonnes Pratiques de Laboratoire) described in the following issues: Organization for Economic Cooperation and Development (O.E.C.D.): Principles of Good Laboratory Practice, 1997 (January 26, 1998); Article Annexe II à l'article D523-8 du Code de l'Environnement (French GLP Legislation); Deviation(s): not specified
GLP:	yes

Report:	[redacted]; 2014; M-490181-01-1
Title:	Evaluation of thiacloprid-thiadiazine in the H295R steroidogenesis assay
Report No.:	SA 13333
Document No.:	M-490181-01-1
Guidelines:	US-EPA, OPPTS Series 890, Endocrine Disruptor Screening Program test guidelines, No. 890.1550: Steroidogenesis (Human Cell Line – H295R) (October 2009) Deviation(s): none
GLP:	yes

I. Materials and methods

A. Materials

1. Test material:	thiacloprid-thiadiazine
Description:	light brown solid
Lot/Batch no:	SES 11969-11-8
Purity:	99.1%
Stability of test compound:	guaranteed for study duration; expiry date: 2014-09-11

2. Vehicle and positive controls:	vehicle: 0.1% dimethylsulfoxid (DMSO) positive controls: Forskolin – for sex steroid hormone biosynthesis stimulation Prochloraz – for sex steroid hormone biosynthesis inhibition
3. Test organism / cells:	
Species:	human
Cell line	adrenal carcinoma immortal cell line H295R
Source:	[redacted] (Batch No. 58660579)
4. Culture maintenance:	
Medium:	DMEM:F12, supplemented with 1% ITS + premix and 2.5% Nu-Serum I and 0.1 % Penicillin-Streptomycin
Conditions:	37°C ± 1°C and 5% CO_2

B. Study design and methods

1. Test conditions:	
Cell isolation:	H295R cells isolated from flasks of ≥ 75 % confluency
Medium:	DMEM: F12, supplemented with ITS + premix and 2.5 % Nu-serum I and 1 % Penicillin-Streptomycin
Test substance concentrations:	thiacloprid-thiadiazine: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M (equivalent to 100 - 0.0001 µM) 100 µM, 500 µM and 1 mM for cytotoxicity assessment Forskolin: 0.03, 0.1, 0.3, 1, 3, 10 µM; Prochloraz: 0.01, 0.03, 0.1, 0,3, 1, 3 µM
Cell density:	H295R cells seeded into 24-well plates at a density of $0.3x10^6$ cells/mL and a final volume of 1 mL/well and cultured for approx. 24 h prior to treatment
Group size:	3 wells per compound/vehicle control, concentration and treatment period
Treatment time:	48 h
Treatment conditions:	37°C ± 1°C and 5% CO_2
2. Sample collection and analysis:	
Sampling:	four aliquots of culture medium / well
Analyses:	using specific enzyme-immunoassay kits (Enzo Life Science Inc., USA); method detection limits: 5.67 pg/mL for testosterone, and 14 pg/mL for estradiol
Replicates:	3 per dose level

II. Results and discussion

A. Interference evaluation

Thiacloprid-thiadiazine did not interfere with the EIA kit for testosterone or estradiol at any concentration evaluated.

B. Cytotoxicity

Cytotoxicity was not observed following treatment of the H295R cells with concentrations up to 1 mM thiacloprid-thiadiazine when evaluated using the XTT method. In addition, staining of the cells with trypan blue at the end of the second, third and fourth evaluation demonstrated the viability of the cells following treatment with 10^{-4} M - 10^{-10} M thiacloprid-thiadiazine.

C. Hormone evaluations

Testosterone concentrations

The variability (CV) between the runs for the solvent controls was within the guideline recommendation (~19% compared to the recommended guideline of 30%).

Thiacloprid-thiadiazine was considered not to interfere with testosterone secretion in the H295R steroidogenesis assay as no concentration-related effects could be established in any of the three evaluations of the test item. The slight changes recorded for testosterone secretion ranging from –20.6% to +8.5% were considered to be within the normal variability of the assay.

Estradiol concentrations

The variability (CV) between the runs for the solvent controls was outside the guideline recommendation (~47 % compared to the recommended guideline of 30%) due to the increased concentration recorded for all samples of the fourth evaluation. This increased CV was considered as having no impact on the overall evaluation of thiacloprid-thiadiazine in the H295R steroidogenesis assay.

Thiacloprid-thiadiazine was considered not to interfere with estradiol secretion in the H295R steroidogenesis assay. The slight changes in estradiol concentration ranging from -12% at 10^{-7} M to +30 % at 10^{-5} M were considered to be within the normal variability of the assay.

Table 5.8.1/24-1: Mean hormone concentrations, standard deviation and % change in comparison to controls after incubation of H295R cells with thiacloprid-thiadiazine for 24 h (mean of three evaluations)

Thiacloprid-thiadiazine concentration	Testosterone (pg/mL)			Estradiol (pg/mL)		
	Mean	SD	% change	Mean	SD	% change
DMSO	7912.9	1510.86	--	221.0	103.50	--
10^{-10} M	7113.1	1250.53	-10.1%	217.7	80.90	-1.5%
10^{-9} M	7503.9	964.75	-5.2%	213.7	84.52	-3.3%
10^{-8} M	7189.7	778.05	-9.1%	217.1	79.63	-1.8%
10^{-7} M	7127.0	668.36	-9.9%	214.9	77.04	-2.8%
10^{-6} M	7295.6	981.31	-7.8%	218.0	79.95	-1.4%
10^{-5} M	7981.7	1231.05	nc	249.3	92.94	+12.8%
10^{-4} M	7074.0	909.12	-10.6%	251.1	104.85	+13.6%

Statistical evaluation conducted on overall data only (based on second, third and fourth evaluation; the first evaluation was invalid due to cytotoxicity). Data have been rounded up.
SD: standard deviation
nc: no change compared to controls

Table 5.8.1/24-2: Mean fold change and standard deviation of hormone concentrations relative to DMSO controls after incubation of H295R cells with thiacloprid-thadiazine for 24 h (mean of three evaluations)

Thiacloprid-thiadiazine concentration	Testosterone		Estradiol	
	Mean fold change	SD	Mean fold change	SD
10^{-10} M	0.90	0.16	0.99	0.37
10^{-9} M	0.95	0.12	0.97	0.38
10^{-8} M	0.91	0.10	0.98	0.36
10^{-7} M	0.90	0.08	0.97	0.35
10^{-6} M	0.92	0.12	0.99	0.36
10^{-5} M	1.01	0.16	1.13	0.42
10^{-4} M	0.89	0.11	1.14	0.47

SD: standard deviation
Data have been rounded up.

Positive controls

The comparison of the data generated in the concurrent positive control study (M-490174-01-1) with the guideline criteria are given in the table below. These data indicate that, with the exception of the testosterone increases induced by forskolin in the first and fourth evaluations, which were <2-times the solvent control, all other criteria were met. It should be borne in mind that steroidogenesis in the H295R assay is dynamic and the limited increase in testosterone secretion following forskolin treatment could, therefore, be a reflection of the marked increase in estradiol secretion (~23- to ~44-fold increase compared to 7.5-fold increase proposed in the guideline).

Table 5.8.1/24-3: Mean hormone concentrations and standard deviation of concurrent positive controls (mean of four evaluations)

		Testosterone		Estradiol	
		Mean	SD	Mean	SD
Minimum Basal Production (pg/mL) (Testosterone: 500 pg/mL; Estradiol: 40 pg/mL)	1st evaluation	12093	1224.5	272	22.4
	2nd evaluation	7616	458.1	250	13.8
	3rd evaluation	6905	398.7	298	16.4
	4thevaluation	9041	891.2	421	29.8
	Overall	8914	2170.8	310	70.4
		Testosterone		Estradiol	
Basal Production (Testosterone :≥ 5-times MDL; Estradiol: ≥ 2.5-times MDL)	1st evaluation	2133-times MDL		19-times MDL	
	2nd evaluation	1343-times MDL		18-times MDL	
	3rd evaluation	1218-times MDL		21-times MDL	
	4thevaluation	1595-times MDL		30-times MDL	
	Overall	1372-times MDL		22-times MDL	
Induction (10 µM Forskolin) (Testosterone: ≥ 2-times SC; Estradiol: ≥ 7.5-times SC)	1st evaluation	1.8-times SC		41.4-times SC	
	2nd evaluation	2.1-times SC		44.3-times SC	
	3rd evaluation	2.0-times SC		28.1-times SC	
	4thevaluation	1.9-times SC		23.4-times SC	
	Overall	1.9-times SC		32.9-times SC	
Inhibition (1 µM Prochloraz) (Testosterone: ≤ 0.5-times SC; Estradiol: ≤ 0.5-times SC)	1st evaluation	0.04-times SC		Complete inhibition	
	2nd evaluation	0.05-times SC		Complete inhibition	
	3rd evaluation	0.05-times SC		Complete inhibition	
	4thevaluation	0.06-times SC		Complete inhibition	
	Overall	0.05-times SC		Complete inhibition	

MDL: minimum detection limit (5.67 pg/mL for testosterone, 14 pg/mL for estradiol)
SC: solvent control
Note: Four positive control evaluations were conducted to cover the assessment of several test items. Evaluations 2 – 4 were conducted concurrently with the three evaluations of thiacloprid-thiadiazine.

III. Conclusion

Overall, thiacloprid-thiadiazine, tested at concentrations between 10^{-10} M and 10^{-4} M, was considered not to interfere with testosterone or estradiol secretion in the H295R steroidogenesis assay.

Report: [redacted]§; [redacted]; 2014; M-490186-01-1
Title: Evaluation of thiacloprid in the H295R steroidogenesis assay
Report No.: SA 13334 [M-490186-01-1]
Guidelines: US-EPA, OPPTS Series 890, Endocrine Disruptor Screening Program test guidelines, No. 890.1550: Steroidogenesis (Human Cell Line – H295R) (October 2009)
Deviation(s): none
GLP: yes

I. Materials and methods

A. Materials

1. Test material:	thiacloprid
Description:	light grey solid
Lot/Batch no:	PFHCA-2013-07-01
Purity:	98.9%
Stability of test compound:	guaranteed for study duration; expiry date: 2015-01-11
2. Vehicle and positive controls:	vehicle: 0.1% dimethylsulfoxid (DMSO) positive controls: Forskolin – for sex steroid hormone biosynthesis stimulation Prochloraz – for sex steroid hormone biosynthesis inhibition
3. Test organism / cells:	
Species:	human
Cell line	adrenal carcinoma immortal cell line H295R
Source:	[redacted] (Batch No. 58660579)
4. Culture maintenance:	
Medium:	DMEM:F12, supplemented with ITS+ premix and 2.5% Nu-Serum I and 0.1% Penicillin-Streptomycin
Conditions:	37°C ± 1°C and 5% CO_2

B. Study design and methods

1. Test conditions:	
Cell isolation:	H295R cells isolated from flasks of ≥ 75 % confluency
Medium:	DMEM: F12, supplemented with ITS + premix and 2.5 % Nu-serum I and 1 % Penicillin-Streptomycin
Test substance concentrations:	Thiacloprid: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10} M 100 µM,500 µM and 1 mM for cytotoxicity assessment Forskolin: 0.03, 0.1, 0.3, 1, 3, 10 µM; Prochloraz: 0.01, 0.03, 0.1, 0,3, 1, 3 µM
Cell density:	H295R cells seeded into 24-well plates at a density of $0.3x10^6$ cells/mL and a final volume of 1 mL/well and cultured for approx. 24 h prior to treatment
Group size:	3 wells per compound/vehicle control, concentration and treatment period
Treatment time:	48 h
Treatment conditions:	37°C± 1°C and 5% CO_2

2. Sample collection and analysis:

Sampling:	four aliquots of culture medium / well
Analyses:	using specific enzyme-immunoassay kits (Enzo Life Science Inc., USA), method detection limits: 5.67 pg/mL for testosterone, 14 pg/mL for estradiol

II. Results and discussion

A. Interference evaluation

Thiacloprid did not interfere with the EIA kit for testosterone or estradiol at any concentration evaluated.

B. Cytotoxicity

Cytotoxicity was not observed following treatment of the H295R cells at concentrations of 500 µM thiacloprid when evaluated using the XTT method. In addition, staining of the cells with trypan blue at the end of the second, third and fourth evaluation demonstrated the viability of the cells following treatment with 10^{-4} M - 10^{-10} M thiacloprid.

C. Hormone evaluations

Due to a technical error during the first evaluation of thiacloprid, a non-treatment related cytotoxicity was observed in several wells of the 24-well culture plate following staining with trypan blue. The data from this first evaluation have not been exploited and an additional assay (designated as the fourth evaluation) was conducted. Thus only the data from the second, third and fourth evaluations have been analyzed and are presented.

<u>Testosterone concentrations</u>

The variability (CV) between the runs for the solvent controls was within the guideline recommendation (~24% compared to the recommended guideline of 30%).

In each evaluation, the highest concentration of thiacloprid tested (10^{-4} M) induced a slight but consistent treatment-related decrease in testosterone concentration, which resulted in an overall statistically significant reduction of testosterone by ~25% at this concentration compared to the solvent controls. No clear treatment-related effects on testosterone levels were observed in any evaluation when considering thiacloprid at all other concentrations (10^{-10} M - 10^{-5} M). Specifically, the slight changes recorded for testosterone secretion ranged from a ~23% increase at 10^{-7} M in the third evaluation to a ~19% reduction at 10^{-10} M in the fourth evaluation. These changes were considered to be within the normal variability of the assay.

<u>Estradiol concentrations</u>

The variability (CV) between the runs for the solvent controls was within the guideline recommendation (~24 % compared to the recommended guideline of 30%).

In each evaluation, the highest concentration of thiacloprid tested (10^{-4} M) induced a slight but consistent treatment-related decrease in estradiol concentration, which resulted in an overall statistically significant reduction of estradiol by ~31% at this concentration compared to the solvent controls. No clear treatment-related effects on estradiol levels were observed in any evaluation when

considering thiacloprid at all other concentrations (10^{-10} M - 10^{-5} M). The ~46% increase in estradiol secretion at 10^{-9} M in the third evaluation was not considered relevant due to the variability observed at that concentration among the three evaluations (0.84- to 1.45-fold).

Table 5.8.1/25-1: Mean hormone concentrations, standard deviation and % change in comparison to controls after incubation of H295R cells with thiacloprid for 48 h (mean of three evaluations)

Thiacloprid concentration	Testosterone (pg/mL)			Estradiol (pg/mL)		
	Mean	SD	% change	Mean	SD	% change
DMSO	7518.8	1824.04		268.2	65.53	--
10^{-10} M	6944.9	921.85	-7.6%	289.6	65.79	+8.0%
10^{-9} M	7389.2	1434.00	-1.7%	290.6	69.53	+8.4%
10^{-8} M	7107.3	1336.31	-5.5%	259.8	58.29	-3.1%
10^{-7} M	7944.9	846.05	+5.7%	240.6	54.58	-10.3%
10^{-6} M	7438.1	949.47	-1.1%	256.2	35.09	-4.5%
10^{-5} M	6966.2	1344.16	-7.3%	232.0	36.79	-13.5%
10^{-4} M	**5657.6***	**1367.8***	**-24.8%**	**184.4*[D]**	**27.28*[D]**	**-31.2%**

Statistical evaluation conducted on overall data only (based on second, third, and fourth evaluation; the first evaluation was invalid due to cytotoxicity). Data have been rounded up.
SD: standard deviation
*: significantly different to controls, $p \leq 0.05$
[C]: data based on 7/9 samples
[D]: data based on 8/9 samples

Table 5.8.1/25-2: Mean fold change and standard deviation of hormone concentrations relative to DMSO controls after incubation of H295R cells with thiacloprid for 48 h (mean of three evaluations)

Thiacloprid concentration	Testosterone		Estradiol	
	Mean fold change	SD	Mean fold change	SD
10^{-10} M	0.92	0.12	1.08	0.25
10^{-9} M	0.98	0.19	1.08	0.26
10^{-8} M	0.95	0.18	0.97	0.22
10^{-7} M	1.06	0.11	0.90[C]	0.20[C]
10^{-6} M	0.99	0.13	0.96	0.13
10^{-5} M	0.93	0.18	0.86	0.14
10^{-4} M	0.75	0.18	0.69[D]	0.10[D]

SD: standard deviation
[C]: data based on 7/9 samples
[D]: data based on 8/9 samples
Data have been rounded up.

Positive controls

The comparison of the data generated in the concurrent positive control study with the guideline criteria are given in the table below. These data have been taken from the results section of the report and indicate that, with the exception of the testosterone increases induced by forskolin, all other

criteria were met. It should be borne in mind that steroidogenesis in the H295R assay is dynamic and the limited increase in testosterone secretion following forskolin treatment could, therefore, be a reflection of the marked increase in estradiol secretion (~23- to ~44-fold increase compared to 7.5-fold increase proposed in the guideline).

Table 5.8.1/25-3: Mean hormone concentrations and standard deviation of concurrent positive controls (mean of four evaluations)

		Testosterone		Estradiol	
		Mean	**SD**	**Mean**	**SD**
Minimum Basal Production (pg/mL) (Testosterone: 500 pg/mL; Estradiol: 40 pg/mL)	1st evaluation	12093	1224.5	272	22.4
	2nd evaluation	7616	458.1	250	13.8
	3rd evaluation	6905	398.7	298	16.4
	4th evaluation	9041	891.2	421	29.8
	Overall	8914	2170.8	310	70.4
		Testosterone		**Estradiol**	
Basal Production (Testosterone : ≥ 5-times MDL; Estradiol: ≥ 2.5-times MDL)	1st evaluation	2133-times MDL		19-times MDL	
	2nd evaluation	1343-times MDL		18-times MDL	
	3rd evaluation	1218-times MDL		21-times MDL	
	4th evaluation	1595-times MDL		30-times MDL	
	Overall	1572-times MDL		22-times MDL	
Induction (10 µM Forskolin) (Testosterone: ≥ 2-times SC; Estradiol: ≥ 7.5-times SC)	1st evaluation	1.8-times SC		41.4-times SC	
	2nd evaluation	2.1-times SC		44.3-times SC	
	3rd evaluation	2.0-times SC		28.1-times SC	
	4th evaluation	1.9-times SC		23.4-times SC	
	Overall	1.9-times SC		32.9-times SC	
Inhibition (1 µM Prochloraz) (Testosterone: ≤ 0.5-times SC; Estradiol: ≤ 0.5-times SC)	1st evaluation	0.04-times SC		Complete inhibition	
	2nd evaluation	0.05-times SC		Complete inhibition	
	3rd evaluation	0.05-times SC		Complete inhibition	
	4th evaluation	0.06-times SC		Complete inhibition	
	Overall	0.05-times SC		Complete inhibition	

MDL: minimum detection limit (5.67 pg/mL for testosterone, 14 pg/mL for estradiol)
SC: solvent control
Note: Four positive control evaluations were conducted to cover the assessment of several test items. Evaluations 2 – 4 were conducted concurrently with the three evaluations of thiacloprid.

III. Conclusion

Overall, thiacloprid tested at concentrations between 10^{-10} M and 10^{-4} M, was considered to induce a slight, though statistically significant treatment-related reduction in both testosterone and estradiol secretion in the H295R steroidogenesis assay at the highest concentration tested (10^{-4} M or 100 µM).

CA 5.8.2 Supplementary studies on the active substance

Summary of supplementary studies

Supplementary studies on thiacloprid comprise previously and recently conducted studies on toxicokinetics, an immunotoxicity study as well as mechanistic studies investigating the modes of action of the observed tumors as well of the finding of dystocia.

Toxicokinetic studies

Toxicokinetic studies in rats revealed dose proportional increases in plasma concentrations in males at high doses of 1000 ppm, while increases were over-proportional in females indicating an overload of the metabolic capacity of the liver. A decrease of plasma concentrations over time due to enzyme induction and increased metabolisation of thiacloprid was not visible. Therefore, a possible inhibitory effect of thiacloprid on CYP450 dependent monooxigenases was investigated in an additional study. Thiacloprid had only a weak inhibitory effect on 7-ethoxycoumarin-deethylation in liver microsomes of rat and dog. However, this is not very relevant *in vivo* because the necessary concentrations will not be reached. A study comparing toxicokinetics in pregnant to non-pregnant rats revealed higher plasma concentrations of thiacloprid in pregnant animals. This finding was most pronounced at the end of gestation. ***Protein binding of thiacloprid in plasma of humans and rats, investigated in a newly submitted study, was low and of similar magnitude in both species (40.7% in human and 54.7% in rat plasma). Newly submitted were also toxicokinetic determinations in plasma of dogs from the 15-week dietary toxicity study. Compared with the administered doses the plasma levels were very high indicating a high oral absorption of thiacloprid. Insufficient oral absorption of thiacloprid as a possible reason for the fact that toxicity of thiacloprid in dogs is not very pronounced, can therefore be excluded.***

Immunotoxicity study

An immunotoxicity study in rats, conducted for US EPA, revealed that thiacloprid has no immuno-suppressive potential.

Supplementary studies to elucidate the tumor mode of actions

Additional work on thyroid tumors: An *in vitro* study showed that thiacloprid and its metabolites are no inhibitors of the thyroid peroxidase (TPO). A 3-week dietary study was especially designed to investigate the effects of thiacloprid on the thyroid in rats. It was shown that thyroid findings (changes of thyroid hormones and TSH as well as thyroid follicular cell hypertrophy occurred only at doses linked with marked liver enzyme induction including pronounced UDP-GT increases. This indicates that the mode of action of thyroid effects including thyroid follicular cell adenoma in male rats after long-term treatment with thiacloprid is secondary to liver enzyme induction, a mode of action which is rat specific and not relevant to humans.

<u>Additional work to elucidate the mode of action of tumors of the female reproductive tract (uterine adenocarcinoma in rat, ovarian luteoma in mice):</u>

In the 1990ies studies in rats and mice have been conducted, in which aromatase (CYP19a1, catalysing the conversion from testosterone to estradiol or from androstenedione to estrone, respectively) levels have been determined mostly in liver tissue and ovaries. This was done in the framework of the mode of action work for the tumors of the female reproductive tract or dystocia, observed after thiacloprid treatment in animals. An induction of aromatase could have been a possible reason for an increased estradiol/progesterone ratio which after long-term treatment would lead to the observed tumors or dystocia, respectively. However, due to the recent work by [redacted] (2009, M-360757-02-1) it is known that the observed increases of aromatase in the old studies were artifacts caused by the unspecificity of the tritiated water assay used at that time and that thiacloprid is no inducer of aromatase (at high dietary thiacloprid doses ≥ 1000 ppm even a marginal inhibition of aromatase in the ovaries was seen).

In a 13-week dietary study in female mice for aromatase induction also slight changes of sex steroid hormones were observed. There were slight decreases of plasma estradiol and increases of plasma progesterone, which resulted in a sligthly decreased estradiol/progesterone ratio. The NOAEL for the hormone changes (based on the estradiol/ progesterone ratio) was 139 mg/kg bw/day. Further findings, besides changes in motility and reduced reactivity and increased liver weight, were increased vacuolization and hypertrophy of the adrenal X-zone. The overall NOAEL in this study was 18 mg/kg bw/day.

Several mode of action studies on development of uterine adenocarcinoma in rats have been conducted by [redacted] between 2007 and 2010 in order to improve the Q Star risk assessment in the US. In these studies it was shown that thiacloprid treatment leads to slight changes of estradiol or progesterone plasma levels. These are accompanied by a slightly increased expression of genes associated with sex steroid hormone biosynthesis in ovary, liver & adrenal gland. It is assumed that this results in an increase of CYP450 enzymes involved in steroid biosynthesis with the magnitude of this effect not being known. However, also moderation of the steroidogenic effects was evident from the data due to an increased expression of genes associated with the metabolism of sex steroid hormones. While the observed slight hormonal changes in young adult rats do not lead to an effect on the estrous cycle or on other sex steroid hormone sensitive organs or tissues (see 2-generation study and short term toxicity studies in rats), effects on the estrous cycle were noted in 72-week old, aging female rats. In comparison to untreated controls fewer thiacloprid treated females were in pseudopregnancy and more in the ambiguous phase. In addition, thiacloprid treated rats displayed a lower level of vaginal mucification and marginal, non significant increases of plasma estradiol, which were more pronounced in females in pseudopregnancy in comparison to those in the ambiguous phase.

In depth mode of action work showed that thiacloprid has no direct estrogenic effect, since it was negative in an uterotrophic assay. However, it was positive for an effect on steroidogenesis in a H295R assay. Increased progesterone secretion was observed at 100 µM (equivalent to an unbound concentration of about 25 mg/L), while unbound plasma concentrations in female rats after dietary exposure with 1000 ppm (high dose of the 2-year rat study, clearly exceeding the MTD) with up to 11.5 mg/L are still in the range of the NOAEC of 50 µM or 12.5 mg/L for progesterone in this

assay. Incubation of rat preantral follicles with thiacloprid led to increased estradiol and progesterone secretion at 500 µM, indicating that preantral follicles are a cellular target for thiacloprid treatment. The NOAEC was 100 µM, indicating again that hormone changes and tumour development in rats occurred at unbound plasma concentrations in the range of the NOEC of this assay. However, the observed effects on sex steroid hormones as well as tumour development in rodents occurred always at dose levels showing pronounced enzyme induction as well as increased expression of genes associated with sex steroid hormone biosynthesis. Therefore, these effects are believed to be secondary to enzyme induction. The mode of action work by [redacted] on uterine adenocarcinoma in rat as well as the comparison of in vitro and in vivo concentrations of thiacloprid are presented in the position papers by [redacted] (2010, M-362441-01-1) and [redacted] (2014, M-498558-01-1).

<u>Supplementary studies to elucidate the mode of action for dystocia</u>

In the 1990ies several special 1-generation and mechanistic studies have been conducted to elucidate the mode of action for dystocia in the Sasco Sprague-Dawley rat (used before in the two-generation study on thiacloprid). Dystocia occurred repeatedly, in incidences between 3.3 and 13.3%, after dietary intake of of 300 to 1000 ppm thiacloprid for 10 weeks during premating and during gestation, while it was not seen in the concurrent controls. An overview on incidences of dystocia observed in the generation studies on thiacloprid is presented in Table 5.8.2-1 below.

In contrast, thiacloprid related dystocia was not seen after short-term treatment with oral gavage doses of 17.5 to 100 mg/kg bw on gestation days 18 to 21. In a further dietary study no effect of thiacloprid on direct birth functions could be shown. However, further data showed that thiacloprid treated animals had increased estradiol levels, slightly increased progesterone and LH levels as well as increased corticosterone levels in plasma during premating, gestation and lactation shortly after parturition.

In an additional special 1-generation study the mode of action of dystocia was further investigated using video-recording of parturition as well as sex homone determinations in plasma shortly before and after parturition. Already in two feasibility studies (conducted before the start of the main study to develop and optimize the procedure of video recording and blood sampling during parturition) several cases of dystocia were observed in untreated animals. This indicates that stress alone can cause dystocia in Sasco Sprague-Dawley rats. In the main study dystocia was noted in 3/28 dams treated with thiacloprid and in none of the controls. In one dam dystocia was due to a missing progesterone decrease, which in rodents is mandatory for a normal parturition. This is not the case in humans, in which progesterone withdrawal is regulated differently and plasma progesterone levels stay high during parturition. In a second dam dystocia there were no changes of hormone levels. Dystocia was obviously due to stress by blood sampling and the high general toxicity of thiacloprid together with the increased sensitivity of the Sasco Sprague-Daley rat towards such effects. A third dam with dystocia was found dead, blood sampling was not possible. Furthermore,in thiacloprid treated animals the levels of progesterone (slightly increased mean value at GD 20, absence of normal decrease prior to parturition in one rat with dystocia) and estradiol (increased mean values at GD 21 and 22) and the respective balance between these hormones in the days before and during parturition were affected. There was no effect on onset and duration of parturition in all other treated rats. In addition, also the known effects of thiacloprid toxicity were

present in treated rats, i.e. reduced body weight and food consumption, increased liver and thyroid weights, hepatocellular hypertrophy and thyroid follicular cell hypertrophy.
In conclusion, dystocia in rats is considered to be either due to hormonal perturbations (a missing progesterone decrease before start of parturition, which is mandatory for normal birth in rat, but not in humans) or due to stress by blood sampling and thiacloprid toxicity together with the relatively high sensitivity of the Sasco Sprague Dawley rat towards stress. The first mode of action is rat-specific and not relevant for humans, the second mode of action is unspecific and secondary to stress.

Table 5.8.2 -1: **Incidences of dystocia observed in several generation studies on thiacloprid in Sasco Sprague-Dawley rats (ordered by increasing dose)**

Author, Year Reference	Dose [ppm]	Dose* [mg/kg bw/day]	Incidences [% (cases) per pregnant dams]
[redacted], 1998, M-003820-01-1	300	20	0 (0/25)
[redacted], 1997, M-001304-01-1	300	22	13.3 (4/30)
[redacted], 1997, M-001304-01-1	600	43	10.0 (3/30)
[redacted]-[redacted], 2011, M-403763-01-1	800	54	11.5 (3/26)
[redacted], 1998, M-004253-01-1	800	61**	8.3 (1/12)#
[redacted], 1998, M-003820-01-1	1000	68	4.5 (1/22)
[redacted], 1998, M-004291-01-1	1000	73***	3.3 (1/30)
Historical control data in Sasco Sprague Dawley rats##			Range: 0 - 11.5 (0/30 - 3/26) Mean incidence: 1.21 (11/906)

*: dose intake determined during gestation
** dose intake determined for pre-mating, gestation and lactation
**: dose intake determined during premating, not determined during gestation
#: There was one additional case of dystocia, but this was obviously caused by big pups (one pup stuck in the birth canal) and is therefore not considered to be related to thiacloprid treatment.
##: Historical control data on dystocia in Sprague-Dawley rats from the breeder Sasco compiled from 26 one- and two-generation studies (comprising 40 generations) conducted at BCS Toxicology in Stilwell U.S. between 1988 and 1997 (in 1997: switch to Wistar rats) (for details please refer to [redacted], 2014, M-498539-01-1)

Publications:
In addition, three articles on thiacloprid or thiacloprid containing formulations were published in 2011 and 2012.
One publication, considered not to be reliable, described changes of thyroid hormone levels in rat serum after treatment of the animals with thiacloprid containing formulations. However, thyroid hormone changes after thiacloprid treatment of rats is a well investigated phenomenon and the reported results of the publication do not change existing endpoints.
In the second publication, which was considered as reliable with restrictions, thiacloprid was reported to induce human CYP 1A1 and 1A2 in HepG2 cells. Because the determined enzyme induction was not pronounced and observed at a concentration exceeding the maximal unbound

plasma concentrations of thiacloprid at the high dose of the 2-year rat study, the finding was considered to be non-relevant for human safety.
The third publication was also assessed as reliable with restrictions. It provides supplemental information on oxidative stress in lymphoid organs, polymorphonuclear leucocytes and plasma of rats after treatment with thiacloprid containing formulations. Also this publication has no influence on existing endpoints.

Table 5.8.2 -2: Summary of additional studies on the active substance

Study ***Doses tested***	**NO(A)EL** (mg/kg bw/day)	**LO(A)EL**	**Result / Main effects seen at LOAEL**	**Reference**
Rat, 4-week oral (diet), Mechanistic study on aromatase induction & toxicokinetics *Study 1: M/F: 0-100-1000 ppm* *Study 2: F: 0-100-200-500 ppm*	Study 1 &2: 6.6 *(100 ppm)*	Study 1: 60.4 (1000 ppm) Study 2: 47.5 *(500 ppm)*	↓ body weight gain, ↑ liver weights ≥ 500 ppm Thiacloprid plasma concentrations: dose proportional ↑ from 100 to 1000 ppm in M, more than dose proportional ↑ in F (overload of metabolic liver capacity in F at higher doses), no ↓ of thiacloprid concentrations due to enzyme induction over time visible Aromatase determinations: no assessment possible due to the unspecificity of the assay used	[redacted], 1998 M-003766-03-1
Rat, subacute oral (diet), special study on toxicokinetics in pregnant / non-pregnant rats *0-1000 ppm*	--	1000 ppm	Plasma levels of thiacloprid ↑ in pregnant rats in comparison to non-pregnant rats during gestation (difference most pronounced at the end of gestation).	[redacted] & [redacted], 1998 M-003821-01-1
Determination of plasma protein binding in rat and human plasma	***na***	***na***	***Low protein binding of [^{14}C]-YRC 2894 of 40.7% in human plasma and 54.7% in rat plasma; no relevant differences between both species***	***[redacted], 1998 (translated 2014) M-075786-01-2***
Toxicokinetic study, 15-weeks dog (diet) *0-250-1000-2000 ppm*	***na***	***na***	***Peak mean plasma concentrations of thiacloprid measured 4 to 6 hours after feeding were approx. 2, 6, and 14 µg/mL at 250, 1000 and 2000 ppm. Compared with the administered doses the plasma levels are very high indicating a high absorption of thiacloprid.***	***[redacted], 1995 M-000760-01-1***
28-day, diet immuno-toxicity, rat (female)	***General toxicity*** **5.78** ***(100 ppm)***	**25.7** ***(300 ppm)***	***≥ 25.7 mg/kg bw/day: ↑ relative spleen weight*** ***80.7 mg/kg bw/day: ↓ body weight /gain & food consumption, ↑ absolute & relative spleen weight***	***[redacted]-[redacted], 2012 M-428958-01-1***
F: 0-100-300-	***Immunotoxicity*** **80.7** ***(1000 ppm)***	**> 80.7** ***(>1000 ppm)***	***No effects on SRBC-specific IgM-response (no immuno-suppressive effect)***	

Study ***Doses tested***	NO(A)EL (mg/kg bw/day)	LO(A)EL	Result / Main effects seen at LOAEL	Reference
1000 ppm				
Determination of aromatase activity in liver and ovaries from a special 1-generation study in rats *0-800 ppm*	--	61 *(800 ppm)*	Aromatase determinations: no assessment possible due to the unspecificity of the assay used	[redacted], 1998 M-003794-01-1
In vitro study on a possible inhibition of CYP450 dependent monooxigenases in liver of rat & dog	--	--	Thiacloprid had a weak inhibitory effect (IC_{50} = 100 µM or 25.3 mg/L) on 7-ethoxycoumarin-decthylation (ECOD, catalyses the transformation of testosterone to estradiol). There was no effect on testosterone hydroxylation (IC_{50} > 1000 µM or 252.7 mg/L) in the liver of rats.	[redacted], 1998 M-003796-01-2
Mice, 13-week oral (diet), mechanistic study for aromatase induction *F: 0-10-30-250-2500 ppm & 2500 ppm + mecamylamine (nicotinic mimicking agent)*	18 *(30 ppm)*	139 *(250 ppm)*	↑ motility & ↓ reactivity, ↑ liver weight (at interim sacrifice after 4 weeks only), ↑ vacuolization (at higher doses also hypertrophy) of the adrenal X-zone NOAEL for sex hormone levels in plasma: 139 mg/kg bw/day *(250 ppm)* Aromatase determinations: no assessment possible due to the unspecificity of the assay used	[redacted], 1998 M-003764-01-1
In vitro study on TPO-inhibition	--	--	No direct inhibitory effect of thiacloprid or its metabolites on the thyroid peroxidase (TPO) was evident.	[redacted], 1994 M-000690-02-1
3-week, rat (diet), special study for the investigation of thyroid effects *0 - 25 - 100 - 400 - 1600*	9.0/12.3 *(100 ppm)*	36.9/44.6 (M/F) *(400 ppm)*	Induction of UDP-glucuronyl-transferase (UDP-GT), ↑liver weight (males), ↑ incidence of minimal to slight thyroid follicular cell hypertrophy (5/10 males)	[redacted], 2000 M-030427-03-1
Uterotrophic assay, rat ***0, 70 mg/kg bw s.c., once daily for 3 days***	***< 70***	***70***	***↓ motor activity (n = 1), ↓ body weight, cumulative body weight loss,*** ***↓ absolute liver weight &absolute ovary weights, no uterotrophic response***	[redacted], ***2007*** ***M-293209-01-1***

Study *Doses tested*	NO(A)EL (mg/kg bw/day)	LO(A)EL	Result / Main effects seen at LOAEL	Reference
Hormone levels in female rats after single oral dose *0, 60 mg/kg bw*	*< 60*	*60*	*Significant ↑ of plasma progesterone (8 & 24 h after dosing)* *Significantly ↑ expression of several genes associated with the regulation of steroid hormone synthesis (24 h after dosing)*	[redacted], *2009* *M-359235-01-1*
Hormone levels in female rats 2 and 8 h after 4 oral doses *0, 60 mg/kg bw*	*< 60*	*60*	*No feces (n =1 on study days 2 & 3), ↓ body weights, cumulative body weight loss* *↑ plasma progesterone (2, 8 h after dosing)* *↓ absolute liver weight, ↑ absolute & relative adrenal weights & ↓ absolute ovary weights without morphological changes*	[redacted], *2009* *M-360362-01-1*
Hormone levels in female rats 24 h after 4 oral doses *0, 60 mg/kg bw*	*< 60*	*60*	*Mortalities: 2/15, few / no faeces, hunched posture, ↓ motor activity, soiled fur (head/mouth region), piloerection* *↓ body weight, cumulative body weight loss,* *significant ↑ in plasma progesterone, non-significant ↑ of estradiol & FSH,* *↑ relative liver weights, ↑ absolute & relative adrenal weights, ↓ absolute & relative ovary & uterus weights,* *↑ expression of genes associated with sex steroid hormone biosynthesis in ovary, liver & adrenal gland.* *Moderation of the steroidogenic effects due to ↑ expression of genes associated with the metabolism.*	[redacted], *2009* *M-360349-01-1*
28-day, diet, rat (female) *F: 0-100-1000-1600 ppm*	*8.0 (100 ppm)*	*75.2 (1000 ppm)*	*↓ body weight, body weight gain & food consumption,* *significant ↑ in plasma estrdiol, marginal, non-significant ↑ of progesterone,* *↑ liver weights, enlarged & dark livers, phenobarbital like liver enzyme induction, changes in expression of genes associated with steroidogenesis & metabolism of sex steroid hormones*	[redacted], *2009* *M-360757-02-1*
28-day, diet, rat (female, aged)	*< 31.5*	*31.5*	*Sacrifice for humane reasons (1/25), ocular discharge, wasted appearance, soiled anogenital region, few / no faeces, ↓ motor activity,* *↓ body weight, body weight loss, ↓ food consumption,* *marginal, non significant ↑ of plasma estradiol (more pronounced in females in pseudopregnancy in comparison to those in the ambiguous phase), fewer females in pseudopregnancy (more in*	[redacted], *2009* *M-359926-01-1*

Study *Doses tested*	NO(A)EL (mg/kg bw/day)	LO(A)EL	Result / Main effects seen at LOAEL	Reference
F: 0-1000 ppm			***ambiguous phase), lower level of vaginal mucification, ↑ relative liver weight***	
H295R steroidogenesis assay	***< 50 µM***	***50 µM***	***Dose-related inhibition of testosterone after incubation for 24 & 48 hrs, at 100 µM: ↑ progesterone secretion after 24 h incubation***	[redacted], ***2010 M-361492-01-1***
In vitro steroid hormone secretion, rat preantral follicles	***100 µM***	***500 µM***	***↑ progesterone & estradiol levels after incubation for 24 & 48 hrs***	[redacted], ***2010 M-361609-01-1***
Special 1-generation study, rat *0-25-300-1000 ppm*	Pre-mating: 20 / 23 (M/F) *(300 ppm)* Gestation: 20	Pre-mating: 69 / 75 (M/F) *(1000 ppm)* *gestation: 68*	Mortality, dystochia (3/30 or 4/30?*), ↓ body weight (females), ↑ liver & thyroid weight, ↓ pup viability	[redacted] 1998 M-003820-01-1
Special 1-generation study in rat for the investigation of the cause for dystochia *0-1000 ppm*	Pre-mating: < 73 *(< 1000 ppm)*	Pre-mating 73 *(1000 ppm)*	Dystocia (1/30), ↓ body weight (F, premating) no direct effect of thiacloprid on birth functions could be detected	[redacted], 1998 M-004291-01-1
Special study in pregnant rats, investigation, if treatment with high oral gavage doses of thiacloprid on GD 18-21 can cause dystocia 0-17.5-35-60	17.5 (general toxicity) 60 (dystochia)	17.5 (general toxicity, not determined for dystocia)	No dystochia up to 60 mg/kg bw/day; 17.5 mg/kg bw/day: ↓ body weight gain & food intake, higher doses: mortality, hypoactivity, ↑ number of stillbirths	[redacted], 1998 M-002127-01-1
Special study in pregnant rats, investigation, if high oral gavage doses of thiacloprid on GD 18-20 can caus dystocia 0-100 (due to marked toxicity reduced to 50 on GD 20)	< 50 (general toxicity)	50 (general toxicity, not determined for thiaclorpid related dystocia)	Mortality (4/26), hypoactivity, tremor, reduced / no faeces, ↓ body weight, One case of dystochia occurred, but this was considered to be related to the marked toxicity and a necrosis of the pregnant uterus horn.	[redacted], 1998 M-016564-02-1

Study *Doses tested*	NO(A)EL (mg/kg bw/day)	LO(A)EL	Result / Main effects seen at LOAEL	Reference
Special 1-generation study, rat Mechanistic study for dystochia *0-800 ppm*	< 54.0 / 61.0 (M/F) *(< 800 ppm)*	54.0 / 61.0 (M/F) *(800 ppm)*	Dystocia (2/12, one case obviously caused by a big pup stuck in the birth canal), ↓ body weight gain, liver enzyme induction, ↑ liver weight, centrilobular hepatocytomegaly, proliferation of smooth endoplasmatic reticulum ↑ plasma levels of estradiol, progesterone & LH (both: slight) & corticosterone	[redacted], 1998 M-004253-01-1
Special 1-generation study, rat, including video recording of parturition &hormone determinations ***0-800 ppm***	***< 50.5 / 60.9 / 54.0 (M/F premating/F gestation) (< 800 ppm)***	***50.5 / 60.9 /54.0 (M/F premating/F gestation) (800 ppm)***	***Dystocia: 3/28, 1 due to a missing progesterone decrease, 1 due to stress (by blood sampling, thiacloprid toxicity & the high sensitivity of the rat strain used - dam had normal hormone levels), 1 found dead (blood sampling not possible) ↓ body weight / gain & food consumption, ↑ progesterone & estradiol levels, changes of estradiol/progesterone ratio,↑ liver & thyroid weight, hepatocelular hypertrophy, thyroid follicular cell hypertrophy***	***[redacted]-[redacted], 2011 M-403763-01-1***

M: male   F: female   ↑: increase(d)   ↓: decrease(d)
Mat: maternal   Dev: developmental
bw: body weight   Neurotox: neurotoxicity
TPO: thyroid peroxidase
*: 3 dams died with signs of dystocia on gestation days (GD) 23 or 24, the 4th dam died on GD 22
na: not applicable

Report: KCA 5.8.2/14[redacted]D; 1998; M-075786-01-2
Title: Letter Report on Plasma Protein Binding of [^{14}C]-YRC 2894
Report No.: M-075786-01-2
Document No.: M-075786-01-2
Guidelines: No applicable guideline
Deviation(s): not applicable
GLP: no

I. Materials and methods

A. Materials

1. Test material: [Methylen-^{14}C]-YRC 2894
- Specific activity: 3.77 MBq/mg
- Lot/Batch no: Not reported
- Radiochemical purity: 99%

2. Sample material: plasma

Species:	human and rat
Sex:	human: male rat: female

B. Study design and methods

1. Purity and stability assessment:

The radiochemical purity and stability of [^{14}C]-thiacloprid were evaluated by HPLC analysis.

2. Determination of plasma protein binding:

For the determination of plasma protein binding a nominal concentration of 60 µM [^{14}C]-thiacloprid was added to plasma. The applied concentration of 60 µM corresponds to plasma concentrations, which had been measured in non-pregnant female rats after application of 1000 ppm thiacloprid in the diet (corresponds to the high dose in the 2-year rat study). After application of the plasma into the ultrafiltration unit, the samples were incubated for 10 minutes before ultracentrifugation (= ultrafiltration). Subsequently, the concentration of [^{14}C]-thiacloprid was determined in the ultracentrifugate by liquid scintillation counting.

II. Results and discussion

1. Purity and stability of [^{14}C]-YRC 2894:

The radiochemical purity of [^{14}C]-thiacloprid was 99%. The stability of [^{14}C]-thiacloprid in PBS, human and rat plasma was sufficient, since there was no change in concentration within 1 h.

2. Determination of plasma protein binding:

Protein binding of [^{14}C]-thiacloprid with 40.7% in human plasma and 54.7% in rat plasma is low, relevant differences between human and rat do not exist. The results are summarised in the following table.

Table 5.8.2/14-1: Binding of [^{14}C]-thiacloprid to human and rat plasma

Sample	Concentration		
	Nominal [µM]	Actual [µM]	Relative [%]
Human plasma (male)	60	59.9	100
Corresponding ultrafiltrate	--	35.5 ± 0.4*	59.3
Corresponding bound fraction	--	--	40.7
Rat plasma (female)	60	65.1	100
Corresponding ultrafiltrate	--	29.5 ± 0.6*	45.3
Corresponding bound fraction	--	--	54.7

*: Mean value ± standard deviation (n=3)

III. Conclusion

Binding of [^{14}C]-thiacloprid with 40.7 or 54.7 % to human and rat plasma is low. Relevant differences between rat and human regarding plasma protein binding do not exist.

The toxicokinetic measurements summarised in the following were determined in plasma samples obtained in the subchronic feeding study in dogs by [redacted] & [redacted], 1998 (M-003814-01-1), which was already evaluated for the Annex I listing of thiacloprid (see DAR B.6.3.1.3), while this was not the case for the report presented below. Therefore, only relevant data of the subchronic feeding study are presented here. For details on the subchronic feeding study in dogs please refer to the DAR and baseline dossier of thiacloprid.

Report:	KCA 5.8.2/15[redacted],; 1995; M-000760-01-1
Title:	YRC 2894 – Concentration of YRC 2894 in the plasma of dogs in a subchronic feeding study
Report No.:	24572
Document No.:	M-000760-01-1
Guidelines:	**No applicable guideline** **Deviation(s): not applicable**
GLP:	**Non GLP**

I. Materials and methods

A. Materials

1. Test material: YRC 2894

Synonym	thiacloprid; NTN 33894
Lot/Batch no:	290894
Purity:	not reported in this report on toxicokinetics according to M-003814-01-1: 97.2% (analysis of 1994-09-06), 96.8% (analysis of 1995-02-28)
Stability of test compound:	guaranteed for study duration; expiry date: 1995-08-27

2. Vehicle: plain diet ("[redacted]HH sole diet for dog husbandry, double ground" from [redacted], Germany), mixed 1:1 with hand-warm tap water immediately before administration to the animals to a homogenous feed paste

3. Test animals:

Species:	dog
Strain:	Beagle dog, Bor. Beag.
Age:	19-20 weeks
Sex:	males and females
Weight at dosing:	6.1 – 8.9 kg
Source:	[redacted], Germany
Acclimatisation period:	18 days

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0-250-1000-2000 ppm equivalent to: 0-8.5-34.9-68.0 mg/kg bw/day (males) equivalent to: 0-8.9-34.7-65.3 mg/kg bw/day (females)
Application route:	oral (diet)
Group size:	4/sex/dose
Blood sampling times:	after 13 weeks of treatment at 0, 2h, 4h, 6h and 24 h after feeding in 1 male dog of each treatment group and at 2h, 4 h and 6 h in all other male and female dogs
Extraction of plasma samples:	1 mL plasma was extracted three times with 3 mL ethyl-acetate. For separation of the organic layer samples were centrifuged 10 min. at 2000 g. The combined extracts were evaporated to dryness at about 40°C under a stream of nitrogen and stored at -20°C. For HPLC measurement the residue was taken up in 500 uL methanol.
Analysis:	measurement of thiacloprid concentrations in plasma by HPLC with UV detection at 242 nm
Evaluation:	determination of plasma concentrations and C_{max} values

II. Results and discussion

A. YRC 2894 concentration in plasma of male dogs

Profile of thiacloprid plasma concentrations in male dogs

Initially plasma concentrations of thiacloprid were determined for one dog per dose level at 0, 2, 4, 6 and 24 h after feeding to find out the time point of the maximal concentration (C_{max}). At the time points 0 and 24 h thiacloprid concentrations were very low (< 0.5 µg/mL). Between 0 and 6 h concentrations increased to reach a C_{max} in the 6 h samples. Thiacloprid concentrations at this time point were clearly dose proportional (see
Table 5.8.2/15-1 below). Based on these results plasma concentrations in all other male and female dogs were investigated 2, 4 and 6 h after feeding.

The analyses for all four male dogs revealed the same result. Mean plasma concentrations increased up to 6 h and the concentrations increased dose proportionally. In the low dose group there was a plateau between 2 and 6 h after feeding, while in the high dose group the concentration increased by a factor of 2 during this time period.
Overall the relatively high plasma levels showed an efficient absorption of thiacloprid after dietary exposure.

Table 5.8.2/15-1: Plasma concentrations of thiacloprid in male dogs obtained after 13 weeks of dietary administration in a subchronic toxicity study ([redacted] & [redacted], 1986, M-003814-01-1)

Dose level (ppm)	Dog No.	Plasma concentrations of thiacloprid (µg/mL)				
		0 h	2 h	4 h	6 h	24 h
250	969	0.05	1.45	1.88	1.80	n.d.
	973	n.s.	1.01	1.22	1.00	n.s.
	983	n.s.	1.05	1.64	1.46	n.s.
	989	n.s.	1.30	1.62	1.66	n.s.
	Mean	**n.c.**	**1.20**	**1.59**	**1.48**	**n.c.**
	SD	**n.c.**	**0.18**	**0.24**	**0.30**	**n.c.**
1000	963	0.04	3.26	4.78	6.19	0.04
	979	n.s.	6.65	9.31	8.99	n.s.
	981	n.s.	3.81	6.27	6.29	n.s.
	985	n.s.	3.26	6.35	7.61	n.s.
	Mean	**n.c.**	**4.25**	**6.68**	**7.24**	**n.c.**
	SD	**n.c.**	**1.41**	**1.65**	**1.16**	**n.c.**
2000	961	0.48	6.60	8.49	13.56	0.38
	977	n.s.	3.69	8.34	10.05	n.s.
	991	n.s.	11.55	23.56	25.22	n.s.
	997	n.s.	5.75	10.03	16.14	n.s.
	Mean	**n.c.**	**6.90**	**12.60**	**16.24**	**n.c.**
	SD	**n.c.**	**2.89**	**6.36**	**5.61**	**n.c.**

n.d.: not detected n.s.: no sample n.c.: not calculated

Profile of thiacloprid plasma concentrations in female dogs

There was no indication for a sex-difference with regard to the absorption and plasma concentrations of thiacloprid.

C_{max} was measured in 8 of 12 female dogs 6 h, in 3 dogs 4 h after feeding. Mean plasma concentrations increased proportional to dose. In the low dose group there was again a plateau between 2 and 6 h.

The plasma concentration vs. time course of female no. 980 was completely different to the data of the other dogs (C_{max} at time point 0, then concentrations decreased up to 6 h). Therefore, the values of this dog were not used for the calculation of the respective mean values.

The plasma concentrations in female dogs are summarised in Table 5.8.2/15-2 below.

Table 5.8.2/15-2: Plasma concentrations of thiacloprid in female dogs obtained after 13 weeks of dietary administration in a subchronic toxicity study ([redacted] & [redacted], 1986, M-003814-01-1)

Dose level (ppm)	Dog No.	Plasma concentrations of thiacloprid (µg/mL)				
		0 h	2 h	4 h	6 h	24 h
250	962	0.08	1.03	1.76	1.62	n.d.
	970	n.s.	1.01	1.28	1.13	n.s.
	984	n.s.	1.28	1.85	1.54	n.s.
	986	n.s.	1.03	1.55	1.61	n.s.
	Mean	**n.c.**	**1.09**	**1.61**	**1.48**	**n.c.**
	SD	**n.c.**	**0.11**	**0.22**	**0.20**	**n.c.**
1000	980*	1.89	1.10	0.63	0.39	0.94
	982	n.s.	7.57	10.72	11.15	n.s.
	988	n.s.	6.50	9.05	9.22	n.s.
	990	n.s.	4.37	5.12	6.13	n.s.
	Mean	**n.c.**	**6.15**	**8.30**	**8.83**	**n.c.**
	SD	**n.c.**	**1.33**	**2.35**	**2.07**	**n.c.**
2000	964	0.19	4.14	7.39	13.87	0.14
	966	n.s.	8.43	9.79	16.56	n.s.
	972	n.s.	2.95	5.72	4.50	n.s.
	978	n.s.	9.29	15.47	22.78	n.s.
	Mean	**n.c.**	**6.20**	**9.64**	**14.43**	**n.c.**
	SD	**n.c.**	**2.71**	**3.66**	**6.58**	**n.c.**

*: values were not considered for calculation of mean
n.d.: not detected n.s. no sample n.c.: not calculated

Overall the relatively high plasma levels showed an efficient absorption of thiacloprid in dogs after dietary exposure.

III. Conclusion

These toxicokinetic data in the plasma of dogs revealed an efficient absorption of thiacloprid after 13 weeks of dietary treatment. The peak concentration was in most cases observed 6 h after feeding. C_{max} values in male and female animals increased proportionally to dose.

Thus, it can be concluded that the marginal toxic effects of thiacloprid observed in the subchronic feeding study are not a consequence of inefficient absorption and low plasma concentrations of the test substance.

Immunotoxicity

The immunotoxicity study on thiacloprid presented in the following was conducted to fulfill an U.S. data requirement.

Report:	KCA 5.8.2/16 [redacted] M; 2012; M-428958-01-1
Title:	Thiacloprid – 28-day immunotoxicity study in the female Wistar rat by dietary administration
Report No.:	SA 10362
Document No.:	M-428958-01-1
Guidelines:	US-EPA OPPTS 870.7800 (1998) Deviation(s): none
GLP:	Yes

I. Materials and methods

A. Materials

1. Test material: Thiacloprid

Description:	light beige solid
Lot/Batch no:	EDE 0011099
Purity:	98.7% (w/w)
Stability of test compound:	guaranteed for study duration, expiry date: 2011-11-11

2. Vehicle and positive controls: vehicle: plain diet
positive control: cyclophosphamide at 3.5 mg/kg bw/day

3. Test animals:

Species:	rat
Strain:	Wistar, RjHan:WI (IOPS HAN)
Age:	approximately 7 weeks
Sex:	females
Weight at dosing:	163 – 199 g
Source:	[redacted], France
Acclimatisation period:	12 days
Diet:	certified rodent pelleted and irradiated diet "A04CP1-10" from [redacted], *ad libitum*
Water:	tap water (filtered and softened), *ad libitum*
Housing:	individually in suspended stainless steel wire mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0-100-300-1000 ppm equivalent to approx. 0-5.78-25.7-80.7 mg/kg bw/day positive control cyclophosphamide: 3.5 mg/kg bw/day
Application route:	oral (diet) positive control: oral (gavage, 5 mL/kg bw)
Duration:	at least 28 days

Group size:	10 females
Antigen stimulation:	sheep red blood cell (SRBC) sensitization
Identification:	sheep red blood cell (SRBC)
Source of SRBC:	[redacted], France
Preparation of SRBC:	On the day of injection, Sheep Red Blood Cells were washed in PBS (Phosphate Buffered Saline), counted using a cell counting instrument (Siemens Advia 120) and diluted in PBS in order to obtain a 5×10^8 cells/mL preparation. The SRBC preparation was kept on ice until use.
Administration of SRBC:	On Study Day 26, all animals in all groups were immunized by intravenous injection in the tail vein (0.5 mL/animal). Prior to intravenous injection, animals were anesthetized with Isoflurane (Virbac, Carros, France).
Observations:	Mortality, clinical signs, body weight, food consumption, organ weights (spleen, thymus), blood (blood sampling from the retro-orbital venous plexus 4 days after SRBC immunization at terminal sacrifice): determination of SRBC-specific IgM with an ELISA.

Methods

Thiacloprid was administered *via* diet to groups of 10 female Wistar rats at concentrations of 0, 100, 300, and 1000 ppm (equivalent to approximately 0, 5.78, 25.7, and 80.7 mg/kg bw/day) for at least 28 days. An additional group of 10 females received 3.5 mg/kg body weight/day cyclophosphamide (immunosuppressive agent) daily by oral gavage for at least 28 days to serve as a as positive control group. Animals were observed daily for mortality and clinical signs. Body weight and food consumption were recorded once weekly. A detailed physical examination was performed once during the acclimatization phase and at least weekly throughout the study. On Study Day 26, four days before necropsy, all animals were immunized with Sheep Red Blood Cell (SRBC) antigen by intravenous injection of 2.5×10^8 SRBC/animal *via* the tail vein. On Study Day 30 (just before necropsy), blood samples were collected from the retro-orbital venous plexus of each animal for specific anti-SRBC immunoglobulin M (IgM) analysis. All animals were necropsied, gross pathology observations were performed and spleen and thymus were weighed.

II. Results and discussion

A. Mortality

No mortality occurred during the study.

B. Clinical signs

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

C. Body weight

Mean body weight was reduced by 7 to 9% (statistically significant, $p \leq 0.05$) after treatment with 1000 ppm thiacloprid in comparison to control group animals from Study Day 9 to the end of the study. Overall mean body weight gain in the high dose group was approximately 32% lower (statistically significant, $p \leq 0.01$) than in the control group at the end of the study.

D. Food consumption and dietary intake of thiacloprid

At 1000 ppm, mean food consumption was 33% lower than in the control group (statistically significant, $p \leq 0.01$) on Study Day 8 and remained approximately 9% lower until end of the study (Study Day 15 to 29, not statistically significant).

Table 5.8.2/16-1: Mean achieved dietary intake of thiacloprid (weeks 1-4)

Dietary concentration of thiacloprid (ppm)	Mean thiacloprid intake (mg/kg bw/day)
100	3.78
300	25.7
1000	80.2

E. Immune response – SRBC-specific IgM response

Treatment with thacloprid had no statistically significant effect on the SRBC-specific IgM response (see Table 5.8.2/16-2 below).

Table 5.8.2/16-2: Thiacloprid-induced SRBC-specific IgM response

SRBS-specific IgM (u/mL), mean ± SD (% change compared to controls)				
Thiacloprid dose level (ppm)	0	100	300	1000
Study Day 30	9724 ± 4824	11753 ± 6078 (+21%)	8521 ± 3041 (-12%)	8468 ± 3725 (-13%)

SD: standard deviation

F. Necropsy

Organ weights

In the high-dose group, the mean terminal body weight was significantly reduced (-10%, $p \leq 0.01$) in comparison to the controls. In addition, the mean absolute and relative spleen weight was significantly increased by +18% ($p \leq 0.01$) and +31% ($p \leq 0.01$), respectively. In the mid-dose group relative spleen weights were also significantly increased (+16%, $p \leq 0.01$).

These changes were considered to be treatment-related.

Table 5.8.2/16-3: Spleen weights in female rats

Mean spleen weight ± SD (% change compared to controls)				
Dose level of thiacloprid (ppm)	**0**	**100**	**300**	**1000**
Mean absolute spleen weight (g)	0.749 ± 0.085	0.784 ± 0.080 (+5%)	0.840 ± 0.117 (+12%)	0.884** ± 0.084 (+18%)
Mean relative spleen weight (%)	0.2991 ± 0.0246	0.3164 ± 0.0357 (+6%)	0.3464** ± 0.0335 (+16%)	0.3929** ± 0.0326 (+31%)

SD: standard deviation
**: statistically significantly different from control, $p \leq 0.01$.

Treatment with the positive control compound cyclophosphamide at 3.5 mg/kg bw/day significantly reduced the mean anti-SRBC IgM concentration by -88% ($p \leq 0.01$) in comparison to the controls. In addition, cyclophosphamide treated animals displayed a lower mean terminal body weight (-5 %, not statistically significant) and statistically significantly lower mean absolute and relative spleen and thymus weights (-17 to -24%, see Table 5.8.2/16-4 below).

Table 5.8.2/16-4: Mean spleen and thymus weights of the positive control group treated with cyclophosphamide

Cyclophosphamide (mg/kg bw/day)	Mean spleen weight ± SD		Mean thymus weight ± SD	
	Absolute (g)	Relative (%)	Absolute (g)	Relative (%)
0	0.749 ± 0.085	0.2991 ± 0.0246	0.544 ± 0.111	0.2168 ± 0.0355
3.5	0.567** ± 0.073 (-24%)	0.2379** ± 0.0274 (-20%)	0.428* ± 0.082 (-21%)	0.1805* ± 0.0366 (-17%)

SD: standard deviation
*: statistically significantly different from control, $p \leq 0.05$
**: statistically significantly different from control, $p \leq 0.01$

Gross pathology

No treatment-related macroscopic changes were observed in any of the thiacloprid treated animals, while in the positive control group atrophic/small spleens and thymus were observed in 8/10 and 3/10 animals, respectively.

III. Conclusion

Dietary treatment with up to 1000 ppm thiacloprid (equivalent to approximately 80.7 mg/kg bw/day) for at least 28 days did not impair the immunological IgM response after immunization with SRBC in female Wistar rats. Therefore, thiacloprid was considered have no immuno-suppressive potential.

Analytical methods

Analytical methods for the determination of thiacloprid by HPLC analysis in rodent diet (+1% corn oil) were developed. The references of the study reports are presented under KCA 5.8.2/31, M-392957-01-1 and KCA 5.8.2/32, M-425259-01-1.

Report:	KCA 5.8.2/17 [redacted],; 2007; M-293209-01-1
Title:	Thiacloprid – Evaluation in the immature rat uterotrophic assay
Report No.:	SA 06252
Document No.:	M-293209-01-1
Guidelines:	Prior to guideline, but in general accordance to OECD 440 (2007) Deviation(s): only one dose tested; wet uterine weight not determined; Each female was assessed for vaginal opening during necropsy (Due to the opinion of the Study Director this deviation did not affect the results of the study).
GLP:	yes

I. Materials and methods

A. Materials

1. Test material:	thiacloprid
Description:	light brown powder
Lot/Batch no:	EDE 0011099
Purity:	99.0%
Stability of test compound:	guaranteed for study duration; expiry date: 2008-02-25
2. Control compounds / nucleic acid analog:	
Denomination:	4-androstene-3,17-dione (male steroid hormone and aromatase inducer)
Description:	off-white powder
Lot/Batch no:	016K1420
Purity:	99%
Stability of test compound:	guaranteed for study duration; expiry date: January 2011
Denomination:	β-estradiol (17 β-estradiol, estrogen receptor agonist)
Description:	white powder
Lot/Batch no:	026K1806
Purity:	99%
Stability of test compound:	guaranteed for study duration; expiry date: March 2009
Denomination:	(+)-5-bromo-2′-deoxyyuridine (BrdU)
Description:	off-white crystalline powder
Lot/Batch no:	03029CE
Purity:	99.9%
Stability of test compound:	quality control acceptance date: March 2006 (no expiry date mentioned)
3. Vehicle:	arachis oil
4. Test animals:	

Species:	rat
Strain:	Wistar rat, Rj:WI (IOPS HAN)
Age:	19 days
Sex:	females
Weight at dosing:	36.4 – 51.2 g
Source:	[redacted], France
Acclimatisation period:	6 days
Diet:	certified rodent pelleted and irradiated diet "A04CP1-10" from [redacted], France), *ad libitum*
Water:	tap water (filtered and softened), *ad libitum* during acclimatisation; commencing on the afternoon before the first day of treatment: solution of 0.8 mg BrdU/mL drinking water *ad libitum* during treatment
Housing:	During acclimatization, each dam with litter was housed in an individual Makrolon cage containing soft wood bedding. Following acclimatization, the immature females were group-housed (7/cage) in Makrolon cages.

B. Study design and methods

1. Animal assignment and treatment:

Test substance dose:	70 mg/kg bw/day
Application:	subcutaneous injection (s.c.) as suspension in arachis oil, 4 mL/kg bw, once daily for three days
Androstenedione dose:	30 mg/kg bw
Application:	s.c., as suspension in arachis oil, 4 mL/kg bw, once daily for three days
17β-estradiol dose:	10 µg/kg bw
Application:	s.c., as suspension in arachis oil, 4 mL/kg bw, once daily for three days
Vehicle dose:	5 mL/kg by oral gavage

Table 5.8.2/17-1: Group size and treatment

Group	First dose by s.c. injection		Second dose by oral gavage	
	Compound	Dose level	Compound	Dose level
1	AO	0 mg/kg bw	AO	0 mg/kg bw
2	thiacloprid	70 mg/kg bw	AO	0 mg/kg bw
3	17β-estradiol	10 µg/kg bw	AO	0 mg/kg bw
4	androstenedione	30 mg/kg bw	AO	0 mg/kg bw
5	AO	0 mg/kg bw	-	-
6	thiacloprid	70 mg/kg bw	-	-
7	17β-estradiol	10 µg/kg bw	-	-
8	androstenedione	30 mg/kg bw	-	-

AO: arachis oil

Group size: 7 females/group

Number of applications: 3 (once daily for 3 days)

Post-treatment observation period: 24 h after last dose

Observations: mortality, clinical signs, body weight, vaginal opening (prior to necropsy), plasma concentrations of estradiol, testosterone and progesterone (blood sampling prior to necropsy), gross necropsy, organ weights (uterus, liver, ovaries), histopathology (uterus; sample of the duodenum as quality control for immunohistochemical staining), mitotic index for epithelial cells (lumen and glands) and endometrial stromal cells, height of endometrial epithelium and thickness of endometrium, assessment of cell proliferation by immunohistochemical staining for BrdU (uterus and duodenum as positive control)

II. Results and discussion

A. Mortality

No mortalities occurred during the study.

B. Clinical signs

One animal in Group 2 (70 mg/kg bw/day thiacloprid s.c.) exhibited reduced motor activity on the last day of dosing. Reduced motor activity or reduced motility, resp., is a typical sign of thiacloprid intoxication in rats and also the applied dose of 70 mg/kg bw thiacloprid s.c. for 3 days could be high enough to lead to first signs of intoxication. Therefore, the finding is considered to be treatment-related (although it was considered to be incidental by the author of the report, since it was not observed in any other animal in this study).

No treatment-related signs were observed in any other animal.

Physical examination prior to necropsy revealed no vaginal opening in any female of any dose group.

C. Body weight

The following body weight effects were noted in the thiacloprid dose groups:
Mean body weight of Group 2 females (70 mg/kg/day thiacloprid) was statistically significantly lower than that of the corresponding Group 1 control females on Day 2 (-12.8%; $p<0.05$) and Day 3 (-19.8%; $p<0.01$) of treatment. Similarly, mean body weight of Group 6 females (70 mg/kg bw/day thiacloprid) was statistically significantly lower than that of the corresponding Group 5 control females on Day 3 (-18.2%; $p<0.01$).
Overall, a mean cumulative body weight loss of -1.5 g (-22 %, $p \leq 0.01$) was observed for Group 2 females treated with thiacloprid compared to a cumulative body weight gain of 9.1 g for the corresponding Group 1 controls, and a mean body weight loss of -3.0 g (- 25 %, $p \leq 0.01$) was recorded for Group 6 females treated with thiacloprid compared to a cumulative mean body weight gain of 8.7 g for the corresponding Group 5 controls.

There were no effects on body weight in the androstenedione or 17β-estradiol treated groups.

D. Hormone analyses
The majority of the data generated from the determination of estradiol, testosterone and progesterone levels proved inconclusive for two reasons. First, as the females used in this study were immature (22 days old at sacrifice), the volume of blood required to evaluate all three hormones was not achieved for several animals (3/56, 14/56 and 9/56 samples were not available for the evaluation of estradiol, testosterone and progesterone, respectively). This was further complicated for estradiol and testosterone by the fact that the concentrations of these hormones were below the limits of detection for many samples (37/56 for estradiol and 23/56 for testosterone).
Overall, estradiol was detected in 5/7 females of Group 7 (17β-estradiol treatment; 16.85 ± 8.85 pg/mL). All other values were considered incidental.
Low levels of testosterone were detected in available samples from animals treated with androstenedione (0.84 ± 0.91 ng/mL for Group 4 and 0.85 ± 0.35 ng/mL for Group 8). All other values were considered incidental.
Progesterone could be detected in all samples evaluated. There were no statistically significant differences observed between the treatment groups and the corresponding control groups.

E. Organ weight
Liver:
The mean absolute liver weight was unaffected by treatment with either 17β-estradiol (Groups 3 and 7) or androstenedione (Groups 4 and 8). However, mean absolute liver weight of thiacloprid treated animals was lower (in Group 6 even statistically significantly lower) than in controls (Group 2: -17%; Group 6: -19.2% ($p \leq 0.05$)). As there were no significant differences in the liver weights relative to body weight, this reduction in absolute liver weight is considered to be due to the body weight losses induced by thiacloprid.
Ovaries:
The mean absolute ovarian weight was unaffected by treatment with either 17β-estradiol (Groups 3 and 7) or androstenedione (Groups 4 and 8). This applies also to the mean absolute ovarian weight of animals dosed with thiacloprid in Group 2. Only the mean absolute ovarian weight of the animals dosed with thiacloprid in Group 6 was statistically significantly lower than the control weight (-34%; $p \leq 0.01$)

Uterus:

The mean absolute uterine weight was significantly higher than the control weight for animals dosed with 17β-estradiol (Group 3: 154% and Group 7: 152%; $p \leq 0.01$) as well as for those treated with androstenedione (Group 4: 145% and Group 8: 142%; $p \leq 0.01$). A marginal increase in absolute uterine weight was recorded for animals in Group 2 dosed with thiacloprid (14%; not statistically significant). However, this was not observed for thiacloprid-treated animals in Group 6. As there were no histopathological changes associated with the increased uterine weight, it is considered as incidental and not treatment-related.

Table 5.8.2/17-2: Mean organ weights at terminal sacrifice (% change *vs.* control)

Group **Substance**	**1** **Control**	**2** **Thiaclo-prid**	**3** **17β-Estradiol**	**4** **Andros-tene-dione**	**5** **Control**	**6** **Thiaclo-prid**	**7** **17β-Estra-diol**	**8** **Andros-tenedione**
Terminal bw (g)	55.7	43.4** (-22%)	56.4	56.2	56.2	42.1** (-25%)	55.6	56.4
Mean abs. liver wt. (g)	2.4	2.0 (-17%)	2.4	2.5	2.6	2.1* (-19.2%)	2.4	2.5
Mean liver to bw ratio	4.4	4.6 (+4.5%)	4.2	4.4	4.6	4.9 (+6.5%)	4.2	4.5
Mean abs. ovarian wt. (mg)	27	26	25	28	29	19** (-34%)	32	26
Mean abs. blotted uterine wt. (mg)	18.1	20.7 (+14%)	46** (+154%)	44.4** (+145%)	17.9	17.6	45.1** (+152%)	43.3** (+142%)

bw: body weight wt.: weight abs.: absolute
*: statistically significantly different form control ($p \leq 0.05$)
**: statistically significantly different form control ($p \leq 0.01$)

F. Microscopic post mortem evaluation

Microscopic examination of the uterus from all females treated with the positive controls 17β-estradiol and androstenedione indicated minimal to moderate diffuse endometrial hyperplasia and cellular debris in the endometrial epithelium. In addition, minimal interstitial mixed cell infiltrate was observed in animals treated with 17β-estradiol (both treatment groups) when compared to the control animals.

No treatment-related effects were observed in the animals dosed with thiacloprid.

Mitotic index:

The mean mitotic index observed in the endometrial stroma and the epithelial cells was higher in the positive control treatment animals when compared to the control animals. No differences were observed between animals treated with thiacloprid (both treatment groups) and control animals.

Endometrium:

The height of the epithelium and the endometrial thickness were higher for animals dosed with 17β-estradiol when compared to control animals. Treatment with androstenedione did not affect the epithelial cell height or the endometrial thickness. No differences were observed between animals dosed with thiacloprid and control animals.

Table 5.8.2/17-3: Mean values of height of the uterus epithelium and the endometrial thickness

Group Substance	1 Control	2 Thiaclo-prid	3 17β-estradiol	4 Andros-tenedione	5 Control	6 Thiaclo-prid	7 17β-estradiol	8 Andros-tenedione
Height of the uterus epithelium (µm)								
Mean	12.39	12.69	15.63	12.68	10.58	11.86	14.97	10.66
SD	1.15	1.67	2.58	2.37	1.19	2.47	1.69	1.22
Endometrial height (µm)								
Mean	80.96	86.70	117.93	106.40	87.29	103.58	115.99	85.13
SD	26.13	24.08	32.35	18.92	13.68	21.02	21.86	15.97

G. Cell proliferation assessment

In the endometrial stromal cells a higher proliferative index was observed for animals dosed with 17β-estradiol (both treatment groups) as well as for those treated with androstenedione (both treatment groups) when compared to control animals. No differences were observed between animals dosed with thiacloprid and control animals.

In the luminal epithelial cells a higher proliferative index was determined mainly for animals dosed with 17β-estradiol but also for animals treated with androstenedione when compared to control animals. There were no differences between animals dosed with thiacloprid and control animals.

Table 5.8.2/17-4: Labelling index after BrdU detection (*)

Group Substance	1 Control	2 Thiaclo-prid	3 17β-estradiol	4 Andros-tenedione	5 Control	6 Thiaclo-prid	7 17β-estradiol	8 Andros-tenedione
Endometrial stromal cells (%)								
Mean	3.62	2.31	48.15	23.31	3.91	8.97	48.77	33.89
SD	1.13	2.41	16.64	10.49	3.18	15.14	6.42	9.67
Luminal epithelial cells (%)								
Mean	0.28	0.21	10.92	5.08	0.40	0.49	8.63	2.86
SD	0.23	0.08	13.87	0.95	0.47	0.81	3.23	1.32

(*): number of BrdU positive cells per 500 cells counted

III. Conclusion

Thiacloprid does not elicit an uterotrophic response (gravimetically and microscopically) in immature female rats. Under the same test conditions, significant gravimetrical and microscopical uterine effects were recorded for the positive control compounds 17β-estradiol and androstenedione.

Report:	KCA 5.8.2/18[redacted],; 2009; M-359235-01-1
Title:	Thiacloprid – Investigation of effects on hormone levels in adult female Wistar rats following a single oral dose
Report No.:	SA 07125
Document No.:	M-359235-01-1
Guidelines:	No applicable guideline Deviation(s): not applicable
GLP:	Non-GLP (no specific Quality Assurance inspections were conducted and dose formulations were not analysed, but performed according to standard operating procedures, which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material: thiacloprid

Description:	light brown powder
Lot/Batch no:	EDE 0011099
Purity:	99%
Stability of test compound:	guaranteed for study duration; expiry date: 2008-02-25

2. Vehicle: aqueous 0.5 % methylcellulose 400

3. Test animals:

Species:	rat
Strain:	Wistar rat, Rj:WI (IOPS HAN)
Age:	approximately 11 weeks
Sex:	females
Weight at dosing:	217 – 269 g
Source:	[redacted], France
Acclimatisation period:	at least 12 days
Diet:	certified rodent pelleted and irradiated diet “A04CP1-10” from [redacted], France), *ad libitum*
Water:	tap water (filtered and softened), *ad libitum*
Housing:	individually in suspended stainless steel wire mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose levels:	0, 60 mg/kg bw
Application route:	oral gavage
Application volume:	5 mL/kg bw

Table 5.8.2/18-1: Group size and treatment

Group	Test substance	Dose levels (mg/kg bw)	Sacrifice time (h after treatment)	Number of rats per group
1	Control	0	2	15
2	Thiacloprid	60		15
3	Control	0	8	15
4	Thiacloprid	60		15
5	Control	0	24	15
6	Thiacloprid	60		15

Group size: 15 females

Number of applications: single application

Post-treatment observation period: 2, 8, 24 h after dosing

Observations: all groups: mortality, clinical signs, body weight (during acclimatization and prior to dosing), blood samples (all animals prior to necropsy, determination of estradiol, testosterone and progesterone), gross necropsy, organ weights (uterus (incl. cervix), liver, ovaries, adrenal gland, pituitary gland).

24 h group: in addition to the above observations: terminal bodyweight, clinical signs at least once after dosing, vaginal smear prior to sacrifice for estrous cycle staging, gene expression analysis by quantitative PCR of isolated total cytoplasmic RNA of ovary and liver samples of individual control and treated animals of Groups 5 and 6.

Table 5.8.2/18-2: Gene expression analysed by quantitative PCR in 24 h animals

Gene (Major function)	Abbreviation
Steroidogenesis	
Steroidogenic acute regulatory protein (Cholesterol transport to inner mitochondrial membrane)	StAR
Cytochrome P450 11a1 (Cholesterol side-chain cleavage to form pregnenolone)	Cyp11a1
Cytochrome P450 17a1 (Pregnenolone → 17αhydroxypregnenolone) (Progesterone → androstenedione)	Cyp17a1
Hydroxysteroid dehydrogenase 3b1 (Pregnenolone → Progesterone)	Hsd3b1
Hydroxysteroid dehydrogenase 17b3 (Androstenedione → testosterone)	Hsd17b3
Hydroxysteroid dehydrogenase 17b1 (Estrone → estradiol)	Hsd17b1
Cytochrome P450 19a1 (aromatase) (Testosterone → estradiol)	Cyp19a1
Nuclear receptor subfamily 5a1 (Transcription factor controlling expression of the steroidogenic cytochrome P450 genes in endocrine tissue)	Nr5a1
Insulin-like 3 (associated with ovarian thecal cells)	Insl3
Metabolism	

Cytochrome P450 1a1	Cyp1a1
Cytochrome P450 3a3 (Inactivation: testosterone → 6βhydroxytestosterone)	Cyp3a3
Aldo-keto reductase 1c18 (Inactivation: progesterone →20-α-hydroxy-progesterone)	Akr1c18
Steroid 5-alpha reductase 2 (Conversion of testosterone → DHT)	Srd5a2

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

$$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$$

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

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of ≥ 35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

Each RQ value obtained for a given gene was normalized by dividing by the RQ value obtained for a randomly chosen control animal. Thus, control rat RT5F3111 was used for the ovary evaluations and control rat RT5F3110 for the liver evaluations.

II. Results and discussion

A. Mortality

There were no mortalities during the course of the study.

B. Clinical signs

No clinical signs were recorded for any animal during the study.

Analyses of vaginal smears prepared for the 24 h sacrifice group prior to termination revealed that thiacloprid treatment did not lead to any relevant changes of the estrous cycle.

Table 5.8.2/18-3: Results of estrous cycle staging conducted prior to necropsy

Treatment	Incidence of observations* (%)			
	Pre-estrous	Estrous	Post-estrous	Di-estrous
Control	3/15 (20)	4/15 (27)	5/15 (33)	3/15 (20)
60 mg/kg bw thiacloprid	1/15 (7)	3/15 (20)	8/15 (53)	3/15 (20)

*: (x/y): number of animals affected / total number of animals

C. Body weight

There were no effects on terminal body weight 24 h after single oral treatment with thiacloprid.

D. Hormone analyses

To minimise variability in hormone measurements, the time of dosing was adjusted so as to ensure that all blood samples were obtained between 9 am and 10:30 am.

A significant increase in plasma progesterone concentration compared to the relevant control concentration was recorded 8 h (+56.7%; $p \leq 0.01$) and 24 h (+81.4%; $p \leq 0.01$) after treatment with thiacloprid. These increases were considered biologically relevant.

The results for testosterone were inconclusive due to the fact that this hormone could only be detected in the plasma of relatively few control females (7/15, 3/15 or 4/15 at 2 h, 8 h or 24 h after treatment, respectively).

This hormone could, however, be detected more readily in the plasma of treated females (12/15, 9/15 or 5/15 at 2 h, 8 h or 24 h after treatment, respectively).

There were no significant changes in plasma estradiol concentration following treatment with thiacloprid.

Hormone data are summarized in Table 5.8.2/18-4 below.

Table 5.8.2/18-4: Hormone data

60 mg/kg thiacloprid		% Change compared to control		
		Progesterone	Testosterone	Estradiol
Sample times after dosing	2 h	+10.7	+14.3	-22.1
	8 h	+56.7**	+66.7	-16.1
	24 h	+81.4**	-13.3	-8.0

**: statistically significant, $p \leq 0.01$

E. Organ weight

There were no effects on absolute and relative organ weights of liver, ovary, uterus, adrenal and pituitary gland 24 h after single oral administration of thiacloprid.

F. qPCR analysis

Ovary:

In the ovary there were no statistically significant changes in gene expression, only marginal increases were recorded for Hsd17b3 (+57%, conversion of androstenedione to testosterone), Hsd3b1 (+70%, conversion of pregnenolone to progesteron) and Insl3 (+125%, associated with thecal cell activity) expression. The large increase in Cyp3a3 expression was due mainly to one animal (RT6F3135), which had an RQ of 535.07 and was considered to be non-relevant as the average threshold cycles (CTs) were 36.1 and 34.8 for control and treated samples respectively.

Table 5.8.2/18-5: Gene expression in the ovary analysed by quantitative PCR (RT-PCR)

Ovarian gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% Change compared to control mean values)	
	Control	60 mg/kg bw thiacloprid
Steroidogenesis		
StAR	1.1 ± 0.4	1.2 ± 0.2 (+9%)
Cyp11a1	1.4 ± 0.5	1.3 ± 0.3 (-7.1%)
Cyp17a1	0.4 ± 0.3	0.3 ± 0.3 (-25%)
Cyp19a	0.3 ± 0.3	0.4 ± 0.4 (+33%)
Hsd17b1	0.9 ± 0.2	0.9 ± 0.3
Hsd17b3	**0.7 ± 0.2**	**1.1 ± 1.4 (+57%)**
Hsd3b1	**1.0 ± 0.3**	**1.7 ± 2.4 (+70%)**
Nr5a1	0.7 ± 0.2	0.7 ± 0.1
Insl3	**0.4 ± 0.3**	**0.9 ± 1.0 (+125%)**
Metabolism		
Cyp1a1	0.8 ± 0.3	1.0 ± 1.5 (+25%)
Cyp3a3	1.2 ± 1.3	40.6 ± 137.9 (+3275%)*
Akr1c18	0.8 ± 0.4	0.7 ± 0.3 (-12.5%)
Srd5a2	17.1 ± 10.2	13.4 ± 7.7 (-36.4%)

*: change mainly due to one animal, considered to be non-relevant (for details see paragraph above)

Liver:

A number of gene transcripts, particularly those associated with metabolism were up-regulated in the liver of females treated with thiacloprid. Cyp1a1 ($p \leq 0.01$) and Cyp3a3 ($p \leq 0.01$) were statistically significantly up-regulated. Akr1c18, which is responsible for the metabolism of progesterone to its inactive metabolite 20-α-hydroxyprogesterone, was also up-regulated but not statistically significant. Cyp17a1 ($p \leq 0.01$; conversion of progesterone to androstenedione) and Hsd17b3 ($p \leq 0.05$; conversion of androstenedione to testosterone), were up-regulated. StAR ($p \leq 0.01$) was statistically significantly up-regulated, although the fold-change was only marginal compared to the other significantly up-regulated genes.

Cyp 19a1 and Insl3 were not expressed in the liver samples and Srd5a2 was only very weakly expressed as evidenced by the CT > 40.

Table 5.8.2/18-6: Gene expression in the liver analysed by quantitative PCR (RT-PCR)

Hepatic gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% Change compared to control mean values)	
	Control	60 mg/kg bw thiacloprid
Steroidogenesis		
StAR	**0.9 ± 0.1**	**1.1 ± 0.3** (+22%)**
Cyp11a1	0.7 ± 0.5	1.1 ± 1.7 (+57%)
Cyp17a1	**0.9 ± 0.5**	**1.8 ± 1.0** (+100%)**
Cyp19a1	ND	ND
Hsd17b1	0.8 ± 0.2	0.8 ± 0.2
Hsd17b3	**2.8 ± 1.6**	**4.5 ± 2.3* (+61%)**
Hsd3b1	0.8 ± 0.4	1.2 ± 1.2 (+50%)
Nr5a1	1.0 ± 0.5	1.3 ± 1.1 (+30%)
Insl3	ND	ND
Metabolism		
Cyp1a1	**2.1 ± 2.7**	**6.9 ± 4.1** (+229%)**
Cyp3a3	**0.85 ± 0.7**	**26.4 ± 25.7** (+3006%)**
Akr1c18	0.8 ± 0.4	5.3 ± 9.1 (+563%)
Srd5a2	0.15 ± 0.3	0.08 ± 0.1 (-46%)

*: statistically different from the control group ($p \leq 0.05$)
**: statistically different from the control group ($p \leq 0.01$)

III. Conclusion

Treatment of adult female rats with a single oral dose of 60 mg/kg bw thiacloprid caused an increase in plasma progesterone concentration and a significantly increased expression of several genes associated with the regulation of steroid hormone synthesis 24 h after dosing. These findings were considered biologically relevant.

Report: KCA 5.8.2/19 [redacted]; 2009; M-360362-01-1
Title: Thiacloprid – Evaluation of hormone levels in female rats 2 and 8 hours after 4 days exposure by oral gavage
Report No.: SA 07011
Document No.: M-360362-01-1
Guidelines: No applicable guideline
Deviation(s): not applicable
GLP: Non-GLP (no specific Quality Assurance inspections were conducted and dose formulations were not analysed, but performed according to standard operating procedures, which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material: thiacloprid

Description: light brown solid

Lot/Batch no: EDE 0011099

Purity: 99%

Stability of test compound: guaranteed for study duration; expiry date: 2008-02-25

2. Vehicle: 0.5% aqueous methyl cellulose 400 (dosing suspensions were prepared freshly for each administration)

3. Test animals:

Species: rat

Strain: Wistar rat, Rj:WI(IOPS HAN)

Age: approximately 11 weeks

Sex: females

Weight at dosing: 236 – 270 g

Source: [redacted], France

Acclimatisation period: at least 13 days

Diet: certified rodent pelleted and irradiated diet "A04CP1-10" from [redacted], France), *ad libitum*

Water: tap water (filtered and softened), *ad libitum*

Housing: individually in suspended stainless steel wire mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose: 0 and 60 mg/kg bw/day

Application route: oral (gavage)

Application volume: 5 mL/kg bw

Duration: 4 days

Group size: 15 females

Sacrifice: 2 and 8 hours after the last application

Table 5.8.2/19-1: Group size and treatment

Group	Test substance	Dose levels (mg/kg bw)	Sacrifice time (h after last treatment)	Number of rats per group
1	Control	0	8	15
2	Thiacloprid	60		15
3	Control	0	2	15
4	Thiacloprid	60		15

Observations: mortality, clinical signs, body weight, food intake, test substance intake, hormonal analyses (progesterone, testosterone, estradiol, FSH), estrus cycle staging (by vaginal smear analyses prior to sacrifice), gross necropsy, organ weight (liver, ovary, uterus, adrenal glands), histopathology (liver, ovary, uterus, adrenal gland, vagina)

II. Results and discussion

A. Mortality

No mortalities occurred during the course of the study.

C. In life observations

There were no faeces observed on Study Days 2 and 3 for one female that was treated with thiacloprid (Group 2, 8 h sacrifice group). Constipation and reduced defecation are known signs of thiacloprid toxicity, which were observed in female rats on treatment days 1-4 after oral gavageadministration of 60 mg/kg bw/day of thiacloprid also in the pilot subacute toxicity study by [redacted] (1995, M-000703-01-4).

Thiacloprid treatment significantly reduced mean body weights of all animals when compared to the appropriate controls. On Day 2 mean body weight was significantly reduced by 3.95% ($p \leq 0.01$) in Group 2 (8 h sacrifice) and by 3.6% ($p \leq 0.05$) in Group 4 (2 h sacrifice), while on Day 3 mean body weight was significantly reduced by 7.9% ($p \leq 0.01$) in Group 2 (8 h sacrifice) and by 6.8% ($p \leq 0.01$) in Group 4 (2 h sacrifice).

Overall, between Study Days 1 and 3, thiacloprid treatment resulted in a significant ($p \leq 0.01$) cumulative body weight loss of 17 g (Group 2, 8 h sacrifice) or 15 g (Group 4, 2 h sacrifice), respectively.

D. Vaginal smears

Vaginal smears were taken from all animals before sacrifice to determine the stage of the estrous cycle.

There were no indications that thiacloprid treatment led to relevant changes of the estrous cycle. The results of the investigation of the vaginal smears are presented in the following table.

Table 5.8.2/19-2: Results of estrous cycle staging conducted in vaginal smears prior to necropsy

Thiacloprid (mg/kg bw/day)	Sacrifice time (h**)	Incidence of observation* (%)			
		Pre-estrous	Estrous	Post-estrous	Di-estrous
0	2	0/15 (0)	4/15 (26.7)	5/15 (33.3)	6/15 (40)
60		1/15 (6.7)	2/15 (13.3)	8/15 (53.3)	4/15 (26.7)
0	4	1/15 (6.7)	5/15 (33.3)	4/15 (26.7)	5/15 (33.3)
60		0/15 (0)	4/15 (26.7)	5/15 (33.3)	6/15 (40)

*: (x/y): number of animals affected / total number of animals
**: after the last of 4 daily doses

E. Hormone analysis

To minimize the interanimal variability in the hormone measurements, the time of dosing was adjusted for the final dose so as to ensure that all rats were sacrificed and all blood samples were obtained between 9 am and 10:30 am.

Thiacloprid treatment significantly (p ≤ 0.01) increased the progesterone plasma concentration of all animals 2 h (+ 70%) or 8 h (54.2%) after the last administration. These increases were considered biologically relevant.

The results for testosterone were inconclusive, because it was detected only in one animal of the control groups but in 9/15 or 8/15 animals 2 h or 8 h after the last treatment with thiacloprid, respectively.

While FSH plasma concentrations were unaffected by treatment, estradiol was not detected in the plasma of any female rat. Re-analysis of the plasma samples for estradiol by an external laboratory using a different RIA kit confirmed that the estradiol concentrations were below or close to the limit of detection for all females in all groups. No conclusions could, therefore, be drawn concerning the effect of thiacloprid treatment on this hormone in the present study. Technical problems are considered to be responsible for this occurrence as it is unlikely that the estradiol levels in all the adult females in all four groups would, under normal circumstances, be below the limit of detection.

Table 5.8.2/19-3: Hormone concentrations in plasma

Thiacloprid dose (mg/kg bw)	Sacrifice time (h)[A]	Hormone concentration (% change compared to control)			
		Progesterone (ng/mL)	Testosterone (ng/mL)	Estradiol (pg/mL)	FSH (ng/mL)
0	2	23.69 ± 12.96	nd	nd	5.8 ± 1.8
60		40.34 ± 13.29** (+70.3)	0.07 ± 0.04	nd	5.8 ± 1.0 (nc)
0	8	20.98 ± 9.52	0.05[B]	nd	6.5 ± 1.4
60		32.35 ± 10.58** (+54.2)	0.05 ± 0.04 (nc)	nd	6.5 ± 1.4 (nc)

**: p ≤ 0.01
A: sacrifice time after the last of four daily oral doses
B: testosterone was detected in the plasma of only one control female
nd: not detected as concentrations were below the limit of detection
nc: no change

F. Terminal body weight and organ weight

Thiacloprid treatment significantly (p ≤ 0.01) reduced the mean terminal body weight of the animals sacrificed at the 2 h (-11.7%) and the 8 h (-9%) time-point compared to control females.

This reduction was reflected in the absolute liver weight, which was also significantly (p ≤ 0.01) reduced at both time points (-14.7% or -16.5% for females sacrificed 2 or 8 h, respectively, after the last thiacloprid dose). The relevance of these changes is unclear as the liver was not subjected to microscopic examination.

Thiacloprid treatment significantly (p ≤ 0.01) increased the absolute and relative weight of the adrenal gland for females sacrificed 2 and 8 h after the last dose of thiacloprid (absolute and relative weights: 36.1% and 53.7% after 2 h, 20.8% and 32.1% after 8 h).

Thiacloprid treatment significantly (p ≤ 0.05) reduced the absolute ovary weight by 12.2% and 15.1% at the 2 h and 8 h time-points, respectively.

The weight changes of adrenal gland and ovary were not associated with any morphological changes.

The following table depicts thiacloprid-induced effects on relative terminal body and organ weights.

Table 5.8.2/19-4: Mean terminal body weights and organ weights

Sample time (h after the last dose)	Mean terminal body weight / mean organ weight ± SD (% change in comparison to controls)		
	Thiacloprid dose (mg/kg bw/day)	**0**	**60**
2	**Terminal Body Weight (g)**	**257.9 ± 10.3**	**227.8 ± 8.9** (-11.7%)**
	Absolute liver weight (g)	**8.51 ± 0.80**	**7.26 ± 0.78** (-14.7%)**
	Liver to body weight ratio (%)	3.295 ± 0.215	3.184 ± 0.289 (-3.4%)
	Absolute adrenal weight (g)	**0.0692 ± 0.0072**	**0.0942 ± 0.015** (+36.1%)**
	Adrenal to body weight ratio (%)	**0.02691 ± 0.00317**	**0.04135 ± 0.00616** (+53.7%)**
	Absolute ovary weight (g)	**0.082 ± 0.011**	**0.072 ± 0.012* (-12.2%)**
	Ovary to body weight ratio (%)	0.0320 ± 0.0043	0.0318 ± 0.0052 (nc)
8	**Terminal Body Weight (g)**	**250.1 ± 10.4**	**227.6 ± 5.8** (-9.0%)**
	Absolute liver weight (g)	**8.49 ± 0.86**	**7.09 ± 0.49** (-16.5%)**
	Liver to body weight ratio (%)	**3.389 ± 0.243**	**3.113 ± 0.193** (-8.1%)**
	Absolute adrenal weight (g)	**0.0745 ± 0.0083**	**0.0900 ± 0.0103** (+20.8%)**
	Adrenal to body weight ratio (%)	**0.02994 ± 0.00428**	**0.03955 ± 0.00454** (+32.1%)**
	Absolute ovary weight (g)	**0.086 ± 0.020**	**0.073 ± 0.014* (-15.1%)**
	Ovary to body weight ratio (%)	0.0344 ± 0.0084	0.0320 ± 0.0057 (-7%)

*: statistically significantly different from control, $p \leq 0.05$
**: statistically significantly different from control, $p \leq 0.01$
nc: no change

G. Gross pathology

No treatment-related gross pathological alterations were detected.

H. Micropathology

No morphological changes were detected in the adrenal glands and ovaries following treatment with thiacloprid.

The uterus and vagina from each surviving female was investigated to establish the stage of the estrous cycle at sacrifice. There no effects on the estrous cycle phase due to four daily oral doses of thiacloprid between the groups.

Table 5.8.2/19-5: Results of estrous cycle staging conducted by *post mortem* histology

Thiacloprid dose (mg/kg bw)	Sacrifice time (h after last dose)	Incidence of observations* (%)		
		Proestrous	Estrous	Metestrous / Diestrous
0	2	0/15 (0)	4/15 (26.7)	11/15 (73.3)
60		0/15 (0)	2/15 (13.3)	13/15 (86.7)
0	8	1/15 (6.7)	5/15 (33.3)	9/15 (60)
60		1/15 (6.7)	5/15 (33.3)	9/15 (60)

*: (x/y): number of animals affected / total number of animals

III. Conclusion

The clear increase in plasma progesterone concentration observed at both 2 h and 8 h after the last of four daily treatments with 60 mg/kg/day thiacloprid was considered treatment-related and biologically relevant.

Report: KCA 5.8.2/20[redacted].; 2009; M-360349-01-1
Title: Thiacloprid – Evaluation of hormone levels in female rats 24 hours after 4 days exposure by oral gavage
Report No.: SA 07010
Document No.: M-360349-01-1
Guidelines: No applicable guideline
Deviation(s): not applicable
GLP: Non-GLP (no specific Quality Assurance inspections were conducted and dose formulations were not analysed, but performed according to standard operating procedures, which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material: thiacloprid
Description: light brown powder
Material No.: 000550971
Lot/Batch no.: EDE 0011099
Purity: 99%
Stability of test compound: guaranteed for study duration; expiry date: 2008-02-25
2. Vehicle: 0.5 % aqueous methyl cellulose 400
3. Test animals:
Species: rat

Strain:	Wistar rat, Rj:WI (IOPS HAN)
Age:	approximately 11 weeks
Sex:	females
Weight at dosing:	231 – 264 g
Source:	[redacted], France
Acclimatisation period:	at least 7 days
Diet:	certified rodent pelleted and irradiated diet "A04C-10" from [redacted], France), *ad libitum*
Water:	tap water (filtered and softened), *ad libitum*
Housing:	individually in suspended stainless steel wire mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0 and 60 mg/kg bw/day
Application route:	oral gavage
Application volume:	5 mL/kg bw
Duration:	once daily for 4 days
Group size:	15 females

Table 5.8.2/20-1: Group size and treatment

Group	Test substance	Dose levels (mg/kg bw)	Sacrifice time (h after the last of 4 treatments)	Number of rats per group
1	Control	0	24	15
2	Thiacloprid	60		15

Sacrifice: 24 hours after the last application

Observations: mortality, clinical signs, body weight, hormonal analyses (progesterone, testosterone, estradiol, FSH), determination of estrous cycle (by vaginal smear analyses prior to sacrifice), gross necropsy, organ weight (liver, ovary, uterus, adrenal glands), gene expression (in ovary, liver, and adrenal gland)

Table 5.8.2/20-2: Gene expression analysed by quantitative PCR

Gene (Major function)	Abbreviation
Steroidogenesis	
Steroidogenic acute regulatory protein (Cholesterol transport to inner mitochondrial membrane)	StAR
Cytochrome P450 11a1 (Cholesterol side-chain cleavage to form pregnenolone)	Cyp11a1
Cytochrome P450 17a1 (Pregnenolone → 17αhydroxypregnenolone)	Cyp17a1

(Progesterone → androstenedione)	
Hydroxysteroid dehydrogenase 3b1 (Pregnenolone → Progesterone)	Hsd3b1
Hydroxysteroid dehydrogenase 17b3 (Androstenedione → testosterone)	Hsd17b3
Hydroxysteroid dehydrogenase 17b1 (Estrone → estradiol)	Hsd17b1
Cytochrome P450 19a1 (aromatase) (Testosterone → estradiol)	Cyp19a1
Nuclear receptor subfamily 5a1 (Transcription factor controlling expression of the steroidogenic cytochrome P450 genes in endocrine tissue)	Nr5a1
Insulin-like 3 (associated with ovarian thecal cells)	Insl3
Metabolism	
Cytochrome P450 1a1	Cyp1a1
Cytochrome P450 3a3 (Inactivation: testosterone → 6βhydroxytestosterone)	Cyp3a3
Aldo-keto reductase 1c18 (Inactivation: progesterone → 20-α-hydroxy-progesterone)	Akr1c18
Steroid 5-alpha reductase 2 (Conversion of testosterone → DHT)	Srd5a2

Gapdh and beta-2 microglobulin (B2m) were selected as reference genes for the quantitative calculations of transcripts in the liver and beta-actin (Actb) was used as the reference gene for the ovary and adrenal gland calculations. The relative quantity (RQ) value of each test transcript was calculated using the following formula:

$$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$$

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

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of > 35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

Thus for the ovary evaluations, the control female used was RT1F0042. RT1F0051 was used for the liver evaluations and RT1F0043 was used for the adrenal evaluations.

II. Results and discussion

A. Mortality

Two females of the thiacloprid treatment group were found dead on study days 4 and 5. These deaths were considered treatment-related.

B. In life observations

The animal that was found dead on study day 4 exhibited the following clinical signs on day 3: no faeces and hunched posture.

The rat that was found dead prior to sacrifice had soiled fur in the mouth region, reduced motor activity and no faeces on study day 4.

In surviving animals clinical signs were recorded on Study Days 3 and 4 for all females treated with thiacloprid. These rats had either few or no faeces and several had soiled fur in the head/mouth region (40%) and/or a hunched posture (33%). Three of the 15 thiacloprid treated females exhibited piloerection (20%) and on Study Day 4 one female had reduced motor activity.

All of these clinical findings are well-known signs of acute thiacloprid intoxication.

Table 5.8.2/20-3: Summary of clinical signs

Clinical sign	Control group*	60 mg/kg bw Thiacloprid*#
Soiled fur, localized, head/mouth	0/15	6/15
Piloerection	0/15	3/15
Reduced motor activity	0/15	1/15
Few or no faeces	0/15	15/15
Wasted general appearance	1/15	0/15
Hunched posture	0/15	5/15

*: (x/y): number of animals affected / total number of animals
#: 60 mg/kg bw thiacloprid were administered once daily for 4 days by oral gavage

C. Body weight

Thiacloprid induced treatment related changes of body weight parameters. In comparison to controls mean body weight was reduced by 7.3% ($p \leq 0.01$) on Study Day 3 and by 10.4% ($p \leq 0.01$) on Study Day 4. Between Study Days 1 and 4, there was a cumulative body weight loss of 23 g ($p \leq 0.01$) in females treated with thiacloprid compared to a body weight gain of 5 g in control females.
The body weight parameters are presented in the following table.

Table 5.8.2/20-4: Mean body weight (g) and body weight gain (g)

Study day	1		2		3		4	
Dose (mg/kg bw/day)	**0**	**60**	**0**	**60**	**0**	**60**	**0**	**60**
No. of rats / group	*15*	*15*	*15*	*15*	*15*	*15*	*15*	*14*
Mean bw (g)	244	245	245	240	248	**230****	249	**223****
SD	7	8	8	8	9	7	8	7
Mean bw gain per day (g)	--	--	1	**-6****	3	**-10****	2	**-8****
SD	--	--	3	4	4	4	4	5
Mean absolute body weight gain (g)	--	--	1	**-5****	3	**-15****	5	**-23****
SD	--	--	3	4	5	5	3	5

**: statistically significantly different from control, $p \leq 0.01$
bw: body weight

D. Vaginal smears

Vaginal smears taken 24 hours after the last application were examined to determine the phase of the estrus cycle. There were no relevant changes to the estrus cycle due to thiacloprid treatment.
The results of the investigation of vaginal smears are presented in the following table.

Table 5.8.2/20-5: Results of estrous cycle staging conducted in vaginal smears prior to necropsy

Treatment	Incidence of observations* (%)			
	Pre-estrous	Estrous	Post-estrous	Di-estrous
Control	2/15 (13%)	4/15 (27%)	4/15 (27%)	5/15 (33%)
60 m/kg bw Thiacloprid	1/13 (7.7%)	1/13 (7.7%)	4/13 (30.8%)	7/13 (53.8%)

*: (x/y): number of animals affected / total number of animals

E. Hormone analysis

Thiacloprid treatment resulted in a significant increase (+74.3%; $p \leq 0.05$) in plasma progesterone concentration compared to control 24 h after the last application. This increase was considered biologically relevant.

The results for testosterone were inconclusive due to the fact that this hormone was only detected in the plasma of only 2 of 13 control females. This hormone was, however, detected in the plasma of 9 of 13 thiacloprid-treated females.

Thiacloprid treatment caused a non-significant increase of the plasma concentration of estradiol (+ 28.2%) and FSH (+ 14%).

The mean results of the determination of hormonal plasma concentrations are depicted in the following table.

Table 5.8.2/20-6: Changes of hormonal plasma concentrations

Treatment	% Change compared to control			
	Progesterone	Testosterone	Estradiol	FSH
60 mg/kg bw Thiacloprid	+74.3%*	+50% (ns)	+28.2% (ns)	+14.1% (ns)

*: statistically significantly different from control, $p \leq 0.05$
ns: not significant

F. Terminal body weight and organ weight

Thiacloprid treatment significantly reduced the mean terminal body weight compared to control females (-9.3%).

With regard to organ weights, significantly increased relative liver weight (+ 21.9%) and absolute and relative adrenal weights (+47.4% and +62.5%) were observed in thiacloprid treated animals in comparison to controls.

In contrast, thiacloprid treatment significantly decreased the mean absolute and relative ovary weights (-25.2% and -17.4%) and the absolute (-24.6%) and relative (-16.9%) uterus weights.

The following table gives an overview on terminal body weight and relative organ weights.

Table 5.8.2/20-7: Mean terminal body weight and organ weights

	Mean terminal bw / organ weight ± SD (% change in comparison to controls)	
Thiacloprid dose (mg/kg bw/day)	**0**	**60**
Terminal body weight (g)	250.2 ± 8.0	227.0 ± 6.7** (-9.3%)
Absolute liver weight (g)	8.19 ± 0.64	9.08 ± 1.76 (+10.9%)
Relative liver weight (%)	3.273 ± 0.211	3.989 ± 0.705** (+21.9%)
Absolute adrenal weight (g)	0.0686 ± 0.0105	0.1011 ± 0.0161** (+47.4%)
Relative adrenal weight (%)	0.02743 ± 0.00439	0.04457 ± 0.00725** (+62.5%)
Absolute ovary weight (g)	0.110 ± 0.008	0.083 ± 0.016** (-25.2%)
Relative ovary weight (%)	0.0443 ± 0.003	0.0366 ± 0.0072** (-17.4%)
Absolute uterus weight (g)	0.357 ± 0.062	0.269 ± 0.064** (-24.6%)
Relative uterus weight (%)	0.1429 ± 0.0256	0.1187 ± 0.0290* (-16.9%)

*: statistically significantly different from control, $p \leq 0.05$
**: statistically significantly different from control, $p \leq 0.01$

G. Gene expression

Ovary:

Genes associated with steroidogenesis showed an overall tendency of increased expression due to thiacloprid treatment. The expression of StAR and Hsd3b1 were significantly increased.

Gene associated with metabolism, i.e. Cyp17a1 (+23%), Cyp19a1 (+98%), and Hsd17b3 (39%), were increased, but the increase was not statistically significant due to high inter-animal variability.

In contrast, two genes (Cyp1a1 and Srd5a2) associated with metabolism were only weakly expressed in the ovary (control and treated), as evidenced by threshold cycles (CTs) ≥35. Cyp3a3 was only weakly expressed in the control ovary (average CT = 35.9) but expression in the ovaries of the thiacloprid treated group, though not statistically significant due to large interanimal variability, was clearly increased (+1853.3%). Akr1c18 was clearly detected in the control and treated ovary samples (CTs ~21) and was marginally up-regulated (+17.3%) due to thiacloprid treatment.The following table depicts the results of quantitative PCR analysis in the ovary.

Table 5.8.2/20-8: Gene expression analysed by quantitative PCR (RT-PCR) in ovaries

Ovarian Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values) Control	 60 mg/kg bw thiacloprid
Steroidogenesis		
StAR	**0.76 ± 0.2**	**1.27 ± 0.27** (+ 67.1%)**
Cyp11a1	1.28 ± 0.39	1.44 ± 0.29 (+ 12.5%)
Cyp17a1	4.84 ± 3.47	5.96 ± 8.39 (+ 23.1%)
Cyp19a1	3.32 ± 2.23	6.57 ± 6.20 (+ 97.9%)
Hsd17b1	0.93 ± 0.48	1.06 ± 0.8 (+ 14.0%)
Hsd17b3	0.82 ± 0.36	1.14 ± 0.54 (+ 39%)
Hsd3b1	**0.64 ± 0.34**	**1.13 ± 0.70* (+ 76.6%)**
Nr5a1	1.25 ± 0.13	1.23 ± 0.26 (nc)
Insl3	0.86 ± 0.48	0.63 ± 0.45 (- 26.7%)
Metabolism		
Cyp1a1	0.76 ± 0.60	0.41 ± 0.37 (- 46.1%)
Cyp3a3	1.84 ± 0.76	35.9 ± 67.31 (+ 1853.3%)
Akr1c18	0.98 ± 0.35	1.15 ± 0.45 (+ 17.3%)
Srd5a2	0.68 ± 0.25	0.54 ± 0.33 (- 20.6%)

*: statistically significantly different from control, $p \leq 0.05$
**: statistically significantly different from control, $p \leq 0.01$
nc: no change

Liver:

Concerning genes associated with steroidogenesis, neither Cyp19a1 nor Insl3 gene expression was detected in livers of control or thiacloprid-treated animals. Further, several other genes were only weakly expressed in the liver samples.

For example, thiacloprid treatment clearly increased Nr5a1 ($p \leq 0.01$; 189.5%), Hsd3b1 ($p \leq 0.05$; +112.7%), and Cyp11a1 (+465.7%; not statistically significant) expression in liver, but these genes were only weakly expressed in both control and thiacloprid liver samples (CTs between 34.5 and 36.1). Therefore, the modulation of these genes should probably not be further evaluated.

Thiacloprid treatment significantly ($p \leq 0.01$) increased StAR (+59.3%) and Hsd17b3 (+159.6%) expression. Thiacloprid treatment also strongly increased Cyp17a1 expression (+607%), but this increase was not statistically significant due to large inter-animal variability. Here, the variability was mainly attributed to one animal (RT2P0062). Excluding it's RQ value from the dataset still results in a significant increase of Cyp17a1 expression in the thiacloprid treatment group compared to controls ($p \leq 0.01$; refined new mean RQ for thiacloprid = 7.83 compared to a control value of 2.66).

Cyp1a1 and Cyp3a3 were readily detected in control liver and the expression of these two genes was significantly up-regulated due to thiacloprid treatment. In addition, Akr1c18 expression was also significantly increased due to thiacloprid treatment.

The following table summarizes the results of quantitative PCR analysis in the liver.

Table 5.8.2/20-9: Gene expression analysed by quantitative PCR (real-time RT-PCR) in liver

Hepatic Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values) Control	 60 mg/kg bw thiacloprid
Steroidogenesis		
StAR	**1.23 ± 0.25**	**1.96 ± 0.50** (+ 59.3%)**
Cyp11a1	1.05 ± 0.78	7.94 ± 11.16 (+ 465.7%)
Cyp17a1	2.66 ± 0.84	18.81 ± 40.0 (+ 607%)
Cyp19a1	Not detected	Not detected
Hsd17b1	1.07 ± 0.27	1.26 ± 0.36 (+ 17.8%)
Hsd17b3	**1.51 ± 0.71**	**3.92 ± 3.07** (+ 159.6%)**
Hsd3b1	**1.26 ± 0.73**	**2.68 ± 2.49* (+ 112.7%)**
Nr5a1	**0.86 ± 0.59**	**2.49 ± 2.06** (+ 189.5%)**
Insl3	Not detected	Not detected
Metabolism		
Cyp1a1	**2.24 ± 2.27**	**21.54 ± 24.90** (+ 862%)**
Cyp3a3	**0.59 ± 0.23**	**37.04 ± 17.88** (+ 6178%)**
Akr1c18	**1.08 ± 0.75**	**11.97 ± 16.76* (+ 1008%)**
Srd5a2	Not detected	Not detected

*: statistically significantly different from control, $p \leq 0.05$
**: statistically significantly different from control, $p \leq 0.01$

Adrenal gland:

Four genes associated with steroidogenesis (StAR, Cyp11a1, Hsd3b1 and Nr5a1) were readily detected in both control and thiacloprid treated adrenal gland samples (CTs ≤25). The remaining steroidogenic genes were either only weakly expressed (CTs of approximately 35) or, in the case of Insl3, not detected in the adrenal samples. Concerning genes associated with steroidgenesis, thiacloprid treatment significantly increased Cyp11a1 and Hsd3b1 expression. The relevance of all other changes in steroidogenic gene expression is unclear as the CT values were low (approximately 35).

All genes associated with metabolism were only weakly expressed in the control adrenal samples, with CT values exceeding 33. The relevance of the statistically significant ($p \leq 0.01$) increase in Akr1c18 expression is unclear due to the low average CT values for both the thiacloprid treated group (CT= 34.6) and the control group (CT= 35.1). A wide inter-animal variability was recorded for Cyp3a3 (RQs between 0.73 and 136.9) making the large increase in expression (+ 1229.8%) of this gene recorded for the thiacloprid group difficult to interpret.

The results of quantitative PCR analysis in the adrenal gland are presented in the table below.

Table 5.8.2/20-10: Gene expression analysed by quantitative PCR (real-time RT-PCR) in the adrenal gland

Adrenal gland Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values) Control	 60 mg/kg bw thiacloprid
Steroidogenesis		
StAR	0.62 ± 0.19	0.70 ± 0.17 (+12.9%)
Cyp11a1	**1.10 ± 0.16**	**1.76 ± 0.30** (+60%)**
Cyp17a1	1.92 ± 2.5	4.38 ± 4.40 (+128.1%)
Cyp19a1	11.74 ± 34.5	0.86 ± 0.94 (-92.7%)
Hsd17b1	**1.36 ± 0.57**	**1.94 ± 0.72* (+42.6%)**
Hsd17b3	**1.49 ± 0.60**	**2.33 ± 1.14* (+56.4%)**
Hsd3b1	**0.88 ± 0.16**	**1.03 ± 0.17* (+17.0%)**
Nr5a1	0.82 ± 0.17	0.78 ± 0.18 (-4.9%)
Insl3	Not detected	Not detected
Metabolism		
Cyp1a1	0.91 ± 0.95	0.54 ± 0.73 (-40.7%)
Cyp3a3	1.78 ± 3.11	23.67 ± 42.23 (+1229.8%)
Akr1c18	**1.24 ± 0.74**	**2.34 ± 0.94** (+88.7%)**
Srd5a2	0.46 ± 0.24	0.53 ± 0.31 (+15.2%)

*: statistically significantly different from control, $p \leq 0.05$
**: statistically significantly different from control, $p \leq 0.01$

III. Conclusion

Four daily doses of 60mg/kg/day thiacloprid by oral gavage led to mortality and typical signs of thiacloprid intoxication in this rat study. At this already lethal dose thiacloprid also induced steroid hormone changes, i.e. increases in plasma progesterone and to some extent estradiol, and also the increased expression of genes involved in steroid hormone biosynthesis in the ovary, liver and adrenal gland. Moderation of the steroidogenic effects was apparent, at least at the gene level, due to increased expression of those genes associated with the metabolism of steroid hormones.

Report: KCA 5.8.2/21 [redacted]; 2010; M-360757-02-1
Title: Thiacloprid – Exploratory 28-day toxicity study in the rat by dietary administration – Amendment
Report No.: SA 08054
Document No.: M-360757-02-1
Guidelines: No applicable guideline
Deviation(s): not applicable
GLP: Non-GLP (no specific Quality Assurance inspections were conducted, but performed according to standard operating procedures, which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material:	thiacloprid
Description:	light brown solid
Lot/Batch no:	EDE 0011099
Purity:	98.7%
Stability of test compound:	guaranteed for study duration; expiry date: 2009-08-28
2. Vehicle:	powdered and irradiated diet
3. Test animals:	
Species:	rat
Strain:	Wistar rat, Rj:WI (IOPS HAN)
Age:	approximately 7 weeks
Sex:	females
Weight at dosing:	approximately 180 – 212 g
Source:	[redacted], France
Acclimatisation period:	at least 14 days
Diet:	certified rodent pelleted and irradiated diet "A04CP1-10" from [redacted], France), *ad libitum*
Water:	tap water (filtered and softened), *ad libitum*
Housing:	individually in suspended stainless steel wire mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0-100-1000-1600 ppm equivalent to 0-8.0-75.2-107.7 mg/kg bw/day
Application route:	oral (diet)
Duration:	28 days
Group size:	15 females
Observations:	mortality, clinical signs, body weight, food intake, vaginal smears for estrous cycle staging, blood sampling for hormone measurements, gross necropsy, organ weight (liver, adrenal glands, uterus with cervix, ovaries), histopathology (uterus with cervix and vagina for estrous cycle staging), determination of CYP enzymes in liver samples and of aromatase in liver and ovary samples, qPCR analyses (liver, ovary)

Table 5.8.2/21-1: Gene expression analysed by quantitative PCR

Gene (Major function)	Abbreviation
Steroidogenesis	
Steroidogenic acute regulatory protein (Cholesterol transport to inner mitochondrial membrane)	StAR
Cytochrome P450 11a1 (Cholesterol side-chain cleavage to form pregnenolone)	Cyp11a1
Cytochrome P450 17a1 (Pregnenolone → 17αhydroxypregnenolone) (Progesterone → androstenedione)	Cyp17a1
Cytochrome P450 19a1 (aromatase) (Testosterone → estradiol)	Cyp19a1
Hydroxysteroid dehydrogenase 3b1 (Pregnenolone → Progesterone)	Hsd3b1
Hydroxysteroid dehydrogenase 17b1 (Estrone → estradiol)	Hsd17b1
Nuclear receptor subfamily 5a1 (Transcription factor controlling expression of the steroidogenic cytochrome P450 genes in endocrine tissue)	Nr5a1
Metabolism	
P450 (cytochrome)oxidoreductase	POR
Cytochrome P450 2b1 (ovary)	Cyp2b1
Cytochrome P450 2b2 (liver)	Cyp2b2
Cytochrome P450 3a3 (Inactivation: testosterone → 6βhydroxytestosterone)	Cyp 3a3
Aldo-keto reductase family 1, member D1 (Conversion of testosterone → 5beta dihydrotestosterone)	Akr1d1
Aldo-keto reductase 1c18 (Inactivation: progesterone →20-α-hydroxy-progesterone)	Akr1c18
Steroid 5-alpha reductase 1 (predominantly expressed in liver) (Conversion of testosterone → DHT)	Srd5a1
Steroid 5-alpha reductase 2 (Conversion of testosterone → DHT)	Srd5a2

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

$$\Delta\Delta Ct = (Ct_{test} - Ct_{B2m})_{treated} - (Ct_{test} - Ct_{B2m})_{control}$$

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

where Ct is the threshold cycle at which PCR amplification started to be significantly different from the background signal. As a Ct of ≥35 indicates that a gene is poorly expressed in the tissue investigated, any subsequent RQ data generated from such a Ct are considered as non-relevant due to an increased risk of contamination.

Each RQ value obtained for a given gene was normalized by dividing it by the RQ value obtained for a randomly chosen control animal. Thus, the RQ data for control female RT1F1879 was chosen for both the liver and ovary evaluations in the present study.

II. Results and discussion

A. Mortality

There were no mortalities during the course of the study.

B. Clinical observations

All females in the 1600 ppm high dose group had a wasted appearance. This observation was first recorded on Study Day 6 or 7. For 7/15 females this continued through to the end of the study, for the remaining females in this group, the effect was transient (Study Days 6/7 up to Study Day 22). Hair loss (abdomen, hind limbs and thorax or back) was also recorded for two 1600 ppm females between Study Days 7 and 28. All other observations were not considered to be treatment related.

C. Body weight

At 100 ppm thiacloprid there was no effect on body weight parameters.

At higher doses mean body weight was statistically significantly reduced by between 9.5% and 12.1% at 1000 ppm and between 14.2% and 18.1% at 1600 ppm throughout the treatment period when compared to controls.

Between Study Days 1 and 7, there was a statistically significant mean body weight loss/day in the mid dose group (1 g/day) and high dose group (3 g/day) when compared to the controls.

Overall, between Study Days 1 and 28, there was a significant reduction in body weight gain for females at 1000 and 1600 ppm compared to the controls. More specifically, mean cumulative body weight gains of 35 g and 23 g at 1000 ppm and 1600 ppm, respectively, were recorded compared to a mean control cumulative body weight gain of 56 g.

Table 5.8.2/21-2: Mean body weights (g) and standard deviation (SD)

Thiacloprid dose	Mean body weight (g) and standard deviation (SD)							
	0 ppm (control)		100 ppm		1000 ppm		1600 ppm	
n	*15*		*15*		*15*		*15*	
Day of study	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	197	10	195	7	194	8	194	6
7	213	13	212	10	188**	9	177**	6
14	232	13	229	12	204**	12	190**	8
21	243	16	244	11	220**	10	205**	9
28	253	15	252	14	229**	11	217**	10

n: number of animals
**: significantly different from control $p \leq 0.01$

Table 5.8.2/21-3: Mean absolute body weight gain (g) and standard deviation (SD)

Thiacloprid dose	Mean absolute body weight gain (g) and standard deviation (SD)							
	0 ppm (control)		100 ppm		1000 ppm		1600 ppm	
n	*15*		*15*		*15*		*15*	
Day of study	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7	17	6	17	5	-5**	5	-17**	7
14	35	9	34	9	11**	8	-4**	9
21	46	10	49	9	27**	8	11**	9
28	56	8	57	10	35**	8	23**	10

n: number of animals **: significantly different from control $p \leq 0.01$

D. Food consumption

At 100 ppm thiacloprid food consumption was marginally reduced by between 10.3% ($p \leq 0.05$; Study Days 8-14) and 5.4% (not statistically significant; Study Days 22-28).

Food consumption was significantly affected due to treatment with 1000 ppm and 1600 ppm thiacloprid. At 1000 ppm food consumption was reduced by between 49.7% ($p \leq 0.01$; Study Days 1-7) and 8.4% ($p \leq 0.05$; Study Days 22-28). At 1600 ppm food consumption was reduced by between 59.3% ($p \leq 0.01$; Study Days 1-7) and 21.8% ($p \leq 0.01$; Study Days 22-28).

Thus, thiacloprid treatment dose-dependently reduced food consumption over the observed time period.

Table 5.8.2/21-4: Mean food consumption (g/day) and standard deviation (SD)

Thiacloprid dose	Mean food consumption (g/day) and standard deviation (SD)							
	0 ppm (control)		100 ppm		1000 ppm		1600 ppm	
n	*15*		*15*		*15*		*15*	
Day of study	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7	18.9	2.4	17.6	1.2	9.5*	1.2	7.7*	2.3
14	21.4	8.5	19.2**	1.3	17.2*	2.0	14.4*	1.6
21	20.6	2.7	19.2	1.2	18.7	1.3	15.8*	1.0
28	20.2	1.8	19.1	1.7	18.5**	1.1	15.8*	1.5

n: number of animals
*: significantly different from control $p \leq 0.05$
**: significantly different from control $p \leq 0.01$

Achieved dose:

The mean achieved dose levels of thiacloprid during the study period were 8.034, 75.158 and 107.671 mg/kg bw/day for the low-, mid- and high-dose group, respectively.

Table 5.8.2/21-5: Group mean achieved doses of thiacloprid (mg/kg bw/day)

	Mean achieved doses (mg/kg bw/day)				
Study week	1	2	3	4	1 to 4
100 ppm Thiacloprid	8.302	8.384	7.869	7.579	8.034
1000 ppm Thiacloprid	50.53	84.31	85.00	80.79	75.158
1600 ppm Thiacloprid	69.6	121.3	123.3	116.5	107.671

E. Vaginal smears

No relevant changes to the estrous cycle due to thiacloprid treatment were recorded.

Table 5.8.2/21-6: Results of estrous cycle staging conducted in vaginal smears on the afternoon before necropsy

Thiacloprid dose	Incidence of observations* (%)			
	Pre-estrous	Estrous	Post-estrous	Di-estrous
0 ppm	1/15 (6.7%)	2/15 (13.3%)	7/15 (46.7%)	5/15 (33.3%)
100 ppm	1/15 (6.7%)	5/15 (33.3%)	1/15 (6.7%)	8/15 (53.3%)
1000 ppm	2/15 (13.3%)	4/15 (26.7%)	4/15 (26.7%)	5/15 (33.3%)
1600 ppm	5/15 (33.3%)	1/15 (6.7%)	4/15 (26.7%)	5/15 (33.3%)

*: (x/y): number of animals affected / total number of animals

F. Hormone analyses

To minimise variability in hormone measurements, blood sampling was conducted between 8:30 a.m. and 11 a.m. in the morning of the day of necropsy.

Marginal, though not statistically significant increases in plasma progesterone concentration were recorded for the thiacloprid treated females (26.2%, 40.3% and 34.3% at 100, 1000 and 1600 ppm, respectively).

The results for testosterone were inconclusive due to the fact that this hormone could be detected in the plasma of only 4/15 control females. Similarly, testosterone could only be detected in 6/15 females treated with 100 ppm. This hormone could, however, be detected more readily in the plasma of those females treated with 1000 ppm and 1600 ppm thiacloprid (10/15 and 11/15 treated females respectively).

No change in plasma estradiol concentration was observed for females treated with 100 ppm thiacloprid. However, a significant increase in plasma estradiol of +64.7 % at 1000 ppm and of +59.7 % at 1600 ppm thiacloprid was observed. This increase was considered to be biologically relevant.

Plasma FSH concentration was not affected following treatment with 100 or 1000 ppm thiacloprid , while a marginal, though not statistically significant, increase (+60%) was recorded in rats treated with 1600 ppm thiacloprid.

Table 5.8.2/21-7: Hormone data

	Hormone concentration ± standard deviation (% change compared to control)			
Thiacloprid dose (ppm)	**0**	**100**	**1000**	**1600**
No. of animals examined	*15*	*15*	*15*	*15*
Progesterone (ng/mL)	24.8 ± 11.3	31.3 ± 12.1 (+26.2%)	34.8 ± 14.1 (+40.3%)	33.3 ± 11.1 (+34.3%)
No. of animals examined	*4*	*6*	*10*	*11*
Testosterone (ng/mL)	0.07 ± 0.01	0.1 ± 0.06 (+42.9%)	0.16 ± 0.07 (+128.6%)	0.11 ± 0.07 (+57.1%)
No. of animals examined	*15*	*15*	*15*	*15*
Estradiol (pg/mL)	11.9 ± 3.5	12.3 ± 6.4 (+3.4%)	**19.6 ± 5.7** (+64.7%)**	**19.0 ± 3.8** (+59.7%)**
No. of animals examined	*12*	*12*	*12*	*15*
FSH (ng/mL)	4.0 ± 2.7	4.9 ± 1.7 (+22.5%)	4.3 ± 2.3 (+7.5%)	6.4 ± 3.1 (+60%)

**: significantly different from control $p \leq 0.01$

G. Necropsy

Organ weights

Dietary concentrations of 100 ppm thiacloprid had no effect on terminal body weight or organ weight parameters. At dietary concentrations of 1000 and 1600 ppm thiacloprid mean terminal body weights were significantly lower (-10.3% and -15.1% respectively) than in control females.

In addition, the absolute and relative liver weights were statistically significantly higher for females treated with 1000 ppm and 1600 ppm thiacloprid when compared to the controls.

The increases in mean ovary weight (absolute and relative) are difficult to interpret as there was no dose response and microscopic examination of the ovary was not performed.

The other organ weight changes were considered to be incidental and not treatment-related.

Table 5.8.2/21-8: Mean terminal body weight and mean organ weight

	Mean ± Standard deviation (% change as compared to controls)			
Thiacloprid dose (ppm)	**0**	**100**	**1000**	**1600**
Terminal body weight (g)	257.1 ± 15.8	255.2 ± 14.3 (nc)	**230.6** ± 10.7 (-10.3)**	**218.3** ± 9.7 (-15.1)**
Mean absolute liver weight (g)	8.7 ± 0.95	8.76 ± 0.86 (nc)	**10.08* ± 0.81 (+15.7)**	**10.71** ± 0.99 (+23)**
Mean relative liver weight (%)	3.384 ± 0.244	3.431 ± 0.227 (nc)	**4.370* ± 0.233 (+29.1)**	**4.898* ± 0.263 (+44.7)**
Mean absolute ovary weight (g)	0.077 ± 0.018	0.134 ± 0.182 (+74)	0.088 ± 0.013 (+14.3)	0.079 ± 0.012 (+2.6)
Mean relative ovary weight (%)	0.0298 ± 0.0066	0.0522 ± 0.0703 (+75.2)	**0.0382* ± 0.0061 (+28.2)**	**0.0362* ± 0.0049 (+21.5)**

Thiacloprid dose (ppm)	Mean ± Standard deviation (% change as compared to controls) 0	100	1000	1600
Mean absolute adrenal gland weight (g)	0.0606 ± 0.0062	0.0631 ± 0.0104	0.0582 ± 0.0084	0.0534 ± 0.0079
Mean relative adrenal weight (%)	0.02361 ± 0.00253	0.02474 ± 0.00407	0.2530 ± 0.00391	0.02442 ± 0.00332
Mean absolute uterus weight (g)	0.495 ± 0.200	0.471 ± 0.148	0.477 ± 0.231	0.403 ± 0.159
Mean relative uterus weight (%)	0.1937 ± 0.0780	0.1855 ± 0.0615	0.2066 ± 0.0981	0.1851 ± 0.0736

*: significantly different from control $p \leq 0.05$
**: significantly different from control $p \leq 0.01$
nc : no change

Gross pathology

Enlarged and dark livers were noted in the majority of females in the 1000 and 1600 ppm groups. All other findings were considered incidental.

Table 5.8.2/21-9: Gross pathological findings

Dose (ppm)	Number affected / total number examined 0	100	1000	1600
Enlarged liver	0/15	0/15	**14/15**	**15/15**
Dark liver	0/15	0/15	**14/15**	**11/15**
Atropic/small adrenal glands	0/15	0/15	0/15	1/15
Enlarged ovaries	0/15	1/15	0/15	0/15
Dilation of uterus horns	4/15	3/15	5/15	2/15
Atropic/small uterus	0/15	0/15	2/15	4/15

Microscopic pathology

Microscopic examination of the uterus and vagina was conducted to establish the stage of the estrous cycle. There were no treatment-related changes to the estrus cycle observed in any dose group.

Table 5.8.2/21-10: Results of estrous cycle staging conducted by *post mortem* histology

Thiacloprid dose	Incidence of observations (%)		
	Pre-estrous	Estrous	Metestrous / Diestrous
0 ppm	6/15 (40)	2/15 (13.3)	7/15 (46.7)
100 ppm	4/15 (26.7)	3/15 (20)	8/15 (53.3)
1000 ppm	4/15 (26.7)	3/15 (20)	8/15 (53.3)
1600 ppm	2/15 (13.3)	5/15 (33.3)	8/15 (53.3)

*: (x/y): number of animals affected / total number of animals

H. Hepatotoxicity testing

There were no treatment-related changes at 100 ppm thiacloprid. At 1000 and 1600 ppm thiacloprid there was a dose-related increase in total P-450 content, as well as in BROD and PROD activity, indicating a phenobarbital-like profile for hepatic enzyme induction.

Table 5.8.2/21-11: Hepatotoxicity testing

Thiacloprid dose (ppm)	Mean ± SD (change as compared to control)			
	0	100	1000	1600
Number of animals examined	*6*	*6*	*6*	*6*
Total P-450 content [nmol/mg prot)	1.13 ± 0.11	1.22 ± 0.08 (nc)	1.80 ± 0.10 (x1.6)	1.92 ± 0.26 (x1.7)
PROD [pmol/min/mg protein]	3.00 ± 1.28	3.33 ± 0.56 (nc)	25.29 ± 7.21 (x8.4)	23.20 ± 10.79 (x7.7)
BROD [pmol/min/mg protein]	3.17 ± 1.86	2.42 ± 0.90 (nc)	104.5 ± 64.12 (x33)	187.34 ± 73.26 (x59.1)

nc: no change

I. Aromatase enzyme activity

Aromatase enzyme activity was directly measured by estradiol production and indirectly by tritiated water production. The aromatase assay with the unspecific tritiated water method was used in several previous studies on thiacloprid, in which a dose related increase in aromatase activity in liver samples had been detected. However, since the release of tritiated water could also have been triggered non-specifically by other Cyp 450 enzymes, the tritiated water method was compared in the current study with the new, specific method (measurement of estradiol production) in liver samples in order to see if the old results were valid.

Estradiol measurement

Measurements were conducted in the presence and absence of a specific aromatase inhibitor (anastrozole) so as to determine the background levels of estradiol present in the homogenate and microsome preparations.

At 100 ppm thiacloprid, there was no change in ovarian aromatase activity compared to the control levels. A non-significant reduction in estradiol production, indicating a marginal inhibition of aromatase activity, was recorded for the ovarian samples from the females treated with 1000 ppm (16.7%) and 1600 ppm (45.6%) thiacloprid.
In liver microsomes no aromatase enzyme activity was observed.

Table 5.8.2/21-12: Aromatase enzyme activity in ovary and liver determined by estradiol production *in situ*

Thiacloprid dose (ppm)	Corrected estradiol production (pg/mL) †	
	Ovary	Liver
0	2297.9 ± 1641.5	10.0 ± 9.5
100	2219.2 ± 1957.3	0.1 ± 18.4
1000	1913.6 ± 744.1	-7.1 ± 7.5
1600	1250.3 ± 517.1	-2.3 ± 1.9

†: Estradiol concentration corrected for background estradiol level; i.e. (pg/mL estradiol in absence of anastrozole) – (pg/mL estradiol in presence of anastrozole).

Tritiated water measurement

In liver microsomes of rats treated with 1000 ppm and 1600 ppm thiacloprid a statistically significant increase of tritiated water production was observed. Thus, the tritiated water data indicated, indirectly, an apparent increase in hepatic aromatase activity due to thiacloprid treatment. However, since this is not confirmed by the specific assay (measurement of estradiol production), this is not a valid result. This applies also to the results in the previous rat studies.

Table 5.8.2/21-13: Apparent aromatase enzyme activity in liver microsomes determined by tritiated water production *in situ* (invalid method)

Thiacloprid dose (ppm)	Mean aromatase activity (pmol/min/g)	% of control
0	7.2 ±2.5	--
100	9.1 ±2.1	+26.4%
1000	27.5 ±4.1*	+281.9%
1600	29.7 ±5.4*	+312.5%

*: significantly different from control $p \leq 0.01$

J. qPCR analysis

Ovary

An increase in Cyp17a1 gene expression associated with steroidogenesis was observed in all thiacloprid treatment groups compared to the controls (33.8%, 156.3%, and 93% at 100, 1000 and 1600 ppm). This was more apparent when considering only those females in metestrus/diestrus (63.6%, 156.7% and 237.1% at 100, 1000 and 1600 ppm, the increase at 1600 ppm was statistically

significant). All other changes in expression of genes associated with steroidogenesis were considered to be incidental.

The following table depicts the ovarian gene expression associated with steroidogenesis.

Table 5.8.2/21-14: Gene expression of enzymes associated with steroidogenesis analysed by quantitative PCR in ovaries

Ovarian Steroidgenesis Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values)			
	Control	100 ppm thiacloprid	1000 ppm thiacloprid	1600 ppm thiacloprid
Ovary: all data				
StAR	0.79 ± 0.3	0.87 ± 0.2 (+10.1%)	0.88 ± 0.2 (+11.4%)	0.87 ± 0.2 (+10.1%)
Cyp11a1	0.73 ± 0.3	0.88 ± 0.4 (+20.5%)	0.88 ± 0.4 (+20.5%)	0.68 ± 0.3 (-6.8%)
Cyp17a1	7.1 ± 4.9	9.5 ± 9.4 (+33.8%)	**18.2 ± 15.1* (+156.3%)**	13.7 ± 14.2 (+93%)
Cyp19a1	4.4 ± 3.9	4.1 ± 3.8 (-6.8%)	4.0 ± 2.9 (-9.1%)	3.6 ± 3.2 (-18.2%)
Hsd17b1	0.95 ± 0.3	0.96 ± 0.4 (nc)	**0.74 ± 0.2* (-22.1%)**	0.74 ± 0.4 (-22.1%)
Hsd3b1	0.92 ± 0.3	1.08 ± 0.6 (+17.4%)	1.0 ± 0.3 (+8.7%)	0.92 ± 0.3 (nc)
Ovarian: Metoestrus / dioestrus				
StAR	0.75 ± 0.4	0.89 ± 0.3 (+18.7%)	0.87 ± 0.2 (+16%)	0.85 ± 0.2 (+13.3%)
Cyp11a1	0.83 ± 0.3	1.05 ± 0.3 (+26.5%)	1.11 ± 0.3 (+33.7%)	0.79 ± 0.3 (-4.8%)
Cyp17a1	7.09 ± 4.2	11.6 ± 10.9 (+63.6%)	18.2 ± 14.2 (+156.7%)	**23.9 ± 11.6** (+237.1%)**
Cyp19a1	3.67 ± 5.2	3.07 ± 3.2 (-16.3%)	3.72 ± 3.0 (nc)	4.52 ± 3.2 (+23.2%)
Hsd17b1	0.96 ± 0.4	0.98 ± 0.4 (nc)	0.75 ± 0.2 (-21.9%)	0.87 ± 0.3 (-9.4%)
Hsd3b1	0.85 ± 0.3	1.04 ± 0.6 (+22.4%)	1.14 ± 0.4 (+34.1%)	0.95 ± 0.2 (+11.8%)

*: significantly different from control $p \leq 0.05$
**: significantly different from control $p \leq 0.01$
nc: no change

Akr1c18 gene expression was increased in all thiacloprid treatment groups compared to the control group (25.3%, 23.3% and 32.2% at 100, 1000 and 1600 ppm). This change was more evident when considering only those females in metestrus/diestrus where the increases at 1000 and 1600 ppm (63.6 and 111.1%) were statistically significantly different compared to the appropriate control. The large increase in Cyp3a3 expression observed for the 1600 ppm thiacloprid treatment group was considered as non-relevant due to the high Cts and was mainly due to one female with an RQ of 76.6. All other changes in the expression of genes associated with metabolism were considered to be incidental. Data on ovarian gene expression associated with metabolism are shown in the table below.

Table 5.8.2/21-15: Gene expression of enzymes associated with metabolism analysed by quantitative PCR in ovaries

Ovarian Metabolism Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values) Control	100 ppm thiacloprid	1000 ppm thiacloprid	1600 ppm thiacloprid
Ovary: all data				
Por	1.18 ± 0.4	1.27 ± 0.3 (+7.6%)	1.28 ± 0.3 (+8.5%)	1.21 ± 0.5 (+2.5%)
Cyp2b1	0.74 ± 1.8	0.46 ± 0.5 (-37.8%)	0.45 ± 0.4 (-39.2%)	0.44 ± 0.2 (-40.5%)
Cyp3a3	1.74 ± 1.7	1.55 ± 1.5 (-10.9%)	1.71 ± 1.7 (nc)	8.48 ±19.7 (+387.4%)
Akr1d1	1.21 ± 1.4	0.83 ± 0.9 (-31.4%)	1.4 ± 0.8 (+15.7%)	1.51 ± 1.6 (+24.8%)
Akr1c18	1.46 ± 0.8	1.83 ± 1.1 (+25.3%)	1.8 ± 0.6 (+23.3%)	1.93 ± 0.7 (+32.2%)
Srd5a1	1.12 ± 1.2	1.36 ± 1.1 (+21.4%)	1.16 ± 1.3 (+3.6%)	1.35 ± 1.2 (+20.5%)
Srd5a2	nd	nd	nd	nd
Ovarian: Metoestrus / dioestrus				
Por	1.23 ± 0.4	1.40 ± 0.3 (+13.8%)	1.31 ± 0.2 (+6.5%)	1.47 ± 0.5 (+19.5%)
Cyp2b1	0.31 ± 0.3	0.72 ± 0.6 (+132.3%)	0.55 ± 0.5 (+77.4%)	0.45 ± 0.2 (+45.2%)
Cyp3a3	1.76 ± 1.6	2.05 ± 1.6 (+16.5%)	2.18 ± 1.9 (+23.9%)	11.9 ± 26.3 (+576.1%)
Akr1d1	1.1 ± 1.0	0.57 ± 0.5 (-48.2%)	1.43 ± 0.5 (+30%)	1.95 ± 2.0 (+77.3%)
Akr1c18	0.88 ± 0.3	1.39 ± 1.0 (+58%)	**1.44 ± 0.4* (+63.6%)**	**1.86 ± 0.9* (+111.4%)**
Srd5a1	0.86 ± 0.5	1.25 ± 0.8 (+45.3%)	0.79 ± 0.4 (-8.1%)	0.67 ± 0.5 (-22.1%)
Srd5a2	nd	nd	nd	nd

*: significantly different from control, $p \leq 0.05$
nd: not detected
nc: no change

Liver

Cyp19a1 and Nr5a1gene expression could not be detected in control and thiacloprid treated liver samples and all other genes associated with steroidgenesis were only weakly expressed in the liver samples as evidenced by Cts of between 32.8 and 35.6. At 1600 ppm, marginal, though not statistically significant increases were observed for StAR (+33%), Cyp11a1 (39.2%) and Hsd3b1 (+28.6%) when compared to the controls.

However, with regard to genes associated with metabolism thiacloprid treatment affects several genes in liver. 1000 ppm and 1600 ppm thiacloprid treatment significantly increased the expression of Por (+172.3 and +308.5%, respectively), Cyp3a3 (+3988.7 and +7740.4 %, respectively), and Akr1d1 (+29.9 and +78.2%, respectively). Cyp2b2 expression was already increased at 100 ppm (+1475.4 %) thiacloprid and further dose-dependently at 1000 and 1600 ppm (+39344 and +48507%, respectively). Srd5a1 gene expression was significantly reduced at 1000 and 1600 ppm thiacloprid (-20.6 and -29.9%, respetively).

The following table depicts hepatic gene expression associated with steroidgenesis and metabolism.

Table 5.8.2/21-16: Gene expression analysed by quantitative PCR in liver

Hepatic Gene transcripts	Mean Relative Quantity ± standard deviation of gene transcripts (% change compared to control mean values) Control	100 ppm thiacloprid	1000 ppm thiacloprid	1600 ppm thiacloprid
Steroidgenesis				
StAR	0.94 ± 0.2	1.05 ± 0.2 (+11.7%)	1.05 ± 0.3 (+11.7%)	1.25 ± 0.56 (+33%)
Cyp11a1	17.1 ± 9.6	13.76 ± 5.5 (-19.5%)	17.9 ± 6.4 (+4.7%)	23.81 ± 12.2 (+39.2%)
Cyp17a1	0.51 ± 0.2	0.6 ± 0.3 (+17.6%)	**0.35 ± 0.2* (-31.4%)**	0.52 ± 0.4 (nc)
Cyp19a1	nd	nd	nd	nd
Hsd17b1	1.25 ± 0.5	1.49 ± 0.6 (+19.2%)	1.25 ± 0.7 (nc)	1.29 ± 0.5 (+3.3%)
Hsd3b1	0.77 ± 0.6	0.63 ± 0.3 (-18.2%)	0.69 ± 0.2 (-10.4%)	0.99 ± 0.6 (+28.6%)
Nr5a1	nd	nd	nd	nd
Metabolism				
Por	0.94 ± 0.2	0.91 ± 0.2 (-3.2%)	**2.56 ± 0.6** (+172.3%)**	**3.84 ± 1.2** (+308.5%)**
Cyp2b2	1.26 ± 2.0	**19.85 ± 18.9** (+1475.4 %)**	**497.0 ± 125.2** (+39344 %)**	**612.45 ± 361.2** (+48507%)**
Cyp3a3	1.51 ± 0.7	1.95 ± 0.9 (+29.1%)	**61.74 ± 18.9** (+3988.7%)**	**118.39 ± 22.1** (+7740.4 %)**
Akr1d1	0.87 ± 0.3	0.97 ± 0.4 (+11.5%)	**1.13 ±0.3* (+29.9%)**	**1.55 ± 0.6** (+78.2%)**
Akr1c18	1.01 ± 0.3	1.15 ± 0.7 (+13.9%)	0.80 ± 0.4 (-20.8%)	1.46 ± 1.3 (+44.6%)
Srd5a1	0.97 ± 0.2	1.01 ± 0.2 (+4.1%)	**0.77 ± 0.1** (-20.6%)**	**0.68 ± 0.1** (-29.9%)**
Srd5a2	nd	nd	nd	nd

*: significantly different from control, $p < 0.05$
**: significantly different from control, $p < 0.01$
nd: not detected
nc: no change

III. Conclusion

In conclusion, the increased hepatic enzyme activity, the changes in plasma sex steroid hormone concentrations and the changes in the expression of several genes associated with the regulation of steroid hormone synthesis following dietary exposure to thiacloprid at 1000 ppm and 1600 ppm for at least 28 days were considered treatment-related and biologically relevant.

Report: KCA 5.8.2/22[redacted]; 2009; M-359926-01-1
Title: Thiacloprid – Exploratory 28-day toxicity study in the aged female rat by dietary administration
Report No.: SA 08327
Document No.: M-359926-01-1
Guidelines: No applicable guideline
Deviation(s): not applicable

GLP: Non-GLP (no specific Quality Assurance inspections were conducted, but performed according to standard operating procedures, which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material: thiacloprid

Description:	light brown solid
Lot/Batch no:	EDE 0011099
Purity:	98.7%
Stability of test compound:	guaranteed for study duration, expiry date: 2008-02-25

2. Vehicle: plain diet

3. Test animals:

Species:	rat
Strain:	Wistar rat, Rj:WI (IOPS HAN)
Age:	approximately 72 weeks
Sex:	females
Weight at dosing:	357 – 470 g
Source:	[redacted], France
Acclimatisation period:	The rats were originally used as control animals in a carcinogenicity study. The study was cancelled and the 50 females used in the current study were selected from the 60 control females of the cancelled study.
Diet:	certified rodent pelleted and irradiated diet “A04CP1-10” from [redacted], France), *ad libitum*
Water:	tap water (filtered and softened), *ad libitum;*
Housing:	individually in suspended stainless steel wire mesh cages

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0, 1000 ppm equivalent to 0, 31.5 mg/kg bw/day
Application route:	oral (diet)
Duration:	28 days
Group size:	25 females

Observations:	mortality, clinical signs, body weight, food intake, test substance intake, hormonal determinations (progesterone, testosterone, estradiol), estrus cycle staging (vaginal smear analysis), gross necropsy, organ weight (liver, ovary, uterus (including cervix), adrenal glands), histopathology (uterus, vagina, one of the adrenal glands)

II. Results and discussion

A. Mortality

One female (ST2F4024) of the thiacloprid treatment group was sacrificed on study day 21 for humane reasons.

B. Clinical signs

Rat ST2F4024, which was killed on Study Day 21 for humane reasons, exhibited a wasted general appearance, ocular discharge and a soiled anogenital region. These signs were considered to be a consequence of thiacloprid treatment.

In the 1000 ppm dose group several clinical signs were considered to be a consequence of thiacloprid treatment. Few or no faeces were observed for 24 females between Study Days 8-15 and 22-29, a general wasted appearance was noted in five females between Study Days 15 and 22 and for one of these also on Study Day 29, a soiled anogenital region was seen in one rat (ST2F4024) on Study Day 21 and reduced motor activity occurred in one female on Study Day 29.

All other clinical signs were considered to be associated with the age (72 weeks at start of study) of the females and not treatment-related.

Table 5.8.2/22-1: Summary of clinical signs

Clinical signs	Control group (x/y)	1000 ppm Thiacloprid (x/y)
Reduced motor activity	0/25	1/25
No faeces	0/25	1/25
Few faeces	0/25	2/25
Wasted general appearance	0/25	4/25
Soiled anogenital region	0/25	1/25

x/y: number of animals affected / total number of animals

C. Body weight

Treatment with 1000 ppm thiacloprid had an effect on body weight parameters. Mean body weight was statistically significantly reduced by between 4.9% (Study Day 8) and 13.4% (Study Day 29) throughout the treatment period when compared to controls. At each weekly interval, there was a statistically significant mean body weight loss per day when compared to the controls. The effect was most pronounced between Study Days 1 and 15.

Overall, between Study Days 1 and 29, thiacloprid treated rats experienced a mean cumulative body weight loss of 58 g ($p \leq 0.01$), while control females exhibited a marginal mean cumulative body weight loss (1 g) during the same treatment period reflecting the age of the females used in this study. Mean body weight parameters are presented in the following table.

Table 5.8.2/22-2: Mean body weight (g) and body weight gain (g)

	Mean body weight (g) and body weight gain (g) and standard deviation (SD)									
Study day	**1**		**8**		**15**		**22**		**29**	
Thiacloprid dose (ppm)	**0**	**1000**	**0**	**1000**	**0**	**1000**	**0**	**1000**	**0**	**1000**
n	*25*	*25*	*25*	*25*	*25*	*25*	*25*	*24*	*25*	*24*
Mean bw (g)	412	414	409	**389***	408	**368****	410	**359****	411	**356****
SD	27	29	27	**25**	28	**21**	31	**20**	31	**21**
Mean bw gain per day (g)	-1	0	-1	**-4****	0	**-3****	0	**-1****	0	**-1****
SD	5	4	1	**1**	1	**1**	1	**1**	1	**1**
Mean absolute body weight gain (g)	--	--	-3	**-25****	-4	**-46****	-2	**-54****	-1	**-58****
SD			5	**10**	8	**15**	10	**15**	11	**19**

n: number of rats per group
*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$

D. Food consumption and achieved dose level

Food consumption:

A statistcally significant reduction in food consumption was recorded for thiacloprid treated females at each weekly interval. The effect was most pronounced between Study Days 1 and 15.

Table 5.8.2/22-3: Mean food consumption (g / day)

	Mean food consumption (g / day) and standard deviation (SD)							
Study day	**8**		**15**		**22**		**29**	
Thiacloprid dose (ppm)	**0**	**1000**	**0**	**1000**	**0**	**1000**	**0**	**1000**
n	*25*	*25*	*25*	*25*	*25*	*24*	*25*	*23*
Mean food consumption (g / day)	16.8	8.1**	19.2	9.9**	19.9	13.1**	19.9	14.9**
SD	1.9	2.7	2.7	3.3	2.1	2.2	2.0	2.0

n: number of rats per group
**: significantly different from control, $p \leq 0.01$

Achieved dose:

The mean achieved dose level of thiacloprid during the study period was 31.5 mg/kg bw/day.

Table 5.8.2/22-4: Group mean achieved dose of thiacloprid

Dietary concentration	Mean achieved thiacloprid dose (mg/kg bw/day) 1000 ppm				
Study week	1	2	3	4	1 to 4
Achieved thiacloprid dose (mg/kg bw/day)	20.8	26.9	36.5	41.9	31.5

E. Evaluation of estrous cycle

Comparison of the vaginal smears taken on Study Day 1 with those taken on the day of sacrifice indicated that there were no effects on the estrous cycle due to thiacloprid treatment.

However, as physiological changes in the estradiol/progesterone ratio occur in aging rats there are subsequent changes in the estrous cycle, such as a lengthening of the estrous phase or a prolonged luteal phase (repetitive pseudo pregnancy). Thus, the standard phases of the estrous cycle established for young adult female rats (i.e. pro-estrous, estrous, post-estrous and di-estrous) are not applicable to the older female. Consequently, the assessment of the estrous cycle in aged females requires the histopathological evaluation of the uterus and vagina.

Table 5.8.2/22-5: Results of estrous cycle staging conducted in vaginal smears on Study Day 1 and prior to necropsy

Thiacloprid dose [ppm]	Incidence of observations (%)							
	Study Day 1				Termination			
	Pre-estrous	Estrous	Post-estrous	Di-estrous	Pre-estrus	Estrous	Post-estrous	Di-estrous
0	5/25 (20%)	1/25 (4%)	6/25 (24%)	13/25 (52%)	1/25 (4%)	0/25 (0%)	10/25 (40%)	14/25 (56%)
1000	5/25 (20%)	0/25 (0%)	10/25 (40%)	10/25 (40%)	5/24 (21%)	2/24 (8%)	7/24 (29%)	10/24 (42%)

x/y: number of animals affected / total number of animals

F. Hormone data

To minimise variability in hormone measurements, blood sampling was conducted between 8:30 and and 11:00 a.m. on the day of sacrifice.

The plasma concentrations of testosterone were below the limit of detection for the majority of the control and treated animals.

Since the age related changes in the estrous cycle are associated with changes in circulating estradiol and progesterone levels and, in particular the ratio between these two hormones, hormone data from this study were analysed both independent of the estrous cycle stages and according to the major phases (persistent estrous and repetitive pseudopregnancy combined with ambiguous) of the cycle observed in this study.

The relevance of the increases in plasma progesterone concentrations recorded for the treated females, particularly when considering those females in persistent estrous, was unclear due to the large interanimal variability observed for the progesterone measurements in both the control group (1.79 to 129.1 ng/mL) and the treated group (1.70 to 127.6 ng/mL).

A marginal, though not statistically significant, increase (+13.1%) in plasma estradiol concentration was recorded for the treated females when considering the data independent of the different stages of the estrous cycle. This increase in plasma estradiol concentration was more apparent (+19%; $p \leq 0.05$ using a one-sided t-test) when considering the data from those females in pseudopregnancy combined with those presenting an ambiguous phase. This increase was considered to be treatment related.

Table 5.8.2/22-6: Hormone data

	Progesterone (ng/mL)		Testosterone (ng/mL)		Estradiol (pg/mL)	
Thiacloprid dose (ppm)	**0**	**1000**	**0**	**1000**	**0**	**1000**
Number of animals	*25*	*23*	*10*	*12*	*25*	*23*
Mean	28.73	30.39	0.00	0.00	8.4	9.5
Standard deviation	35.43	41.74	0.00	0.01	2.6	3.1

Table 5.8.2/22-7: Mean estradiol and progesterone levels and E2: P ratios according to stage of estrous cycle

	Estradiol (pg/mL)		Progesterone (ng/mL)		E2:P Ratio x 10^3	
Thiacloprid dose (ppm)	**0**	**1000**	**0**	**1000**	**0**	**1000**
	Persistent estrus					
Number of animals	*9*	*6*	*9*	*6*	--	--
Mean	10.992	10.592	56.565	8.290	2.1962	2.5701
Standard deviation	1.817	3.890	2.378	8.007	0.6426	2.285
	Combined repetitive pseudo-pregnancy/ambiguous stage					
Number of animals	*15*	*12*	*15*	*12*	--	--
Mean	7.025	**8.363***	44.420	31.773	0.678	0.868
Standard deviation	1.572	2.060	38.580	38.742	1.088	0.963

*: significantly different from control, $p \leq 0.05$, by one-sided t-test

Table 5.8.2/22-8: Hormone data (% change compared to control)

1000 ppm thiacloprid (number of controls, number of treated females)		% Change compared to control		
		Progesterone	**Estradiol**	**E2:P Ratio**
Phase of estrous cycle	All phases (25/23)	+ 5.1%	+13.1%	+5.4%
	Persistent estrus (9, 6)	+49.0%	-3.6%	+17.0%
	Pseudopregnancy and ambiguous (15, 12)	-28.5%	**+19.0%***	+28.0%

*: significantly different from control, $p \leq 0.05$, using a one-sided t-test

G. Necropsy

The mean terminal body weight of thiacloprid treated females was significantly lower (-14.5%) than that of the control females. In addition, treated females had a statistically significantly higher relative

liver weight (+14.9%) than the controls. The other organ weight changes were considered to be incidental and not treatment-related.

Table 5.8.2/22-9: Mean terminal body weight and mean organ weight

	Mean ± Standard deviation (% change as compared to controls)	
Thiacloprid dose (ppm)	**0**	**1000**
Number of animals examined	*25*	*24*
Terminal body weight (g)	413.4 ± 31.3	**353.6** ± 23.7 (-14.5%)**
Absolute adrenal weight(g)	0.0781 ± 0.0212	0.0722 ± 0.0196
Relative adrenal weight (%)	0.01917 ± 0.00601	0.02058 ± 0.00600
Absolute liver weight (g)	11.55 ± 1.17	11.38 ± 1.15 (-1.5%)
Relative liver weight(%)	2.804 ± 0.298	**3.222* ± 0.285 (+14.9%)**
Absolute uterus weight (g)	1.137 ± 0.897	0.946 ± 0.431
Relative uterus weight (%)	0.2809 ± 0.2294	0.2692 ± 0.1241
Absolute ovary weight (g)	0.088 ± 0.059	0.082 ± 0.063
Relative ovary weight (%)	0.0215 ± 0.0143	0.0230 ± 0.0174

**: significantly different from control, $p \leq 0.01$

Gross pathology

There were no macroscopic findings in rat ST2F4024, which was killed for humane reasons on Study Day 21, and no treatment-related lesions in the animals killed at scheduled sacrifice.

Microscopic pathology

In order to provide an accurate evaluation of the estrous cycle longitudinal sections of the vagina and cervix, transverse sections of both uterine horns, and medial sections of both ovaries are necessary. Since the ovaries from all females were frozen for possible further evaluations they were not available for histopathological evaluation to support staging. It has also to be considered that histological inconsistencies are incidentally seen between the various components of the reproductive tract in the aging rat. Therefore, staging of the estrous cycle in the aged rat is difficult.

Despite these impediments, the control females were shown to be in phases commonly associated with the physiological changes in the estradiol/progesterone ratio known to occur in aging rats. More specifically, the majority of control females were observed in either persistent estrous (corresponding with low levels of progesterone and, therefore, a high E2:P ratio) or repetitive pseudopregnancy (corresponding with an increase in progesterone levels and, therefore a reduced E2:P ratio compared to the ratio for persistent estrous).

In contrast, there were fewer thiacloprid treated females than controls exhibiting clear repetitive pseudopregnancy (27.3% *vs.* 52% in controls) and there was a higher incidence of thiacloprid treated females exhibiting an ambiguous cycle compared to the controls (27.3% *vs.* 8%).

Table 5.8.2/22-10: Incidence (%) of stages of the estrous cycle in uterus and vagina at scheduled sacrifice [E2:P ratio x 10^3]

Thiacloprid dose (ppm) / Estrous cycle	Incidence (%) of stages of the estrous cycle [E2:P ratio x 10^3]	
	0 (control)	1000
Persistent estrous	9/25 (36%) [E2:P = 2.2]	6/22 (27.3%) [E2:P = 2.6]
Repetitive pseudo-pregnancy	13/25 (52%) [E2:P = 0.65]	6/22 (27.3%) [E2:P = 0.64]
Persistent anestrous	1/25 (4%)	2/22 (9.1%)
Pro-estrous	0/25 (0%)	2/22 (9.1%)
Ambiguous female reproductive cycle	2/25 (8%) [E2:P = 0.9]	6/22 (27.3%) [E2:P = 1.1]

x/y: number of affected animals / number of animals examined

Treatment related changes concerning the level of vaginal mucification were also recorded. In particular, the level of vaginal mucification observed in thiacloprid treated females (minimal to slight) was lower than that observed in the control females (minimal to moderate). As vaginal mucification is a useful criterion to stage the cycle, this could explain the greater incidence of treated females with an ambiguous phase.

Vaginal mucification was observed mainly in those females in repetitive pseudo-pregnancy. Thus, the reduced incidence of treated females with vaginal mucification (31.8% *vs.* 64% in controls) was considered non-relevant as fewer treated than control females were in repetitive pseudo-pregnancy (27% *vs.* 52% in controls).

Table 5.8.2/22-11: Incidence and severity of vaginal mucification at scheduled sacrifice

Thiacloprid dose (ppm) / Vaginal mucification	Incidence of vaginal mucification* (%)	
	0 (control)	1000
Minimal	4/25	5/22
Slight	3/25	2/22
Moderate	9/25	0/22
Total	**16/25 (64%)**	**7/22 (31.8%)**
Incidence of females with vaginal mucification in repetitive pseudo-pregnancy	**13/16 (81.25%)**	**5/7 (71.4%)**

*: number of animals affected / total number of animals examined

III. Conclusion

Treatment of adult (aged) female rats with dietary doses of 1000 ppm (31.5 mg/kg bw/day) thiacloprid for at least 28 days caused a marginal increase in mean plasma estradiol concentration, as well as changes to the estrous cycle and to the level of vaginal mucification.

These findings were considered treatment-related and biologically relevant.

Analytical methods

An analytical method for the determination of thiacloprid by HPLC analysis in rodent diet was developed. The reference of the study report is presented under KCA 5.8.2/33, M-304485-01-1.

Report:	KCA 5.8.2/23 [redacted]; 2010; M-361492-01-1
Title:	Thiacloprid: Investigation of in vitro effects on steroidogenesis using H295R cells - Report of studies: SA 08351, SA 09029
Report No.:	SA 08351
Document No.:	M-361492-01-1
Guidelines:	No applicable guideline Deviation(s): not applicable
GLP:	Non-GLP (not subjected to specific Quality Assurance inspections, dosing solutions were not analysed; however, performed according to standard operating procedures for cell culture and hormone analyses, which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material: thiacloprid

Description:	light brown solid
Lot/Batch no:	EDE-0011099
Purity:	98.7%
Stability of test compound:	guaranteed for study duration; expiry date: 2009-08-28

2. Vehicle and positive controls: vehicle: 1% dimethylsulfoxid (DMSO)
positive controls:
Forskolin – for sex steroid hormone secretion stimulation
ketoconazole – for sex steroid hormone secretion inhibition

3. Test organism / cells:

Species:	human
Cell line:	adrenal carcinoma immortal cell line H295R
Source:	[redacted] (CRL-2128; ATCC-LGC Standarts, Manassas, VA, USA)

4. Culture maintenance:

Medium:	DMEM:F12, supplemented with ITS+ premix and 2.5% Nu-Serum I
Conditions:	75 mL flasks at 37°C and 5% CO_2

B. Study design and methods

1. Test conditions:

Cell isolation:	H295R cells isolated from flasks of ≥ 75 % confluency
Medium:	DMEM: F12, supplemented with ITS + premix and 2.5 % Nu-serum I
Test substance concentrations:	Study 1 Thiacloprid: 50, 100, 500 µM, 1mM; Forskolin: 1 µM; Ketoconazole: not used Study 2 Thiacloprid: 50, 100, 500 µM, 1mM; Forskolin: not used; Ketoconazole: 10 µM
Cell density:	H295R cells seeded into 24-well plates at a density of $3x10^6$ cells/mL and cultured for approx. 2 d h prior to treatment
Group size:	4 wells per compound/vehicle control, concentration and treatment period
Incubation time:	Study 1: 24 h and 48 h Study 2: 48 h
Incubation conditions:	37°C and 5% CO_2

2. Sample collection and analysis:

Sampling:	three aliquots of culture medium / well
Analyses:	using specific radio-immunoassay kits (Diagnostic Systems Laboratories) progesterone: DSL-3900 ACTIVE; testosterone: DSL-4000 ACTIVE; estradiol: DSL-43100 ACTIVE

H. Results and discussion

A. Cytotoxicity

Cytotoxicity was not observed following treatment of the H295R cells with 500 µM thiacloprid for 48h. Treatment of the cells with 1 mM thiacloprid for 48h gave rise to 87% viability, which was considered acceptable for the studies. However, visual inspection of the cells indicated that the cell morphology was slightly altered.

B. Hormone concentrations

Vehicle controls

Control progesterone levels were between 1.4 ng/mL (study 1) and 4.6 ng/mL (study 2) following 48h incubation and between 0.8 ng/mL and 0.92 ng/mL (study 1) following 24h incubation.

Control testosterone levels were between 1.8 ng/mL and 3.8 ng/mL (study 1) following 48h incubation and between 0.7 ng/mL and 1.9 ng/mL (study 1) following 24h incubation.

Control estradiol levels were between 1.8 pg/mL (study 2) and 9.4 pg/mL (study 1) following 48h incubation and between 8.4 pg/mL and 11.2 pg/mL (study 1) following 24h incubation.

Positive controls

Twenty-four hour treatment of the H295R cells with 1 µM forskolin led to significant increases in progesterone (+136%) and estradiol (+190%) secretion compared to the controls but had no effect on testosterone secretion. Treatment of the cells for 48h with 1 µM forskolin also significantly increased the secretion of progesterone (+37.7%) and estradiol (+342%) and marginally increased testosterone secretion (+14.7%).

Treatment of the H295R cells with 10 µM ketoconazole for 48h led to a significant inhibition of both progesterone (-50.7%) and testosterone (-99.8%) secretion and a marginal increase (+19%) in estradiol secretion.

24 h treatment:

A dose-related increase in progesterone secretion and an inhibition in testosterone secretion were recorded following 24h treatment of the cells with thiacloprid. No clear effects on estradiol were recorded following 24h treatment.

Table 5.8.2/23-1: Mean hormone concentrations and standard deviation (SD) after 24h treatment (study 1)

Thiacloprid concentration	Progesterone (ng/mL)		Testosterone (ng/mL)		Estradiol (pg/mL)	
	Mean ± SD	% change	Mean ± SD	% change	Mean ± SD	% change
0	0.9 ± 0.06	--	1.3 ± 0.5	--	9.6 ± 1.2	--
50 µM	0.9 ± 0.09	nc	0.7 ± 0.2	-42.4%	7.8 ± 0.8*	-18.9%
100 µM	**1.0 ± 0.1***	**+16.5%**	**0.6 ± 0.1***	**-56.5%**	8.0 ± 0.6	-16.0%
500 µM	**2.2 ± 0.4****	**+151.5%**	**0.5 ± 0.2***	**-60.8%**	8.6 ± 1.0	-10.3%
1 mM	**2.4 ± 0.3****	**+177.7%**	0.8 ± 0.1	-40.1%	10.9 ± 1.9	+13.8%
Forskolin concentration						
1 µM	**2.1 ± 0.3****	**+135.9%**	1.2 ± 0.5	-2.04%	**27.7 ± 3.8****	**+190.2%**

*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$
nc: no change compared to controls

48 h treatment:

There was a clear dose related and statistically significant inhibition of testosterone secretion following treatment of the cells with 50 µM – 1 mM thiacloprid, with the effects being more apparent than those observed following 24h treatment. There was no clear effect on progesterone concentration following 48h thiacloprid treatment. The effects of thiacloprid on estradiol secretion were difficult to interpret as the control levels were low and variable, particularly for study 2.

Table 5.8.2/23-2: Mean hormone concentrations after 48h treatment (study 1 and 2 combined#)

Thiacloprid concentration	Progesterone (ng/mL)		Testosterone (ng/mL)		Estradiol (pg/mL)	
	Mean ± SD	% change	Mean ± SD	% change	Mean ± SD	% change
0	3.0 ± 1.4	--	2.4 ± 0.7	--	5.5 ± 3.0	--
50 μM	2.5 ± 1.5	-16.6%	**1.1 ± 0.3****	**-53.7%**	6.5 ± 0.8	+17.8%
100 μM	2.8 ± 1.6	-5.6%	**1.1 ± 0.4****	**-55.5%**	6.2 ± 0.4	+13.1%
500 μM	3.7 ± 2.1	+23.1%	**0.8 ± 0.2****	**-68.3%**	5.7 ± 1.6	+4.1%
1 mM	2.7 ± 0.8	-11.0%	**0.8 ± 0.1****	**-69.0%**	9.8 ± 1.0**	+77.3%
Forskolin concentration						
1 μM	**2.3 ± 0.2***	**+37.74%**	3.2 ± 0.8	+14.74%	**36.3 ± 5.1****	**+341.8%**
Ketoconazole concentration						
10 μM	**2.1 ± 0.1****	**-50.7%**	**0.003 ± 0.003****	**-99.8%**	3.4 ± 1.6	+19.0%

#: only values for thiacloprid treatment were combined
*: significantly different from control, p ≤ 0.05
**: significantly different from control p ≤ 0.01
nc: no change compared to controls

III. Conclusion

The most marked effect induced by thiacloprid was a consistent and clear dose related inhibition of testosterone secretion following 24h and 48h treatment. An increase in progesterone secretion following 24h treatment was also recorded. The effects of thiacloprid on estradiol secretion were difficult to interpret as the control levels were low and variable.

Overall, sex steroid hormone secretion in the H295R cells was affected due to thiacloprid treatment, with the effects on testosterone being the most apparent. Under the same conditions of test, the positive controls, forskolin and ketoconazole induced the expected changes in steroid hormone secretion.

Report:	KCA 5.8.2/24 [redacted],; 2010; M-361609-01-1
Title:	Thiacloprid – *In vitro* investigation of steroid hormone secretion in rat ovarian preantral follicles
Report No.:	SA 09062
Document No.:	M-361609-01-1
Guidelines:	No applicable guideline Deviation(s): not applicable
GLP:	Non-GLP (not subjected to specific Quality Assurance inspections, dosing solutions were not analysed; however, performed according to standard operating procedures for cell culture and hormone analyses which were previously accepted and periodically inspected by the Quality Assurance Unit)

I. Materials and methods

A. Materials

1. Test material:	thiacloprid
Description:	light brown solid
Lot/Batch no:	EDE 0011099
Purity:	98.7%
Stability of test compound:	guaranteed for study duration; expiry date: 2009-08-28
2. Vehicle and/or positive control:	vehicle: 1% dimethylsulfoxid (DMSO) positive controls: FSH (follicle stimulating hormone) and forskolin
3. Test animals:	
Species:	rat
Strain:	Wistar rat, Rj:WI (IOPS HAN)
Age:	approximately 7 weeks
Sex:	females
Weight at dosing:	not reported / not applicable
Source:	[redacted] France
Acclimatisation period:	at least 7 days
Diet:	not reported
Water:	not reported
Housing:	not reported
4. Test system:	
Tissues:	ovaries
Maintenance medium:	on ice in DMEM/F12 medium
Follicle isolation:	follicles were released by finely mincing the ovary and mechanically separating the tissue; preantral follicles were distinguished from primordial and secondary follicles on the basis of size
Follicle culture:	24-well plate containing DMEM/F12 medium supplemented with ITS + premix and 2.5% Nu-serum I
Well allocation:	4 follicles /well
Conditions:	37°C and 5% CO_2

B. Study design and methods

1. Test conditions:	
Test cells:	preantral follicles
Medium:	DMEM/F12 medium supplemented with ITS + premix and 2.5% Nu-serum I
Test substance concentrations:	thiacloprid: 50, 100, 500 µM FSH: 5 IU Forskolin: 1 µM

Incubation time:	24 and 48 h
Group size (treatment time):	Study 1: 3 wells per compound/vehicle control (24 h) 2 wells per compound/vehicle control (48 h) Study 2: 4 wells per compound/vehicle control, and concentration (24 h and 48 h)
Incubation conditions:	37°C and 5% CO_2

2. Sample collection and analyses:

Sampling:	two aliquots per well
Analyses:	using specific radio-immunoassay kits (Diagnostic Systems Laboratories) – progesterone: DSL-3900 ACTIVE; estradiol: DSL-43100 ACTIVE

II. Results and discussion

A. Study 1: Hormone concentrations

Progesteron concentrations

An increase in progesterone secretion (+170.2%) was detected following incubation of the preantral follicles with 500 µM thiacloprid for 24 h. Progesterone (+155.3%) could only be detected in one out of three samples treated for 24 h with 5 IU FSH.

After incubation of the preantral follicles for 48 h with 500 µM thiacloprid or 5 IU FSH increases in progesterone secretion were +146.7% or 268.3%, respectively, compared to the vehicle controls.

Table 5.8.2/24-1: Study 1 - Mean progesterone concentrations in rat preantral follicles after incubation with thiacloprid or FSH for 24 or 48 h

Incubation time (h)	Compound	Compound concentration	Mean Progesterone conc. (ng/mL) ± SD	% of control
24	Vehicle control	0	0.47 ± 0.21	--
	FSH	5 IU	1.20#	+155.3%#
	Thiacloprid	50 µM	0.63 ± 0.40	+34%
		100 µM	0.47 ± 0.29	n.c.
		500 µM	1.27 ± 0.61	+170.2%
48	Vehicle control	0	3.66 ± 0.65	--
	FSH	5 IU	13.48 ± 8.41	+268.3%
	Thiacloprid	500 µM	9.03 ± 3.62	+146.7%

conc.: concentration
n.c.: no change
#: progesterone detected in only one out of three samples

Estradiol concentrations

Both thiacloprid and FSH had no clear effect on estradiol levels following 24 h treatment of the follicles. Following incubation of the follicles for 48 h increases of 23.8% and 132.5% compared to the relevant controls were recorded for 500 µM thiacloprid and 5 IU FSH, respectively.

Table 5.8.2/24-2: Study 1: Mean estradiol concentrations in rat preantral follicles after incubation with thiacloprid or FSH for 24 or 48 h

Incubation time (h)	Compound	Compound concentration	Mean estradiol conc. (ng/mL) ± SD	% of control
24	Vehicle control	0	7.15 ± 0.60	--
	FSH	5 IU	4.85 ± 1.94	-32.2%
	Thiacloprid	50 µM	7.02 ± 1.44	-1.8%
		100 µM	7.52 ± 1.45	+5.2%
		500 µM	7.96 ± 0.55	+11.3%
48	Vehicle control	0	14.08 ± 2.32	--
	FSH	5 IU	32.74 ± 12.55	+132.5%
	Thiacloprid	500 µM	17.43 ± 2.71	+23.8%

conc.: concentration

B. Study 2: Hormone concentrations

Progesterone concentrations

An increase in progesterone secretion was detected following treatment of the follicles with 500 µM thiacloprid for 24 h (+178.7%) and for 48 h (+464.7%). The significant increase in progesterone observed for 48 h treatment with 50 µM thiacloprid is difficult to interpret as the increase appeared to be only due to one of four samples (3.26 ng/mL compared to progesterone concentrations being below the limit of detection for 2 samples and 0.33 ng/mL for one sample).

Treatment of the follicles for 24 h and 48 h with 5 IU FSH led to increases in progesterone secretion of +73.8% and +394.1% respectively.

Treatment of the follicles for 48 h with 1 µM forskolin also led to an increase in progesterone concentration (+405.9%). The increase (+262.3%) at 24 h is difficult to interpret as the increase appeared to be due to only one of the four samples.

Table 5.8.2/24-3: Study 2 - Mean progesteron concentrations in rat preantral follicles after incubation with thiacloprid, FSH or forskolin for 24 or 48 h

Incubation time(h)	Compound	Compound concentration	Mean progesterone conc. (ng/mL) ± SD	% of control
24	Vehicle control	0	0.61 ± 0.29	--
	FSH	5 IU	1.06 ± 0.54	+73.8%
	Forskolin	1 µM	2.21 ± 3.11#	+262.3%#
	Thiacloprid	50 µM	0.58 ± 0.36	-4.9%
		100 µM	0.48 ± 0.16	-21.3%
		500 µM	**1.70 ± 0.13****	+178.7%
48	Vehicle control	0	0.34 ± 0.04	--
	FSH	5 IU	1.68 ± 1.14	+394.1%
	Forskolin	1 µM	1.72 ± 1.76	+405.9%
	Thiacloprid	50 µM	1.79 ± 2.07#	+426.5%#
		100 µM	0.29 ± 0.06	-14.7%
		500 µM	1.92 ± 1.45	+464.7%

conc.: concentration
**: significantly different from control, $p \leq 0.01$
#: progesterone increase only due to one out of four samples

Estradiol concentrations

Marginal, dose related increases in estradiol concentration were recorded following incubation with thiacloprid for 24 and 48 h.

Treatment with 1 µM forskolin induced a marginal increase in estradiol concentration, with the effect being more apparent following 48h treatment (+25.8% *vs.* +10.8% following 24 h treatment).

FSH had no effect on estradiol secretion in this study.

Table 5.8.2/24-4: Study 2 - Mean estradiol concentrations in rat preantral follicles after incubation with thiacloprid, FSH or forskolin for 24 or 48 h

Incubation time (h)	Compound	Compound concentration	Mean estradiol conc. (ng/mL) ± SD	% of control
24	Vehicle control	0	8.70 ± 0.32	--
	FSH	5 IU	8.68 ± 0.71	n.c.
	Forskolin	1 µM	9.64 ± 0.67*	+10.8%
	Thiacloprid	50 µM	9.13 ± 0.09*	+4.9%
		100 µM	9.74 ± 0.56**	+11.95%
		500 µM	10.11 ± 0.57**	+16.2%
48	Vehicle control	0	8.83 ± 0.64	--
	FSH	5 IU	8.97 ± 0.21	n.c.
	Forskolin	1 µM	11.11 ± 2.75	+25.8%
	Thiacloprid	50 µM	9.08 ± 1.21	+2.8%
		100 µM	9.79 ± 0.76	+10.9%
		500 µM	**10.22 ± 0.66***	**+15.7%**

conc.: concentration
*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$
n.c.: no change

III. Conclusion

Treatment of the follicles with 500 µM thiacloprid gave a clear and consistent increase in progesterone at both 24 or 48 h (up to +178.7 or +464.7%, respectively) in both studies. The changes in progesterone levels at 50 µM and 100 µM were not considered relevant due to high variability. Estradiol was also consistently increased due to 24 and 48h treatment of the follicles with 500 µM thiacloprid.

In conclusion, the consistent increases in progesterone and estradiol levels following 24 and 48h exposure of ovarian preantral follicles to 500 µM thiacloprid were considered treatment related and biologically relevant.

Reproductive toxicity

Report:	KCA 5.8.2/25[redacted],; 2011; M-403763-01-1
Title:	Thiacloprid – A special one-generation dietary reproduction study in Sprague-Dawley rats
Report No.:	SA 10007
Document No.:	M-403763-01-1
Guidelines:	No applicable guideline Deviation(s): not applicable
GLP:	yes

I. Materials and methods

A. Materials

1. Test material: thiacloprid

Description:	light beige solid
Lot/Batch no:	EDE 0011099
Purity:	98.7%
Stability of test compound:	guaranteed for study duration; expiry date: 2011-11-11

2. Vehicle: plain diet

3. Test animals:

Species:	rat
Strain:	Crl:CD (SD) Sprague-Dawley
Age:	approximately 7 weeks
Sex:	males and females
Weight at dosing:	males: approximately 207 – 255 g females: approximately 151 – 207 g
Source:	[redacted] USA. Sprague-Dawley rats from this breeder were selected since dystocia was only observed in animals of this strain and breeder in previously conducted studies on thiacloprid.
Acclimatisation period:	16 days
Diet:	A04CP1-10 powdered rodent diet from [redacted], France), *ad libitum*
Water:	tap water (filtered and softened), *ad libitum;*

Housing:	premating phase: individual housing in suspended stainless steel wire mesh cages, except during the mating period: polycarbonate cages with bedding material; gestation phase: polycarbonate cages with bedding material, except for the females selected for video-recording, which were free of bedding material from gestation day (GD) 21 until parturition was completed or until sacrifice.

B. Study design and methods

1. Animal assignment and treatment:

Dose:	0-800 ppm equivalent to: 0-50.5 mg/kg bw/day (males) 0-60.9 mg/kg bw/day (females; pre-mating) 0-54 mg/kg bw/day (females; gestation)
Duration:	all animals, daily during pre-mating (10 weeks) and mating; females in addition during pregnancy and gestation until gestation day (GD) 21
Application route:	oral (diet)
Group size:	Dose level 0 ppm (control): 25 males; 43 females Dose level 800 ppm: 25 males; 43 females
Observations:	mortality, clinical signs, body weight, food consumption, , determination of the estrous cycle at the end of pre-mating, video-recording of parturition (to determine onset and duration of parturition, observe dystocia), blood sampling (determination of estradiol, progesterone and thiacloprid levels), terminal sacrifice: in the morning after parturition, gross pathology, organ weights, histopathology, reproductive data

Table 5.8.2/25-1: Study design and assigment of females to treatment groups after mating

Group	Main groups	Satellite groups (1)	Satellite groups (2)	Satellite groups (3)
0 ppm Thiacloprid (controls): *no. of females*	*24*	*5*	*6*	*5*
800 ppm Thiacloprid: *no. of females*	*24*	*6*	*5*	*5*
Clinical signs, body weight & food intake	X	X	X	X
Video recording of parturition	**X**	**X**	--	--
Blood sampling (determination of hormone & thiacloprid levels)	**individually on the morning after parturition** (terminal sacrifice)	**GD 21** (venipunct.) & **individually on the morning after parturition** (terminal sacrifice)	**GD 21.5** (terminal sacrifice)	**GD 22** (terminal sacrifice)
Organ weight	brain, liver, thyroid gland	brain, liver, thyroid gland	--	--
Histopathology	liver, thyroid gland	liver, thyroid gland	--	--

GD: gestation day
venipunct.: venipuncture of the retroorbital plexus under anesthesia

Feasibility studies: Two feasibility studies were conducted before start of the main study in untreated pregnant rats in order to develop and optimize the procedure of video recording during parturition. Already the presence of humans during parturition or the manipulations of blood sampling can *per se* lead to dystocia in rat. Therefore, video recording of parturition and blood sampling had to be performed in a way which avoided unspecific dystocia.

II. Results and discussion

A. Feasibility studies:

In the two feasibility studies in untreated rats also cases of dystocia occurred (in 3/18 dams of the [1]st and 3/8 dams of the [2nd] study). These findings were attributed to the increased level of stress caused by the long presence of technicians in the animal room during parturition (in order to develop/optimize the video recording), the noise of people talking and handling materials, the missing bedding material so that the dams could not build a nest for their pups (necessary to enable video recording of parturition through the transparent bottom of the cage) and the procedure of blood sampling before birth in some of the animals. The findings also gave a first indication for an increased sensitivity of the Sasco Sprague-Dawley rat towards stress-induced disorders of parturition (this was confirmed by historical control data on dystocia in this and other rat strains compiled lateron). The feasibility studies

showed that stress in the main study had to be reduced as much as possible in order to have no cases of dystocia in the untreated animals.

B. Mortality and dystocia

Control group:

There were no mortalities in the control group in either sex during any phase.

800 ppm thiacloprid group:

Premating phase

Thiacloprid treated female UR2F0473 was killed for humane reasons on Day 84, after having been aggressed by the male during mating. The female had a large skin lesion at the head and the left ear was damaged. No other macroscopic findings were noted at necropsy. The male was excluded from pairing.

Gestation phase

In the main group, thiacloprid-treated female UR2F0481was killed for humane reasons on gestation day (GD) 23 (03:42 pm) during parturition. The first pup had been delivered on GD 22 (08:40 pm). Clinical signs consisted of piloerection, reddish soiled anogenital region and reduced motor activity. There were no other remarkable findings during gestation, except for a slightly lower body weight gain between GD 14 and 21 compared to the other pregnant thiacloprid-treated females (58 g compared to a mean value of 76 ± 18 g). Before sacrifice the female had delivered 5 pups but only 4 of them were still present in the cage at the time of sacrifice and one uterine horn was protruding out of the vagina. A blood sample was collected before euthanasia and the hormonal status and thiacloprid concentration were determined. Progesterone concentration was 71687 pg/mL, the estradiol level was below the detection limit of 16.4 pg/mL and the concentration of thiacloprid was 5.8 mg/L. Necropsy confirmed the marked uterus prolapse and revealed 3 live pups in the uterine horn remaining in the abdomen. At the microscopic examination, a moderate centrilobular hepatocellular hypertrophy was noted in the liver and a minimal follicular cell hyperplasia/hypertrophy was observed in the thyroid gland.

Main group animal UR2F0463 was found dead on GD 24 (09:59 am). The first pup had been expelled at 12:25 am on GD 23 and at least 12 other pups had been delivered by 05:19 pm on GD 23. No clinical signs were recorded in the data capture system but reduced motor activity and markedly soiled anogenital region were seen in the video records during parturition. Since the animal was found dead, blood sampling was not possible. At necropsy, a moderate autolysis was recorded and one dead pup was found in the uterus. Histopathology revealed a moderate centrilobular hepatocellular hypertrophy and minimal hepatocellular degeneration/necrosis in the liver.

In the satellite group, piloerection, general pallor and markedly soiled anogenital and abdominal regions were recorded (GD 23, 09:02 am) on female UR2F0451 after parturition. The first pup was expelled on GD 23 at 00:50 am and the parturitional length was 210 min for 14 pups, compared to a mean length of 128 ± 55 min in the treated satellite group. Hormonal values of this animal were 105300 pg/mL progesterone and 22 pg/mL estradiol on GD 20 and 9806 pg/mL progesterone and

21 pg/mL estradiol at terminal sacrifice after parturition. Thiacloprid concentrations were 25.9 and 5.76 mg/L on GD 20 and at terminal sacrifice, respectively. At necropsy, an atrophic and small uterus was noted. At the microscopic examination, a slight centrilobular hepatocellular hypertrophy was noted in the liver and a slight follicular cell hyperplasia/hypertrophy was observed in the thyroid gland.

Table 5.8.2/25-2: Mortality and status of females in the main and satellite groups (gestation phase)

	Main animals	Satellite animals, Blood sampling GD20 & at termination	Satellite animals, Blood sampling GD21	Satellite animals, Blood sampling GD22
0 ppm (controls)				
No. females	*24*	*5*	*6*	*5*
Scheduled death	24	5	6	5
Pregnant	20	5	4	5
Not pregnant	4	0	2	0
Unscheduled death	0	0	0	0
Pregnant	0	0	0	0
Not pregnant	0	0	0	0
800 ppm thiacloprid				
No. females	*24*	*6*	*5*	*4*
Scheduled death	22	6	5	4
Pregnant	21	5	4	4
Not pregnant	1	1	1	0
Unscheduled death	2	0	0	0
Pregnant	2	0	0	0
Not pregnant	0	0	0	0

B. Clinical observations

Premating phase

There were no treatment-related clinical signs.

Two males and 2 females in the control group and 3 males in the treated group showed hyper-reactivity to external stimuli, aggression and/or resistance to handling at one or several occasions between Days 64 and 84. In addition, concomitantly to the hyper-reactivity to external stimuli, the treated male UB2M0428 had clonic convulsions and piloerection on Day 74. These clinical signs, observed in control and treated animals, were attributed to stress and not to the treatment with thiacloprid.

Other clinical signs recorded during the premating phase were those commonly observed in the rat or were consecutive to the implantation of the identification micro-implant (scabs and/or hair loss at the head or neck).

Gestation phase

There were no treatment-related clinical signs, except for the animals that exhibited dystochia.

One thiacloprid-treated female of the main group was killed for humane reasons on GD 23, approximately 19 hours after delivery of the first pup. Clinical signs indicative of pain and difficult parturition (piloerection, reddish soiled anogenital region and reduced motor activity) were noted in this animal.

Another thiacloprid-treated female of the satellite group with dystochia showed clinical signs (piloerection, general pallor and markedly soiled anogenital and abdominal regions) after parturition.

C. Body weights

Premating phase

Between Day 1 and 8, the mean body weight gain per day was reduced by 50 and 46% in treated males and females, respectively, compared to controls ($p \leq 0.01$). Thereafter, the mean body weight gain per day in both sexes was occasionally lower than controls. Overall, the mean body weight gain cumulated over treatment was reduced on all intervals between Day 1 and 71 in both sexes, the effect being more severe in females than in males. The difference to controls was statistically significant ($p \leq 0.01$) in males between Day 1 and 22 (-11 to -49%) and in females between Day 1 and 71 (-15 to -44%). At the end of the premating phase, the mean body weight of females was 4% ($p \leq 0.01$) lower than in controls.

Gestation phase

Between GD 7 and 14 and between GD 14 and 21, the mean body weight gain of treated females was 17 and 16% lower than in controls, respectively ($p \leq 0.01$). The mean body weight gain over gestation (GD 0 to 21) was reduced by 14% ($p \leq 0.01$), when compared to controls.

Mean body weights and mean body weight gain during the different phases of the study are presented in the tables below.

Table 5.8.2/25-3: Mean absolute body weight (g) during pre-mating

Thiacloprid dose (ppm)	Mean absolute body weight gain (g) during pre-mating			
	0 (control)		800	
	Males	**Females**	**Males**	**Females**
Number of rats	*25*	*43*	*25*	*43*
Study day				
1	234	177	235	179
8	273	195	**255****	**189***
15	303	205	**292***	**199***
22	327	215	318	210
29	346	222	339	216
36	361	229	356	223
43	376	235	372	**225****
50	389	239	382	**231***

Thiacloprid dose (ppm)	Mean absolute body weight gain (g) during pre-mating			
	0 (control)		800	
	Males	Females	Males	Females
Number of rats	*25*	*43*	*25*	*43*
Study day				
57	402	243	395	**235***
64	413	248	405	**238****
71	421	251	411	**240****

*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$

Table 5.8.2/25-4: Mean absolute body weight gain (g) during pre-mating

Thiacloprid dose (ppm)	Mean absolute body weight gain (g) during pre-mating			
	0 (control)		800	
	Males	Females	Males	Females
Number of rats	*25*	*43*	*25*	*43*
Study day				
8	39	18	**20****	**10****
15	69	29	**57****	**20****
22	93	38	**83****	**31****
29	112	45	104	**37****
36	127	53	121	**44****
43	142	58	137	**46****
50	155	62	147	**52****
57	169	66	160	**56****
64	179	71	170	**59****
71	187	74	176	**61****

**: significantly different from control, $p \leq 0.01$

Table 5.8.2/25-5: Mean body weight (g) of females during gestation

Thiacloprid dose (ppm)	Mean body weight (g) of females during gestation	
	0 (control)	800
Study day *Number of rats*	*34*	*36*
0	225	**245***
7	285	**273****
14	314	**297****
21	405	**373****

*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$

Table 5.8.2/25-6: Mean body weight gain (g) of females during gestation

Thiacloprid dose (ppm)	Mean body weight (g) of females during gestation 0 (control)	800
Study day *Number of rats*	*34*	*36*
7	30	27
14	59	**52****
21	149	**128****

**: significantly different from control, $p \leq 0.01$

D. Food consumption and test substance intake

Food consumption

Pre-mating phase

When compared to controls, mean food consumption was significantly reduced during the first week of treatment in males and females (-21% and -26% respectively). Thereafter, mean food consumption remained slightly lower in treated females on most intervals throughout the treatment (-6% to -9%).

Table 5.8.2/25-7: Mean food consumption (g/day) during premating

Day	Mean food consumption (g/day) during pre-mating 8	15	22	29	36	43	50	57	64	71
Dose / sex	**0 ppm thiacloprid (control) males**									
N	*25*	*25*	*25*	*25*	*25*	*24*	*21*	*21*	*22*	*21*
Mean intake (g/day)	23.0	22.4	22.7	22.8	22.7	22.9	22.4	22.1	22.3	22.6
Dose / sex	**800 ppm thiacloprid males**									
N	*25*	*25*	*25*	*25*	*25*	*24*	*24*	*23*	*23*	*24*
Mean intake (g/day)	**18.2****	22.6	22.5	22.5	22.5	22.2	22.0	22.1	22.3	22.3
Dose / sex	**0 ppm thiacloprid (control) females**									
N	*43*	*42*	*43*	*43*	*41*	*41*	*37*	*38*	*42*	*42*
Mean intake (g/day)	18.7	18.0	18.8	18.8	18.1	18.4	18.3	18.1	18.3	18.3
Dose / sex	**800 ppm thiacloprid females**									
N	*43*	*43*	*43*	*43*	*43*	*41*	*43*	*42*	*42*	*42*
Mean intake (g/day)	**13.9****	17.2	**17.3****	**17.2****	**16.9****	**16.9****	**17.1****	**17.0***	**16.6****	**17.2****

N: number of animals
*: significantly different from control, $p \leq 0.05$;
**: significantly different from control, $p \leq 0.01$

Gestation phase

When compared to controls, the mean food consumption of thiacloprid-treated females was slightly, but significantly reduced by 8 to 10% throughout the gestation phase ($p \leq 0.01$).

Table 5.8.2/25-8: Mean food consumption (g/day) of females during gestation phase

Day	Mean food consumption (g/day) of female rats during gestation		
	7	14	21
Thiacloprid dose (ppm)	0 ppm		
Number of animals	*34*	*34*	*33*
Mean intake (g/day)	21.6	22.5	25.7
Thiacloprid dose (ppm)	800 ppm		
Number of animals	*35*	*35*	*33*
Mean intake (g/day)	**19.4****	**20.6****	**23.1****

**: significantly different from control, $p \leq 0.01$

Achieved doses:

The mean achieved dose levels of thiacloprid during the pre-mating and gestation period are summarised in the following table.

Table 5.8.2/25-9: Group mean achieved doses of thiacloprid during premating and gestation

Thiacloprid dose (ppm) and sex	Mean achieved dietary intake of thiacloprid (mg/kg bw/day)	
	800 ppm males	800 ppm females
Premating phase (weeks 1 to 10)	50.5	60.9
Gestation phase	--	54.0

E. Thiacloprid concentrations in plasma

The mean plasma concentrations of thiacloprid in treated females were as presented in the table below.

Table 5.8.2/25-10: Mean thiacloprid concentration and standard deviation in plasma (mg/L)

Blood sampling	Mean thiacloprid concentration in plasma (mg/L)				
	Main animals	Satellite animals			
	TS	GD 20	GD 21	GD 22	TS
No. of animals	*22*	*5*	*4*	*4*	*5*
Mean thiacloprid concentration (mg/L)	16.9	22.1	16.1	14.8	9.8
Standard deviation	5.6	3.4	1.5	3.6	3.0

TS: terminal sacrifice (in the morning after parturition)
GD: gestation day

F. Onset of parturition and parturitional length

There was no effect of the treatment with thiacloprid on pregnancy, onset of parturition or parturitional lengths.

In main animals, onset of parturition took part in most of the controls as well as thiacloprid-treated animals on GD 22 (ranged from GD 21 to GD 23). The parturitional length in controls

varied from 58 minutes (4 pups) to 238 minutes (14 pups) and from 51 utes (13 pups) to 199 minutes (15 pups) in thiacloprid-treated animals not displaying dystocia. The mean parturitional length was 120 ± 50 minutes in controls and 106 ± 43 minutes in treated animals.
In satellite animals which had been blood sampled on GD 20, the mean parturitional length was prolonged compared to the main animals, in both control and treated groups. This effect was less marked in treated animals (128 *vs.* 106 minutes) than in control animals (171 *vs.* 120 minutes). In contrast, the onset of parturition was slightly delayed (i.e., the gestation phase was prolonged) in treated satellite animals compared to the satellite control group (22.6 days compared to 21.8 days).

Table 5.8.2/25-11: Mean start of parturition

	Mean start of parturition (gestation day)			
	Main groups		Satellite groups	
Thiacloprid dose (ppm)	**0**	**800**	**0**	**800**
Start of parturition (gestation day)	22.0	22.1	21.8	22.6

Table 5.8.2/25-12: Mean parturitional length and standard deviation in main and satellite group females

	Mean parturitional length and standard deviation (minutes)					
Main groups: thiacloprid dose (ppm)	**0 ppm (control)**			**800 ppm**		
	N	**Mean**	**SD**	*N*	**Mean**	**SD**
Main group animals	*20*	119.8	50.3	*20*	105.6[ns]	42.9
Satellite animals#	*5*	171.2	64.6	*5*	128.2	54.6

[ns] not significantly different from controls
SD: standard deviation
N: no. of animals
#: Statistical analysis was not conducted on these parameters.

There were three thiacloprid-treated females (two main group-, one satellite group animal) that exhibited a prolonged parturition (dystochia).

Main group:
One thiacloprid-treated female was killed for humane reasons on GD 23, approximately 19 hours after having delivered the first pup. At the time of sacrifice, 3 live pups were noted in the uterine horn remaining in the abdomen, which indicates that the parturition was not complete.
Another thiacloprid-treated female was found dead after delivery of 12 pups within 266 minutes. At necropsy one dead pup was found in the uterus indicating that the parturition was not completed.
For comparison, the mean parturitional lentgh in this group was 105.6 minutes.

Satellite group
One thiacloprid-treated female of the satellite group had a parturitional length of 210 minutes, as compared to a mean value of 128.2 minutes in this group.

However, since this animal belonged to the satellite group it is unclear whether the effect was due to the thiacloprid treatment, the stress caused by the blood sampling at GD 20 or both. Hormonal values measured on GD 20 and at terminal sacrifice were within the normal range of values.

G. Hormone analyses

Hormonal levels and the balance between progesterone and estradiol concentrations were affected by thiacloprid treatment.

In control animals, the mean plasma concentration of progesterone was elevated on GD 20 (90220 pg/mL) and decreased by 83% between GD 20 and GD 22, then remaining static during parturition. In thiacloprid-treated animals, the mean plasma concentration of progesterone was 20% higher than in controls on GD 20 (108177 pg/mL, not statistically significant) and decreased by 87% between GD 20 and GD 22, then remaining stable during parturition.

In controls, the level of estradiol decreased between GD 20 and the end of parturition (terminal sacrifice). The estradiol to progesterone ratio (E2/Px1000) gradually increased 5-fold between GD 20 and 22. In animals treated with thiacloprid, the level of estradiol strongly increased between GD 20 and 21, reaching a concentration 88% higher than controls on GD 21, then decreasing but remaining 31% higher than controls on GD 22 (not statistically significant). Overall, the difference in estradiol levels in treated females between terminal sacrifice and GD 20 was lower than in controls (-2.8 pg/mL compared to -11.0 pg/mL). The estradiol to progesterone ratio (E2/Px1000) sharply increased 10-fold between GD 20 and 21 and remained stable between GD 21 and 22.

Table 5.8.2/25-13: Hormone data

Thiacloprid dose (ppm)		0 ppm (control)			800 ppm		
Hormone	Time	*N*	Mean	SD	*N*	Mean	SD
Progesterone [pg/mL]	GD 20	5	90219.8	17336.8	5	108176.8^{ns}	16042.1
	GD 21	4	26687.5	4053.0	4	26334.0^{ns}	13786.0
	GD 22	5	15747.8	6464.5	4	14268.0^{ns}	3087.5
	TS	5	15753.4	2827.6	5	16975.2^{ns}	6265.6
Estradiol [pg/mL]	GD 20	5	27.0	8.1	5	20.2^{ns}	3.2
	GD 21	4	21.0	6.0	4	**39.5***	12.4
	GD 22	5	22.0	5.1	4	28.8^{ns}	14.7
	TS	5	16.0	0.0	5	17.4^{ns}	2.2
Ratio E2/Px1000	GD 20	5	0.31	0.11	5	**0.19****	0.02
	GD 21	4	0.78	0.15	4	1.88^{ns}	0.97
	GD 22	5	1.54	0.62	4	1.93^{ns}	0.70
	TS	5	1.05	0.21	5	1.19^{ns}	0.60

N: no. of animals
GD: gestation day
TS: terminal sacrifice (in the morning after parturition)
*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$
^{ns}: not significantly different from control

H. Necropsy

Terminal body weight and organ weights

Mean terminal body weight was statistically significantly lower when compared to controls. Treatment with 800 ppm thiacloprid led to an increase of absolute and relative liver and thyroid weights as well as to increased liver and thyroid to brain weight ratios. Details are presented in the table below.

Table 5.8.2/25-14: Mean terminal body weight, organ weights and standard deviations

	Mean organ weight ± standard deviation (% change as compared to controls)	
Thiacloprid dose (ppm)	**0**	**800**
Terminal body weight (g)	321.3 ± 50.4	**297.3 ± 48.0* (-7%)**
Number of animals examined#	*25*	*26*
Mean absolute liver weight (g)	11.54 ± 1.01	**14.17 ± 1.73** (+23%)**
Mean relative liver weight (%)	3.875 ± 0.274	**5.108 ± 0.371** (+32%)**
Mean liver to brain weight ratio (%)	615.831 ± 54.111	**773.678 ± 84.212** (+26%)**
Number of animals examined#	*24##*	*26*
Mean absolute thyroid weight (g)	0.0146 ± 0.0032	**0.0167 ± 0.0029* (+14%)**
Mean relative thyroid weight (%)	0.00493 ± 0.00108	**0.00606 ± 0.00114** (+23%)**
Mean thyroid to brain weight ratio (%)	0.78073 ± 0.17753	**0.91517 ± 0.16937** (+17%)**

*: significantly different from control, $p \leq 0.05$
**: significantly different from control, $p \leq 0.01$
#: main animals and satellite animals with blood sampling on GD 20
##: thyroid glands of one satellite animal were missing

Gross pathology

There were no macroscopic lesions observed in male rats after the pre-mating phase.
After gestation gross pathological findings observed in thiacloprid-treated females consisted of enlarged livers (5/40) and prominent lobulation of livers (12/40).

Microscopic pathology

Only pregnant females from the main and the satellite groups sacrificed on GD 20 were histopathologically examined.
Treatment-related effects were observed in the liver and thyroid, as summarised in the following table.
Almost all females at 800 ppm displayed a minimal to moderate diffuse centrilobular hepatocellular hypertrophy in the liver as well as a a minimal to moderate diffuse follicular cell hypertrophy in the thyroid.

Table 5.8.2/25-15: Histopathological findings in liver and thyroid at terminal sacrifice

Thiacloprid dose (ppm)	0	800
	Incidence and severity of liver findings	
Number of pregnant females	34	36
Number of females examined	25	26
Hepatocellular hypertrophy, centrilobular		
Minimal	0	3
Slight	0	18
Moderate	0	4
Total	**0**	**25**
	Incidence and severity of thyroid findings	
Number of pregnant females	34	36
Number of females examined	24#	26
Follicular cell hyperplasia/hypertrophy: diffuse		
Minimal	2	2
Slight	0	15
Moderate	0	3
Total	**2**	**20**

#: thyroid glands of one control animal missing

III. Conclusion

Stress-related clinical signs were noted in male and female control and treated animals during the premating phase, indicating an enhanced sensitivity of this rat strain to environmental stress.

General toxicity of thiacloprid was indicated by changes in body weight, food consumption and liver/thyroid histology in almost all treated females.

Three cases of dystocia were observed in thiacloprid-treated rats:
- one of them showed a very high progesterone level at parturition (whereas the group mean values reflected the normal strong decrease between GD20 and GD21/ parturition, which in rodents is mandatory for a normal parturition. This is not the case in humans, in which progesterone withdrawal is regulated on receptor level in the uterus myometrium, while progesterone levels in plasma stay high during parturition.),
- in a second rat hormone levels could not be determined (animal found dead),
- and in the third animal hormone levels were in the normal range, but since a blood sample had been drawn in this satellite animal on GD20, dystocia could have been a consequence of stress related to blood sampling and/or to general toxicity of thiacloprid. It had been demonstrated in the previously conducted feasibility study that stress can cause dystocia in untreated rats of this strain.

The levels of progesterone (slightly increased mean value at GD 20, absence of normal decrease prior to parturition in the individual rat mentioned above) and estradiol (increased mean values at GD 21 and 22) and the respective balance between these hormones in the days preceding and during parturition were affected. A causal relationship between these changes and the observed cases of

dystocia is obvious only for the individual with the missing normal progesterone decrease.
There was no effect on onset and duration of parturition in all other treated rats.

In summary, these findings support the assumption that the observed dystocia in single animals was caused:

1. either by thiacloprid-induced increases of progesterone (i.e. a missing progesterone decrease before birth)
2. or by stress due to general toxicity of thiacloprid and blood sampling combined with the increased sensitivity of the Sasco Sprague Dawley rat to stress.

Analytical methods

Analytical methods for the determination of thiacloprid by HPLC analysis in rodent diet (+1% corn oil) and rat plasma were developed. The references of the study reports are presented under KCA 5.8.2/31, M-392957-01-1 and KCA 5.8.2/34, M-398883-01-1.

Publication(s)

Report:	KCA 5.8.2/26 [redacted]; 2013, M-462492-01-1
Title:	Effects of commercial formulations of deltamethrin and/or thiacloprid on thyroid hormone levels in rat serum
Reference:	Toxicology and Industrial Health, (2012 Jun 7), p. 1-7
Document No.:	M-462492-01-1
Guidelines:	not applicable deviation(s): not applicable
GLP:	no

I. Materials and methods

A. Materials

1. Test material:	Clypso OD 240; Decis 2.5 EC
Source:	[redacted] (Turkey)
Content of a.s.:	Clypso OD 240: 240 g/L thiacloprid Decis 2.5 EC: 25 g/L deltamethrin
Lot/Batch no:	not reported
Purity:	not reported
Stability of test compound:	not reported
2. Vehicle and positive controls:	vehicle: corn oil positive control: none
3. Test animals:	
Species:	rat
Strain:	Wistar albino rats

Age:	7-8 weeks
Sex:	males
Weight at dosing:	250-300 g
Source:	[redacted], Turkey
Acclimatisation period:	not reported
Diet:	not reported
Water:	not reported
Housing:	in polycarbonate cages with steel wire tops and rice husk bedding

B. Study design and methods

1. Animal assignment and treatment:

Dose:	Acute exposure (single application): vehicle control: corn oil thiacloprid alone: 112.5 mg/kg bw deltamethrin alone: 15 mg/kg bw thiacloprid / deltamethrin mixture: 112.5 / 15 mg/kg bw repeated exposure (once daily for 30 days): vehicle control: corn oil deltamethrin alone: 3 mg/kg bw/day thiacloprid alone: 22.5 mg/kg bw/day thiacloprid / deltamethrin mixture: 22.5 / 3 mg/kg bw/day
Application route:	oral (gavage)
Application volume:	2 mL/kg bw
Duration:	Single dose and repeated administration for 30 days
Group size:	6 male rats per group
Examinations:	TSH, free T3 and free T4
Blood sampling:	At 24 h after the last dose, the rats were euthanized by cervical dislocation and blood samples were collected by cardiac puncture. The blood samples collected into serum tubes were centrifuged (2500 r/min for 20 min), the serum was stored at -80 °C until analysis.
Statistics:	The data are expressed as the mean ± standard error. Differences between the groups were assessed by oneway analysis of variance using the SPSS software package for Windows. The comparisons between the groups were made using a post hoc test, Tukey's test.

II. Results and discussion

A. Thyroid hormone levels

Acute exposure

TSH-levels were increased after acute exposure with thiacloprid and the mixture of thiacloprid and deltamethrin when compared to vehicle control. Levels of free T3 and free T4 were decreased after both acute exposure with thiacloprid and the mixture of thiacloprid and deltamethrin. However, none of the changes reached statistical significance.

Repeated exposure

After repeated exposure of thiacloprid and the thiacloprid-deltamethrin mixture TSH-levels were slightly decreased, whereas free T3 and free T4 was statistically significantly increased when compared to controls.

The results are summarised in the following table.

Table 5.8.2/26-1: Effects on thyroid hormone levels after acute and repeated exposure to thiacloprid containing mono- and combination products

Treatment groups [mg/kg bw/day]		Hormone levels (mean ± SE) TSH [µIU/mL]	Free T3 [ng/mL]	Free T4 [ng/mL]
Acute exposure (single dose)				
Control (corn oil)	0	0.19 ± 0.01	1.78 ± 0.13	1.81 ± 0.18
Deltamethrin	15	0.48 ± 0.11	1.70 ± 0.14	1.56 ± 0.22
Thiacloprid	142.5	0.49 ± 0.16	1.37 ± 0.14	1.42 ± 0.13
Deltamethrin + thiacloprid	15 + 142.5	0.62 ± 0.14	1.54 ± 0.17	1.38 ± 0.13
Repeated exposure (30 days)				
Control (corn oil)		0.20 ± 0.02	2.13 ± 0.17	2.44 ± 0.20
Deltamethrin	3	0.17 ± 0.03	2.24 ± 0.12	2.77 ± 0.26
Thiacloprid	22.5	0.16 ± 0.02	2.89 ± 0.21*	3.27 ± 0.17*
Deltamethrin + thiacloprid	3 + 22.5	0.13 ± 0.01	3.02 ± 0.19*	3.23 ± 0.15*

SE: standard error
µIU: micro international units
*: significantly different to control, $p \leq 0.05$

III. Conclusion

The authors concluded, that single oral exposure to commercial formulations containing thiacloprid as well as to a combination of deltamethrin and thiacloprid caused a non-significant increase of TSH and non-significant decreases of free T3 and free T4, while repeated oral exposure of the same formulations at lower dose levels led to a non-significant decrease of TSH and statistically significant increases of free T3 and free T4.

BCS conclusion: It is known that repeated administration of high thiacloprid doses to rats (in BCS studies observed after ≥ 69 mg/kg bw/day) leads to slight perturbations of thyroid hormone and TSH levels. These changes are secondary to liver enzyme induction leading to an increased metabolism and excretion of T3 and T4 from plasma and subsequently to a stimulation of the hypothalamic-pituitary-thyroid axis. Permanent stimulation of the thyroid follicular cells by TSH

then leads to thyroid follicular cell hypertrophy. BCS data show, that the changes observed after thiacloprid treatment in rats are mostly minimal to slight and that the body up to very high doses approaching the MTD is able to maintain an euthyroid state.
It is difficult to evaluate the hormone levels reported in the publication by [redacted] et al. without any historical control data. After single application there are no significant changes, which could as well all be in the normal range of biological variation. Apparent differences between groups might be due to the order of blood sampling and the circadian fluctuations of the hormones. Since the thyroid effects in rat are secondary to liver enzyme induction, real effects should not occur as early as 24 h even after such a high single dose of 112.5 mg/kg bw. However, slight effects could be visible after 30 days of treatment with 22.5 mg/kg bw/day (in the BCS studies liver enzyme induction in male rats was noted starting at 25 mg/kg bw in studies of up to 1 year duration). However, also here it is not clear, if the observed slight effects are real or e.g. a consequence of the sequence of blood sampling and the circadian rhythm of these hormones. Basically, it could be possible to see slightly increased free T3 and T4 values and a slightly decreased TSH level (after an up-regulation of thyroid hormone production which resulted in slightly higher thyroid hormone levels, TSH secretion will be decreased again).
However, in conclusion, due to species specific differences between rat and man the mode of action for the thyroid effects observed after thiacloprid treatment in rat (secondary to liver enzyme induction) is considered not to be relevant for humans. The study results do not change existing endpoints.
The reliability evaluation of the publication is given below.

Klimisch evaluation

Reliability of study:	Not reliable (Klimisch code: 3)
Comment:	Non-GLP study, conducted according to scientific principles but with major methodical and / or reporting deficiencies. Only one dose-level for individual formulations and the mixtures were used for acute and repeated exposures. The OECD 407 (2008) recommends at least three dose levels. Only 6 rats per dose group used. OECD 407 recommends at least 10 (5/sex) rats per group, in modern hormone studies at least 15 animals per sex and dose are used to get reliable results. Important is also the sequence of blood sampling, since TSH, T3 and T4 show a circadian rhythm, so that apparent dose related changes can be a consequence of sequential blood sampling of the different groups. Stress (i.e. disturbances in the animal room due to blood sampling) can also lead to altered TSH and thyroid hormone levels. Examinations on body weights, general signs of toxicity were not done or not reported. Since there are several factors that can influence thyroid hormone levels (e.g. stress) the assessment of general toxicity would have been useful for the evaluation of the results. No historical control data were provided. Since thyroid hormone levels differ also with the methods (test kits) used, historical

	control data for the applied test method could have proved the validity of the test results. No histopathological investigations of the thyroid were conducted, which could have proven the reliability of the effects.
Relevance of study:	In *in vivo* rat studies conducted by BCS according to valid guidelines and GLP thyroid efects were comprehensively investigated. BCS consider those studies to be more reliable than this published non-guideline, non-GLP study. However, the results of this study are not in contradiction to the results of the BCS studies and do not change existing endpoints.

Report: KCA 5.8.2/27 [redacted]; 2012; M-488996-01-1

Title: The inducibility of human cytochrome P450 1A by environmental-relevant xenobiotics in the human hepatoma derived cell line HepG2

Reference.: Environmental Toxicology and Pharmacology 28 (2009); p 370–378

Document No.: M-488996-01-1

Guidelines: not applicable
Deviation(s): not applicable

GLP: non-GLP

I. Materials and methods

A. Materials

1. Test material: thiacloprid and several other xenobiotics, pesticides

Source: [redacted], Germany

Lot/Batch no: not reported

Purity: 99.9%

Stability of test compound: not reported

2. Vehicle and positive controls: vehicle: dimethylsulfoxide (DMSO)
positive control: omeprazole

3. Test cells:

Cell line: human hepatoma cell line HepG2

Source: [redacted]

Culture medium: Williams medium with 10% (v/v) FCS

Culture conditions: 5% CO_2, 95% humidity, 37°C

B. Study design and methods

1. Treatment:

Thiacloprid stock solutions were prepared in DMSO. Working solutions were prepared in the corresponding media and added to the plates (final DMSO-concentration: 0.05%). Thiacloprid was diluted in log 2 steps.

HepG2 cells were maintained routinely in 75 cm^2 cell culture flasks for 3 days in order to become confluent. The cells were transferred onto 96-well plates, with 2×10^4 cells per well for EROD/MROD assays and for RNA-isolation onto 6-well plates with 5×10^5 cells per well after trypsination (0.25% trypsin and 0.02% EDTA). The cells were allowed to attach themselves for 24 h, afterwards they were exposed to xenobiotics for 24 h.

Exposure duration: 24 hours

Concentrations tested: 1.25×10^{-4} to 3 x 10^{-3} M (corresponding to total concentrations of 31.59 – 758.19 mg/L)

2. Measurements:

CYP1A1/1A2 activity: Cytochrome activity was determined by two spectrofluorometric methods based on a catalysed 7-ethoxyresorufin-O-dethylation (EROD) and 7-methoxyresorufin-O-demethylation (MROD) by CYP1A1 and CYP1A2, respectively.

Immediately after withdrawal of the cell culture medium 100 µL/well EROD/MROD-solution (0.5 µM 7-ethoxyresorufin/7-methoxyresorufin, respectively, 10 µM dicumerole, 0.1 mM NADPH in cell culture medium without FCS) was added followed by an incubation of exactly 90 minutes (37°C, 5% CO_2, 95% humidity). A supernatant of 90 µL/well was transferred into a new plate and the reaction was stopped by adding 150 µL/well absolute ethanol. A resorufin standard in duplicates (31.25 – 1000 nM) served as an activity reference. EROD/MROD activity of CYP1A1/CYP1A2 was measured by fluorescence using an excitation wavelength of 550 nm and an emission wavelength of 585 nm.

Replicates: 3 in 3 independent experiments

Protein determination: After withdrawal of the remaining EROD/MROD solution of the exposed plate the cells were washed twice with 0.1 M PBS. After drying the cells at room temperature the plates were stored at −80°C. Addition of 20 µL/well 0.1 M sodiumhydrogenphosphate buffer (pH 7.8) was followed by a 3-fold freeze-thaw-cycle (−80°C, 25°C).
The protein concentration was measured by the BC assay (modified Lowry protein assay, based on bicinchoninic acid). The protein content was measured spectrophotometrically at 562 nm.

Cytotoxicity assays:

MTT-assay MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide	At the end of the exposure the cell culture medium was withdrawn leaving 50 µL/well. Thereafter, a 10 µL/well MTT stock solution (5 mg/mL) was added followed by incubation for 4 h (37 °C, 5% CO_2, 95% humidity). Subsequently, 100 µL/well stop solution (40 g SDS/200 mL 50% DMF) was added followed by an overnight incubation step (12 h, 37 °C). MTT conversion as a parameter for metabolic activity was spectrophotometrically measured at 570 nm.
Neutral red assay (NRU):	After exposure the cell culture medium was withdrawn and 100 µL/well NRU solution was immediately added (1:66 dilution of NRU stock solution [3.3 mg/mL] in medium) followed by incubation for 3 h (37 °C, 5% CO_2, 95% humidity). After withdrawal of the reaction solution and addition of 100 µL/well fixation solution (0.5%, v/v formaldehyde, 1%, w/v $CaCl_2$ in ddH_2O) for 1 minute, cells were washed once with 100 µL/well 0.1 M PBS for 1 minute followed by an addition of a 100 µL/well extraction solution (1%, v/v acetic acid, 50% ethanol in ddH_2O). The plates were incubated for 30 minutes at 37°C and then shaken at room temperature for additional 30 minutes. Neutral red uptake as a parameter for functional activity of the cells was measured by an absorbance at 540 nm (reference 410 nm). Data points of normalized means of cytotoxicity were sigmoid fitted (regression coefficient $r^2 > 0.9$) and depicted as a single regression curve.
Replicates:	3 in 3 independent experiments
Isolation and amplification of nucleic acid:	Cellular RNA was isolated from HepG2 cells with the peqGold RNAPure™ Isolation protocol (Peqlab, Erlangen, Germany). After a DNAse digestion step, 5 µg of total RNA was taken to synthesize cDNA utilising the RevertAid H Minus First strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany).
Semiquantitative real-time polymerase chain reaction (RT-PCR):	The semi-quantitative CYP1A1/CYP1A2-RT-PCR was done using custom synthesized primer pairs. The PCR was performed at 94°C for 15 minutes, then 40 cycles at 94°C for 30 s, at 58°C for 30 s, at 72°C for 30 s. Specific PCR products were evaluated by the melting curves of the PCR product. HPRT (hypoxanthine-guanine phosphoribosyltransferase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ALB (albumin) served as a housekeeping control to calculate mRNA expression

changes.

Replicates: 1

II. Results and discussion

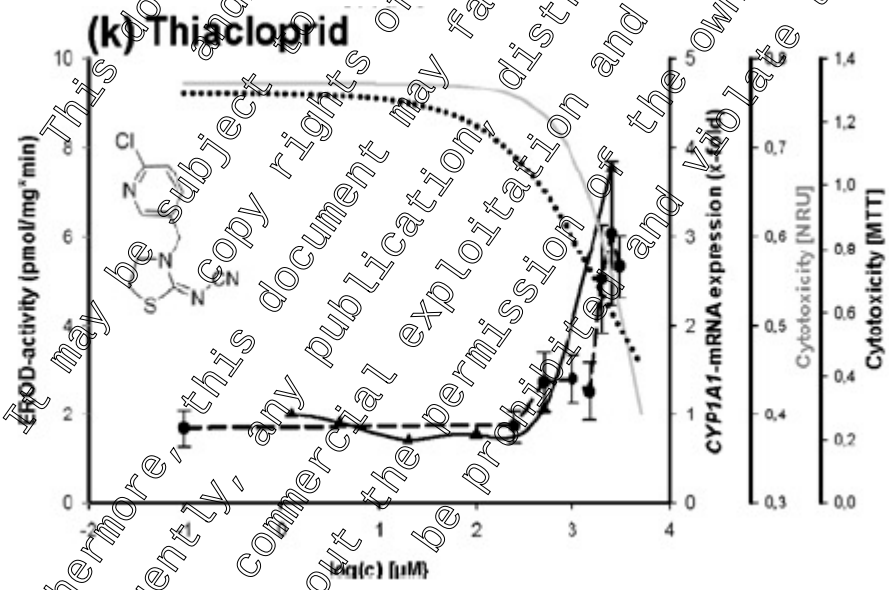
A. Induction of CYP1A1 mRNA transcript in human HepG2 cells

Following a dose-dependent exposure of thiacloprid for 24 hours, the highest CYP1A1-specific mRNA induction was a 3.8-fold increase in comparison to the vehicle control (0.05 % DMSO) observed at 2500 µM (corresponding to a total concentration of 631.8 mg/L). The positive control omeprazole (at 50 µM) caused a 25.5-fold change of the CYP1A1-mRNA induction.

B. Induction of EROD-activity in human HepG2 cells (CYP1A1-activity)

Additionally, xenobiotics were examined for their capability to induce the CYP1A1 enzyme activity measured by EROD-activity. A low level EROD activity was constitutively expressed (vehicle control, DMSO). A dose-response relationship could be detected for the majority of xenobiotics. Comparing the CYP1A1 mRNA and the EROD-activity, the data differs in its degree of magnitude, but an overlay of the CYP1A1 mRNA induction with CYP1A1 enzyme activity profiles for single xenobiotic has shown well conform dose-response patterns.
Also for thiacloprid the peak value for EROD activity was in good correlation to the induction of the respective CYP1A1-mRNA transcript (see Figure below).

Figure 5.8.2/27-1: Comparative dose-response relationship of CYP1A1 mRNA expression (black line) and CYP1A1-activity (EROD, dashed line) after a 24 h exposure in the human hepatoma derived cell line HepG2 in relation to cytotoxicity. The cytotoxicity is depicted by a regression curve of means of the NRU (gray line) and the MTT-(spotted line) assays



The results are summarised in the following table.

Table 5.8.2/27-1: Effects on EROD activity and CYP1A1-mRNA expression

Treatment group	Concentration [M]	Maximum EROD activity Normalized# EROD-activity [fold change]	Maximum CYP1A1-mRNA expression Induction rate [fold change]
Control (0.05% DMSO)	--	1.7	1.0
Thiacloprid	2.5 x 10^{-3}	5.9**	3.8
Positive control (omeprazol)	5.0 x 10^{-5}	18.6**	25.5

**: significantly different to control, $p \leq 0.001$
#: normalized to total protein amount

C. Induction of CYP1A2 mRNA transcript in human HepG2 cells

In general the constitutive and inducible 7-methoxyresorufin activity (MROD) was about 2- to 5-fold lower than that for EROD activity and less xenobiotics showed a significantly increased MROD activity.

Following a dose-dependent exposure of thiacloprid for 24 hours, the highest induction was a 7.8-fold increase of CYP1A2-mRNA in comparison to the vehicle control (0.05 % DMSO) observed at 2500 µM (corresponding to a total concentration of 631.8 mg/L). The positive control omeprazole (at 50 µM) caused a 16.6-fold change of the CYP1A2-mRNA induction.

D. Induction of MROD-activity in human HepG2 cells (CYP1A2-activity)

In contrast to the higher CYP1A2-mRNA induction, the maximum normalised MROD activity for thiacloprid at 2.5 x 10^{-3} M was 2.2-fold.

The results are shown in the following table.

Table 5.8.2/27-2: Effects on MROD activity and CYP1A1-mRNA expression

Treatment group	Concentration [M]	Maximum MROD activity Normalized# MROD-activity [fold change]	Maximum CYP1A2-mRNA expression Induction rate [fold change]
Control (0.05% DMSO)	--	1.0	1.0
Thiacloprid	2.5 x 10^{-3}	2.2**	7.8
Positive control (omeprazol)	5.0 x 10^{-5}	10.6**	16.6

**: significantly different to control, $p \leq 0.001$
#: normalized to total protein amount

III. Conclusion

The data show that thiacloprid exposure of HepG2 cells for 24 hours caused induction of CYP1A1- and CYP1A2-mRNA as well as CYP1A1 and CYP1A2 activity. Induction measured as fold-change of CYP1A2-mRNA was higher than induction of CYP1A2-activity.

BCS opinion: It is known that thiacloprid is an inducer of different CYP450 enzymes. The maximum induction of CYP1A1 and CYP 1A2 determined in this study as normalized EROD and MROD activity in Hep G2 cells as a model for human hepatocytes was not very pronounced (an 5.9 or 2.2 fold increase in comparison to the vehicle control, respectively). In addition, it was observed at a very high concentration of 2500 mM thiacloprid, which corresponds to a total concentration of

631.83 mg thiacloprid/L. This concentration is a factor of 25 higher than the maximum total plasma concentration of thiacloprid in rats treated with 1000 ppm thiacloprid in the diet (this being the high dose of the 2-year rat study, which is exceeding the MTD). Therefore, the finding is not considered to be relevant for human safety. Furthermore, the results do not change existing endpoints.
The reliability evaluation of the publication is given below.

Klimisch evaluation

Reliability of study:	Reliable with restrictions (Klimisch code 2)
Comment:	This *in vitro* study was conducted according to scientific principles, but not according to GLP.
Relevance of study:	The maximum CYP1A1 and CYP1A2 induction caused by thiacloprid was not very pronounced and observed at a very high concentration, which exceeds the maximum total plasma concentration of thiacloprid in rats at the high dose of the 2-year rat study by a factor of 25. The enzyme induction is therefore not regarded as relevant for the situation *in vivo*. Therefore, information is regarded as supplemental.
	Not relevant. Supplemental information **Results do not change existing endpoints**

Report:	KCA 5.8.2/28 [redacted], 2011; M-478667-01-1
Title:	Effects of thiacloprid, deltamethrin and their combination on oxidative stress in lymphoid organs, polymorphonuclear leukocytes and plasma of rats
Reference.:	Pesticide Biochemistry and Physiology 100 (2011) 165-171
Document No.:	M-478667-01-1
Guidelines:	not applicable Deviation(s): not applicable
GLP:	non-GLP

I. Materials and methods

A. Materials

1. Test material: CALYPSO OD 240; DECIS 2,5 EC; Proteus

Source: [redacted]

Lot/Batch no.: not reported

Purity: not reported

Stability of test compound: not reported

2. Vehicle / positive control: negative control: corn oil
positive control: cyclophosphamide (CPA)

3. Test animals:

Species:	Rat
Strain:	Wistar albino
Age:	not reported
Sex:	males
Weight at dosing:	250 – 300 g
Source:	not reported
Acclimatisation period:	not reported
Diet:	standard pellet, *ad libitum*
Water:	tap water, *ad libitum*
Housing:	in laboratory cages
Light/dark cycle:	12 h light / 12 h dark
Temperature:	21 – 1°C

B. Study design and methods

1. Animal assignment and treatment:

Dose:	- acute treatment: 112 mg/kg bw thiacloprid (TCP) - 15 mg/kg bw deltamethrin (DLT) - mixture of 112mg/kg bw TCP + 15 mg/kg bw DLT Subacute treatment: 22.5 mg/kg bw/day TCP - 3 mg/kg bw/day DLT - mixture of 22.5 mg/kg bw/day TCP + 3 mg/kg bw/day DLT - acute treatment positive control: 50 mg/kg bw CPA
Application route:	- oral gavage (negative control& thiacloprid/deltamethrin treatment groups) - i.p. (positive control cyclophosphamide)
Application volume:	not reported
Group size:	6 males per group
Sampling times:	- acute treatment: 24 h post application - subacute treatment: on day 30
Euthanization:	by cervical dislocation
Blood sampling:	A part of the blood was used for serum preparation, another part (obtained by heart puncture with a heparinized disposable syringe) was centrifuged at 2500 rpm for 5 minutes for separation of plasma, mononuclear cells and erythrocytes. For isolation of polymorphonuclear leukocytes (PMNs) the leukocyte-rich ‘‘buffy coat’’ was removed and subjected to gelatin sedimentation by mixing with an equal volume of 2% gelatin in 0.9% NaCl and incubation at 37 °C for 40 min.

After centrifugation at 1000 rpm for 10 minutes, the cell pellet containing the PMNs was resuspended in cold erythrocyte lysing solution (155 mM NH_4Cl, 2 mM $NaHCO_3$, 0.1 mM EDTA). The cell suspension was centrifuged at 275g for 5 minutes, the supernatant was discarded and the pellet was washed tree times with Hank's balanced solution (sodium chloride 138 mmol/L, potassium chloride 2.7 mmol/L, disodium hydrogen phosphate 8.1 mmol/L, potassium dihydrogen phosphate 1.5 mmol/L magnesium chloride 0.6 mmol/L, calcium chloride, 1 mmol/L and glucose 10 mmol/L; pH 7.4). The PMNs suspension was then homogenized using liquid nitrogen. Supernatant obtained from PMNs by mild centrifugation at 250g for 20 minutes was used for biochemical analyses.

Tissue sampling:	Bone marrow was ejected from the femur after cutting the epiphyseal cartilage using 1 mL of ice cold Tris–HCl buffer (0.1 M; pH 7.4). After centrifugation at 1000 rpm for 3 minutes the pelleted marrow cells were kept at −80 °C until used. Packeded marrow cells were homogenized using liquid nitrogen and 1000 g supernatants were used for biochemical analyses. Spleen and thymus were placed in ice cold 0.15 M NaCl solution, perfused with the same solution to remove red blood cells, blotted on filter paper, quickly weighed and stored at −80°C until used. Tissues were centrifuged at 10,000 g for 30 minutes and supernatants were used for the determination of enzyme activity and other biochemical parameters.
Examinations:	
Measurements of liver and renal injury markers	Serum aspartate transaminase (AST), alanine transaminase (ALT), urea and creatinine levels
Antioxidant, phase II enzymes, reduced glutathion assay in lymphoid organs	The catalase (CAT) activity, superoxide dismutase (SOD; EC 1.15.1.1) activity, glutathione peroxidase (GPx; EC 1.11.1.9) activity, glutathione-S-transferase (GST; EC 2.5.1.18) activity, DT-diaphorase (DTD; quinone reductase, EC 1.6.99.2) activity, and glutathion (GSH) in the lymphoid organs (i.e. spleen and thymus)
Determination of thiobarbituric acid reactive substances (TBARS)	The extent of lipid peroxidation in terms of TBARS formation was measured in lymphoid organs, PMNs and plasma.
Myeloperoxidase (MPO) activity	MPO activity was determined in PMNs.

Nitric oxide level	Total NO_x (NO_2 + NO_3) determination in PMNs and plasma
Total antioxidant capacity (TAC)	TAC was measured in plasma.

II. Results and discussion

A. Liver and renal injury markers

Acute treatment

After acute treatment with thiacloprid AST was reduced and ALT was significantly increased, when compared to controls. After a single application of deltamethrin both AST and ALT were significantly increased when compared to controls. In both cases, there were no significant effects on urea and creatinine. After a single application of the thiacloprid – deltamethrin mixture urea and ALT were statistically significant increased when compared to control. No effects were observed for AST and creatinine levels.

Subacute treatment

After subacute treatment with thiacloprid both AST and ALT were significantly decreased and creatinine levels were significantly increased when compared to controls. In contrast, after deltamethrin treatment AST, ALT, urea and creatinine were significantly increased. After subacute treatment with the mixture of thiacloprid and deltamethrin AST was increased and ALT was decreased but without reaching statistical significance. However, urea and creatinine were significantly increased.

Table 5.8.2/28-1: Effects on AST, ALT, urea and creatinine in serum of rats (mean ± SD)

Substance	Dose (mg/kg bw)	AST (U/L)	ALT (U/L)	Urea (mg/dL)	Creatinine (mg/dL)
Single treatment					
Control	--	140.83 ± 8.04	62.87 ± 5.41	24.17 ± 2.56	0.34 ± 0.05
TCP	112	130.39 ± 9.25	74.06 ± 12.31*	27.07 ± 2.87	0.37 ± 0.05
DLT	15	169.72 ± 18.89*	98.15 ± 9.51*	28.52 ± 3.02	0.41 ± 0.06
TCP + DLT	112 + 15	139.72 ± 7.89	135.79 ± 11.70*	30.93 ± 3.28*	0.42 ± 0.06
CPA*	50	167.93 ± 19.00*	85.24 ± 15.65*	41.51± 4.79*	0.60 ± 0.12*
Subacute treatment (30 days)					
Control	--	154.92 ± 8.84	75.44 ± 6.50	24.65 ± 2.61	0.33 ± 0.05
TCP	22.5	125.78 ± 14.14*	52.81 ± 4.55*	30.41 ± 3.22	0.51 ± 0.07*
DLT	3	185.94 ± 12.63*	96.84 ±18.08*	30.56 ± 3.66*	0.49 ± 0.07*
TCP + DLT	22.5 + 3	170.51 ± 12.67	65.63± 18.08	38.01 ±4.03*	0.58 ± 0.08*

Control: corn oil TCP: thiacloprid
DLT: deltamethrin CPA: cyclophosphamide
AST: aspartate transaminase ALT: alanine transaminase
*: significantly different to control, $p < 0.05$

B. Effects on antioxidant and phase II enzymes in lymphoid organs

Spleen - Acute treatment

After acute treatment with thiacloprid or deltamethrin alone, and with the thiacloprid – deltamethrin mixture SOD was significantly increased and CAT, GPX and DTD were significantly decreased, when compared to controls. In addition, after application of the thiacloprid – deltamethrin mixture GSH was statistically significantly decreased when compared to control. No significant effects were observed for GST.

Spleen - Subacute treatment

The decreases, which were seen for CAT, GPX and DTD after acute treatment, were also observed after subacute treatment. However, SOD was significantly decreased after application of thiacloprid alone. In addition, GSH was significantly decreased in all treated groups when compared to control.

Table 5.8.2/28-2: Effects on antioxidant enzymes, phase II enzyme activity and glutathione content in the spleen of rats (mean ± SD)

Substance Dose (mg/kg bw)	SOD	CAT	GPX	GST	DTD	GSH
	(U/mg protein)			(mmol/mg protein)		
Single treatment						
Control	3.91 ±0.32	28.20 ±2.29	88.00 ±8.81	110.50 ±7.18	50.07 ±5.01	8.41 ±0.90
TCP 112	5.87 ±0.47*	18.01 ±1.74*	35.20 ±3.52*	104.98 ±6.82	22.03 ±2.20*	8.03 ±0.68
DLT 15	5.48 ±0.44*	13.54 ±1.34*	44.35 ±4.44*	113.82 ±7.39	41.73 ±7.28	8.06 ±0.63
TCP + DLT 112+15	4.50 ±0.36	16.36 ±1.62*	30.16 ±3.02*	100.02 ±6.01	39.33 ±4.23*	6.39 ±0.61*
CPA 50	4.70 ±0.38*	16.26 ±2.58*	35.42 ±1.71*	88.05 ±8.94*	43.91 ±4.60	6.89 ±0.50*
Subacute treatment (30 days)						
Control	4.39 ±0.35	25.94 ±2.57	82.07 ±6.25	103.67 ±15.87	51.82 ±5.43	8.20 ±0.40
TCP 22.5	2.94 ±0.24*	18.16 ±1.80*	24.16 ±1.44*	89.92 ±10.42	34.69 ±4.59*	6.00 ±0.36*
DLT 3	5.05 ±0.41	10.38 ±1.03*	33.48 ±4.41*	110.92 ±16.98	45.48 ±7.94	5.32 ±0.16*
TCP + DLT 22.5+3	4.83 ±0.39	15.83 ±1.57*	33.47 ±2.55*	111.29 ±18.57	41.33 ±4.50*	4.75 ±0.36*

Control: corn oil
TCP: thiacloprid
DLT: deltamethrin
CPA: cyclophosphamide
SOD: superoxide dismutase
CAT: catalase
GPX: glutathione peroxidase
GST: glutathione-S-transferase
DTD: DT-diaphorase
GSH: glutathione
*: significantly different to control, $p < 0.05$

Thymus - Acute treatment

After acute treatment with thiacloprid or deltamethrin alone, and with the thiacloprid – deltamethrin mixture CAT and GPX and DTD were significantly decreased, when compared to controls. In addition, after application of thiacloprid alone GST and DTD were significantly reduced, whereas GST and GSH were significantly decreased after application of the mixture. SOD was only decreased after application of deltamethrin and the mixture.

Thymus - Subacute treatment

The decreases, which were seen for CAT, GPX and DTD after acute treatment, were also observed after subacute treatment. In addition, SOD and GST were significantly decreased after application of thiacloprid alone. GSH was significantly decreased in all treated groups when compared to control.

Table 5.8.2/28-3: Effects on antioxidant enzymes, phase II enzyme activity and glutathione content in the thymus of rats (mean ± SD)

Substance Dose (mg/kg bw)	SOD	CAT	GPX	GST	DTD	GSH
	(U/mg protein)			(nmol/mg protein)		
Single treatment						
Control	3.56 ±0.29	31.33 ±1.63	74.80 ±7.49	56.00 ±4.47	78.33 ±7.37	5.71 ±0.42
TCP 112	3.88 ±0.31	26.32 ±1.37*	56.85 ±5.69*	46.33 ±6.38*	53.07 ±5.08*	5.65 ±0.22
DLT 15	2.67 ±0.22*	18.80 ±0.98*	32.16 ±3.22*	58.87 ±5.71	68.00 ±7.67	5.30 ±0.33
TCP + DLT 112+15	2.85 ±0.23*	17.23 ±0.90*	30.67 ±3.07*	48.39 ±4.67*	66.93 ±5.29	5.11 ±0.28*
CPA* 50	2.92 ±0.24*	27.26 ±1.42*	28.28 ±2.55*	47.47 ±4.50*	75.57 ±5.36	5.12 ±0.23*
Subacute treatment (30 days)						
Control	3.68 ±0.27	29.77 ±1.55	70.71 ±6.37	53.76 ±4.29	81.30 ±4.81	5.68 ±0.31
TCP 22.5	2.46 ±0.18	25.30 ±1.32*	42.43 ±3.82*	44.57 ±2.51*	52.28±10.77*	4.96 ±0.50*
DLT 3	3.31 ±0.24*	26.19 ±1.37*	31.11 ±2.80*	59.68 ±2.50	72.16 ±5.85	4.79 ±0.32*
TCP + DLT 22.5+3	3.50 ±0.24	16.97 ±0.88*	25.46 ±2.29*	48.95 ±4.37	67.84 ±10.04	3.94 ±0.24*

Control: corn oil
TCP: thiacloprid
DLT: deltamethrin
CPA: cyclophosphamide
SOD: superoxide dismutase
CAT: catalase
GPX: glutathione peroxidase
GST: glutathione-S-transferase
DTD: DT-diaphorase
GSH: glutathione
*: significantly different to control, $p < 0.05$

Bone marrow - Acute treatment

After acute treatment with thiacloprid or deltamethrin as well as with the thiacloprid – deltamethrin mixture CAT and GPX and GSH were decreased, when compared to controls. SOD was significantly increased after application of deltamethrin and the mixture, and slightly increased after application of thiacloprid.

Bone marrow - Subacute treatment

After subacute treatment CAT, GPX and GSH were significantly decreased in all pesticide treatment groups, while SOD was significantly increased.

Table 5.8.2/28-4: Effects on antioxidant enzymes, phase II enzyme activity and glutathione content in bone marrow of rats (mean ± SD)

Substance	Dose (mg/kg bw)	SOD	CAT	GPX	GSH
		(U/mg protein)			(nmol/mg protein)
Single treatment					
Control	--	5.48 ± 0.44	33.33 ± 2.07	80.96 ± 8.10	6.88 ± 0.43
TCP	112	6.63 ± 0.53	20.67 ± 1.28*	35.62 ± 3.57*	6.24 ± 0.44
DLT	15	11.12 ± 0.90*	18.00 ± 1.12*	45.91 ± 8.02*	5.90 ± 0.53*
TCP + DLT	112 + 15	7.50 ± 0.61*	16.53 ± 0.98*	26.80 ± 2.13*	5.80 ± 0.48*
CPA*	50	11.61 ± 0.94*	19.00 ± 1.18*	24.72 ± 2.46*	5.88 ± 0.55*
Subacute treatment (30 days)					
Control	--	5.59 ± 0.45	30.00 ± 1.86	77.72 ± 7.78	6.55 ± 0.55
TCP	22.5	11.73 ± 0.95*	17.40 ± 1.08*	31.09 ± 3.11*	5.51 ± 0.33*
DLT	3	12.85 ± 1.04*	19.42 ± 1.90*	27.22 ± 2.94*	5.45 ± 0.38*
TCP + DLT	22.5 + 3	12.46 ± 1.00*	15.48 ± 0.73*	28.20 ± 3.04*	4.84 ± 0.39*

Control: corn oil TCP: thiacloprid
DLT: deltamethrin CPA: cyclophosphamide
SOD: superoxide dismutase CAT: catalase
GPX: glutathione peroxidase GSH: glutathione
*: significantly different to control, $p < 0.05$

C. Effects on TBARS levels in lymphoid organs

The extent of lipid peroxidation in terms of thiobarbituric acid reactive substances (TBARS) levels were increased in all pesticide treatment groups after acute and subacute application. Except for the acute treatment with deltamethrin all increases were statistically significant. Treatment with the mixture of thiacloprid and deltamethrin were in general more pronounced than for single pesticides.

Table 5.8.2/28-5: Effects on TBARS level in lymphoid organs, PMNs and plasma of rats (mean ± SD)

Substance Dose (mg/kg bw)	Spleen	Thymus	Bone marrow	PMNs	Plasma
	(nmol/mg protein)				
Single treatment					
Control	1.90 ± 0.21	3.43 ± 0.39	2.51 ± 0.28	0.03 ± 0.00	2.64 ± 0.17
TCP 112	2.72 ± 0.41*	4.63 ± 0.53*	4.52 ± 0.51*	0.05 ± 0.00*	6.47 ± 0.77*
DLT 15	2.62 ± 0.44	4.59 ± 0.62*	4.37 ± 0.49*	0.06 ± 0.00*	8.19 ± 0.54*
TCP + DLT 112+15	2.66 ± 0.38*	5.21 ± 0.59*	7.54 ± 0.85*	0.06 ± 0.00*	10.57 ± 0.70*
CPA* 50	2.83 ± 0.38*	4.54 ± 0.46*	5.27 ± 0.59*	0.04 ± 0.00*	6.07 ± 0.40*

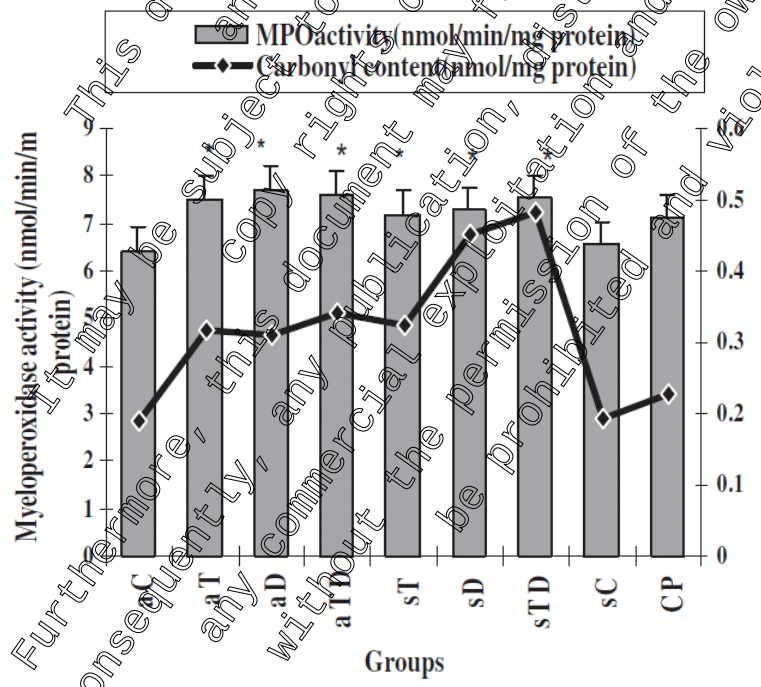
Substance Dose (mg/kg bw)	Spleen	Thymus	Bone marrow	PMNs	Plasma
	(nmol/mg protein)				
Subacute treatment (30 days)					
Control	2.09 ± 0.24	3.64 ± 0.34	2.69 ± 0.23	0.03 ± 0.00	2.77 ± 0.21
TCP 22.5	3.01 ± 0.53*	4.63 ± 0.63*	4.75 ± 0.54*	0.04 ± 0.00*	7.51 ± 1.03*
DLT 3	3.20 ± 0.53*	4.69 ± 0.66*	4.72 ± 0.53*	0.06 ± 0.00*	8.46 ± 0.63*
TCP + DLT 22.5+3	3.31 ± 0.56*	5.90 ± 0.76*	6.97 ± 0.60*	0.07 ± 0.00*	11.91 ± 0.89*

TBARS: extent of lipid peroxidation in terms of thiobarbituric acid reactive substances
PMNs: polymorphonuclear leukocytes
Control: corn oil
DLT: deltamethrin
SOD: superoxide dismutase
GPX: glutathione peroxidase
TCP: thiacloprid
CPA: cyclophosphamide
CAT: catalase
GSH: glutathione
*: significantly different to control, $p < 0.05$

D. Effects on MPO activity and carbonyl content in PMNs

Myeloperoxidase (MPO) activity in the PMNs was significantly increased ($p < 0.05$) in all pesticide treated groups when compared with the corresponding control groups. In addition, a significantly increased carbonyl content was observed in PMNs in all acute pesticide groups and thiacloprid and deltamethrin subacute groups.

Figure 5.8.2/28-1: Effects on myeloperoxidase (MPO) activity and carbonyl content in rat PMNs



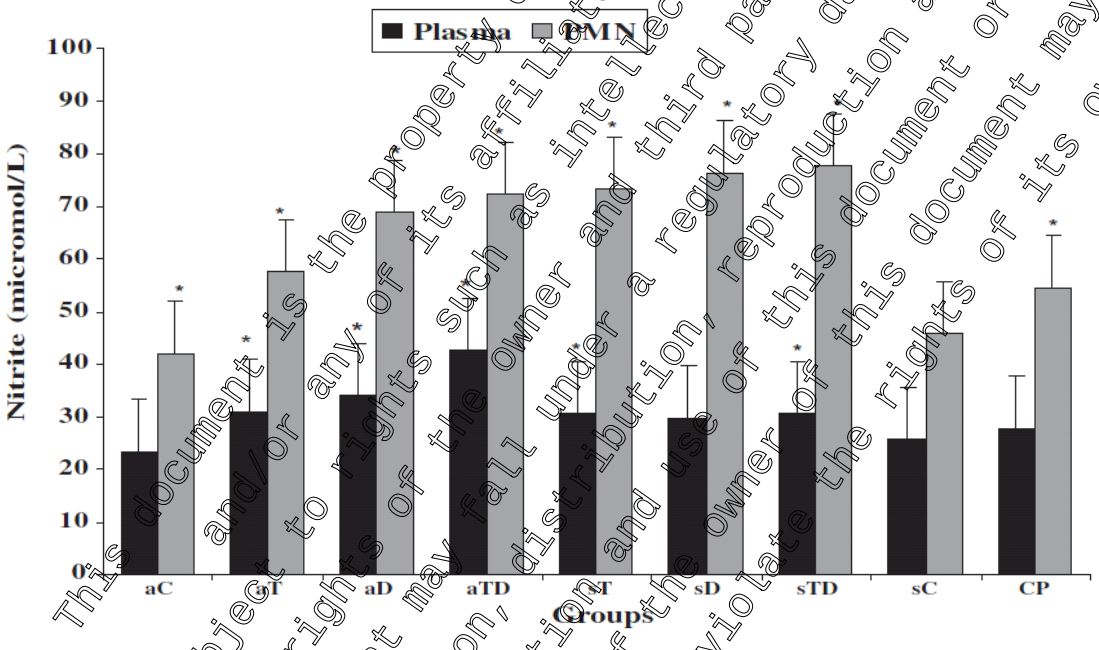
aC / sC: acute / subacute control
aT: single acute dose of 112.5 mg/kg thiacloprid
aD: single acute dose of 15 mg/kg bw deltamethrin
aTD: single acute dose of 112.5 mg/kg thiacloprid + 15 mg/kg deltamethrin;

sT: subacute dose of 22.5 mg/kg/d thiacloprid for 30 days
sD: subacute dose of 3 mg/kg/d deltamethrin for 30 days
sTD: subacute dose of 22.5 mg/kg/d thiacloprid + 3 mg/kg bw/d deltamethrin for 30 days
CP: positive control cyclophosphamide
*: significantly different to controls, $p \leq 0.05$

E. Effects on total nitric oxide levels in PMNs and plasma of rats

A marked elevation in the NO_x level ($p < 0.05$) was observed in all pesticide and CPA treated rats in PMNs and plasma; the increase was higher after acute pesticide exposure than after subacute pesticide exposure.

Figure 5.8.2/28-2: Effects on total nitric oxide levels in PMNs and plasma of rats

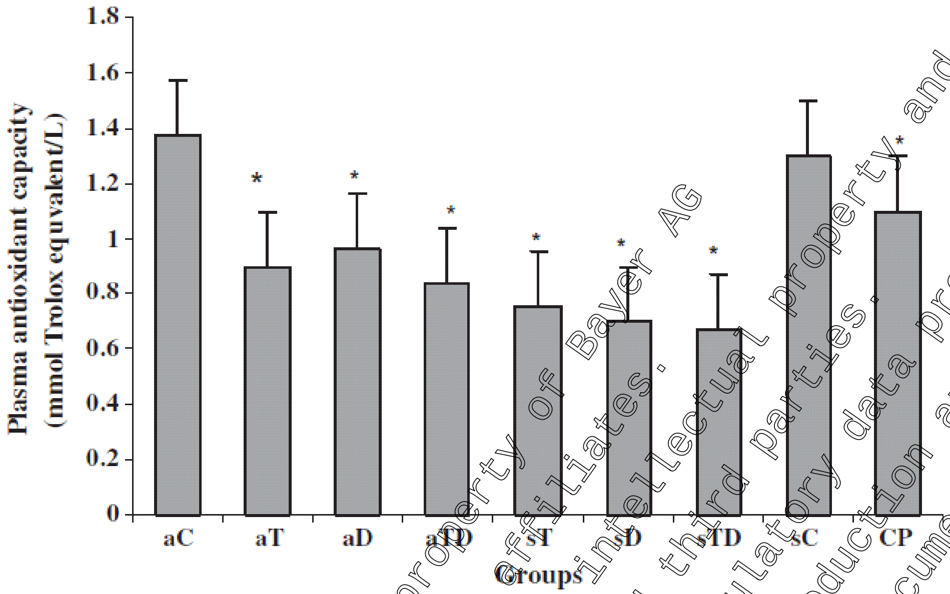


PMN: polymorphonuclear leukocytes
aC / sC: acute / subacute control
aT: single acute dose of 112.5 mg/kg thiacloprid
aD: single acute dose of 15 mg/kg bw deltamethrin
aTD: single acute dose of 112.5 mg/kg thiacloprid + 15 mg/kg deltamethrin;
sT: subacute dose of 22.5 mg/kg/d thiacloprid for 30 days
sD: subacute dose of 3 mg/kg/d deltamethrin for 30 days
sTD: subacute dose of 22.5 mg/kg/d thiacloprid + 3 mg/kg bw/d deltamethrin for 30 days
CP: positive control cyclophosphamide
*: significantly different to the controls, $p < 0.05$

F. Effects on total antioxidant capacity of plasma of rats

Total antioxidant capacity in plasma was significantly decreased in all pesticide treatment groups and in the positive control group.

Figure 5.8.2/28-3: Effects on total antioxidant capacity of plasma of rats



aC / sC: acute / subacute control
aT: single acute dose of 112.5 mg/kg thiacloprid
aD: single acute dose of 15 mg/kg bw deltamethrin
aTD: single acute dose of 112.5 mg/kg thiacloprid + 15 mg/kg deltamethrin;
sT: subacute dose of 22.5 mg/kg/d thiacloprid for 30 days
sD: subacute dose of 3 mg/kg/d deltamethrin for 30 days
sTD: subacute dose of 22.5 mg/kg/d thiacloprid + 3 mg/kg bw/d deltamethrin for 30 days
CP: positive control cyclophosphamide
*: significantly different to the controls, $p \leq 0.05$

III. Conclusion

Acute and subacute pesticide treatments caused significant changes in the levels of AST, ALT, urea and creatinine. Antioxidant enzyme (catalase and glutathione peroxidase), glutathione and plasma antioxidant levels decreased while lipid peroxidation increased in all lymphoid organs and the plasma. Glutathione-S-transferase and especially DT-diaphorase activity decreased after thiacloprid treatment. Myeloperoxidase activity, carbonyl content, lipid peroxidation and total nitrite levels increased in PMNs and plasma. When evaluated as a whole, the oxidative and inflammatory stresses seen in the pesticide combination groups were not much more pronounced than in the groups treated with a single pesticide. In terms of the evaluated biochemical parameters, the pesticides showed similar effects to cyclophosphamide.

BCS opinion: This non-GLP rat study, which was conducted with thiacloprid doses in the range of the known effect levels, provides supplemental information on oxidative stress in lymphoid organs, polymorphonuclear leukocytes and plasma of rats. The reported results do not change existing endpoints and do not change the risk assessment.

The reliability evaluation of the publication is given below.

Klimisch evaluation

Reliability of study	**Reliable with restrictions (Klimisch code 2)**
Comment	Non-GLP study, conducted according to scientific principles with reporting and methodical deficiencies. Only 6 males/group, no positive control for subacute treatment, only one dose for each treatment, only means and no individual values provided; signs of toxicity not reported, no gross and histopathological evaluation, no historical control data provided.
Relevance of study	**Relevant: supplemental information which does not change existing endpoints and does not lead to a more conservative risk assessment.**

Analytical methods

Analytical methods for the determination of thiacloprid by HPLC analysis in rodent diet (+1% corn oil), rodent diet and rat plasma were developed for studies summarised under KCA 5.8.2/16 (M-428958-01-1), KCA 5.8.2/22 (M-359926-01-1) and KCA 5.8.2/25 (M-403763-01-1). The references of the study reports are presented in the following.

Report:	[redacted]6; [redacted]; [redacted];2010;M-392957-01
Title:	Thiacloprid - Determination by high performance liquid chromatography analysis in ground rodent diet (+1 percent corn oil)
Report No:	SA 10217
Document No:	M-392957-01-1
Guidelines:	OECD, 1997; Deviation(s): not specified
GLP/GEP:	**yes**

Report:	[redacted]q; [redacted]; [redacted];2011;M-425259-01
Title:	Thiacloprid - Determination by high performance liquid chromatography analysis in ground rodent diet (+1 percent corn oil)
Report No:	SA 11328
Document No:	M-425259-01-1
Guidelines:	O.E.C.D. Principles of Good Laboratory Practice, 1997 (January 26, 1998) and Article Annexe II à l'article D523-8 du Code de l'Environnement du 16 octobre 2007 (French GLP Legislation).; Deviation(s): not specified
GLP/GEP:	**yes**

Report:	[redacted]t; [redacted]; [redacted];2008;M-304485-01
Title:	Thiacloprid - Determination by high performance liquid chromatography analysis in ground rodent diet
Report No:	SA08077
Document No:	M-304485-01-1
Guidelines:	OECD, 1997; US EPA OPPTS 870.SUPP; Deviation(s): not specified
GLP/GEP:	**yes**

Report:	[redacted]p; [redacted]; [redacted];2011;M-398883-01
Title:	Thiacloprid - Determination by high performance liquid chromatography analysis in Sprague-Dawley rat plasma
Report No:	SA 10347
Document No:	M-398883-01-1
Guidelines:	OECD,1997; Deviation(s): not specified
GLP/GEP:	**yes**

CA 5.8.3 Endocrine disrupting properties

Thiacloprid - Endocrine disruption

A review of the whole data base on thiacloprid was conducted to identify possible effects of thiacloprid on endocrine organs, tissues or parameters.
Thiacloprid treatment led to findings linked to the thyroid as well as to findings linked to steroidogenesis. However, the data give no indications for effects on the pancreas (including blood glucose, urinalysis data or histopathology), pituitary (organ weight, histopathology), thymus (histopathology) or parathyroid (based on blood calcium levels and histopathology) in rat, mouse or dog.

Effects on the thyroid

According to the study reports as well as the evaluation at Annex I inclusion thyroid effects were observed in the rat (including effects on thyroid hormones, thyroid weight and histopathology) and dog (thyroid hormone changes), while there were no effects on the thyroid in the mouse (no histopathological evidence up to 3351 and 873 mg/kg bw/day in the 14-week and the oncogenicity study, respectively). However, thyroid hormone data in rat and dog had to be re-evaluated, since in the original evaluation historical control data collected only from studies run prior to the thiacloprid studies had been used. The re-evaluations can be studied in detail in the respective position papers by [redacted] 2014 (M-496853-01-1 and M-496983-01-1).

Thyroid effects in rats:

Thyroid data in rats and the mode of action of the development of benign thyroid follicular cell adenoma in aging male rats treated with thiacloprid were reconsidered based on a new analysis of the thyroid hormone data in the available rat studies.

By comparison of the hormone data with the historical control data of the year of study conduct it could be shown that changes or tendencies for changes in thyroid hormone levels were only seen after high doses of 69 mg/kg bw/day (equivalent to 1000 ppm in the diet, the high dose of the 2-year rat study, which approaches or exceeds the MTD in males or females, respectively) or higher. These changes comprised increased TSH values as well as decreased T3 and T4 values. In lower doses no changes of thyroid hormones or TSH were observed. Thyroid findings were not very pronounced and included only minimal to slight thyroid follicular cell hypertrophy and colloid changes. The fact that these findings were observed at lower doses than changes of thyroid hormones and TSH indicates that the body maintains an euthyroid state at these dose levels. Thyroid findings were always accompanied by liver enzyme induction with markedly and dose dependently increased UDP-GT levels as well as less increased P450 levels in liver tissue. Further evidences of liver enzyme induction were increased liver weights, minimal to moderate hepatocellular hypertrophy and cytoplasmatic changes. Regarding thyroid follicular cell hypertrophy and liver enzyme induction male rats were markedly more sensitive than female rats, and this was getting even more pronounced after long-term treatment for 1 or 2 years. This fits to the fact that a slightly increased incidence of benign thyroid follicular cell adenoma was only observed in aging male rats and not in females.

With regard to possible modes of action genotoxicity as well as an inhibition of thyroid peroxidase (TPO) can be excluded.

Taking all facts together, the mode of action of thyroid follicular cell adenoma development in aging male rats treated with thiacloprid is secondary to liver enzyme induction (including pronounced and dose dependent UDP-GT increases as well as less pronounced increases of P450), which leads to an increased metabolism and excretion of T3 and T4 from plasma and subsequently to a stimulation of the hypothalamic-pituitary-thyroid axis. Permanent stimulation of the thyroid follicular cells by TSH leads to thyroid follicular cell hypertrophy and, after long-term treatment in aging males (which show a more pronounced liver enzyme induction than females) also to a slightly increased incidence of thyroid follicular cell adenoma. It should be noted that the changes observed under thiacloprid treatment are mostly minimal to slight and that the body up to high doses approaching the MTD is able to maintain an euthyroid state.

Due to species specific differences between rat and man this mode of action of thyroid tumor development (including the mechanism by increased UDP-GT and P450) is considered not to be relevant for humans.

An overview on the thyroid data in rat studies with thiacloprid is given in the tables below.

Table 5.8.3-1: Thyroid effects in rats in toxicological studies on thiacloprid

Study Doc ID Doses [ppm] [mg/kg bw/day]	14-day gavage M-000703-01-4 0-5-10-20-120	14-day dietary M-000785-02-1 0-25-100-500-2000 0/0-2.5/2.3-11/9.6-49/50-188/187	3-week dietary M-030427-03-1 0-25-100-400-1600 0/0-2.6/3.1-9/12-37/45-145/191	13-week dietary + rec. M-000863-01-1 0-25-100-400-1600 0/0-1.9/2.0-7.3/7.6-29/36-123/161	2-year dietary M-003817-02-1 0-25-50-500-1000 0/0-1.2/1.6-2.5/3.3-25/34-52/69
Food intake & body weight (bw)	≥ 60 mg/kg bw/d: ↓ food intake & bw	≥ 49/50 mg/kg bw/d: ↓ food intake & bw	145/191 mg/kg bw/d: ↓ food intake (week 1), ↓ bw	≥ 29/36 mg/kg bw/d: ↓ bw (rec: N.E.) food intake. N.E.	≥ 25/34 mg/kg bw/d: ↓ bw Food intake. N.E.
Protein & albumin levels	N.E.	N.E.	Protein: N.E. Albumin: N.I.	at 123 mg/kg bw/d: ↑ protein (week 11/12, m) (rec: N.E.) Albumin: N.E.	N.E.
Thyroid hormones & thyroid binding capacity	T3. T4, TBC: N.E. TSH: N.I.	188/187 mg/kg bw/d: ↑TSH (m/f, week 2, in 3-s range of HCD, statistically significant in f only) T3, T4, TBC: N.E.	145/191 mg/kg bw/d: tendencially ↓ T3 (day 2) & T4 (day 2, 7 & 21 (m)) ↑TSH (day 14 (m), day 7 & 21/22 (f), in 3-s range of HCD (m), in 2-s range or exceeding the 3-s range in (f) TBC: N.E.	123 mg/kg bw/d: ↑T3 (m; > 3-s range of HCD in week 3 & 11/12) T4, TBC: N.E. TSH: N.I.	69 mg/kg bw/d: TSH: trend for an ↑ (f, weeks 26 & 105) T3, T4, TBC: N.E.
Clinical chemistry in liver tissue	≥ 60 mg/kg bw/d: ↑UDPGT ↑P450	≥ 49/50 mg/kg bw/d: ↑UDPGT P450: N.I.	≥ 37/45 mg/kg bw/d: ↑UDPGT P450: N.I.	≥ 29/36 mg/kg bw/d: ↑UDPGT (rec: N.E.) ↑P450 (rec: N.E.)	<u>After 1 year:</u> ≥ 25/34 mg/kg bw/d: ↑UDPGT P450: N.I. <u>After 2 years</u>: liver enzymes N.I.

Study **Doc ID** Doses [ppm] [mg/kg bw/day]	**14-day gavage** **M-000703-01-4** 0-5-10-20-120	**14-day dietary** **M-000785-02-1** 0-25-100-500-2000 0/0-2.5/2.3-11/9.6-49/50-188/187	**3-week dietary** **M-030427-03-1** 0-25-100-400-1600 0/0-2.6/3.1-9.12-37/45-145/191	**13-week dietary + rec.** **M-000863-01-1** 0-25-100-400-1600 0/0-1.9/2.0-7.3/7.6-29/36-123/161	**2-year dietary** **M-003817-02-1** 0-25-50-500-1000 0/0-1.2/1.6-2.5/3.3-25/34-52/69
Liver findings	≥ 60 mg/kg bw/d: ↑ liver weight (up to +20 & +40% at 60 & 120 mg/kg bw/d), slight cytoplasmatic changes (f) 120 mg/kg bw/d: slight hypertrophy & cytoplasmatic changes in m/f	≥ 49/50 mg/kg bw/d: ↑liver weight (up to +9 & +38% at 49/50 & 188/187 mg/kg), hypertrophy & cytoplasmatic changes 188/187 mg/kg bw/d: ↑ lipid storage (m)	≥ 37/45 mg/kg bw/d: ↑ liver weight (m, up to +14%) 145/191 mg/kg bw/d: ↑ liver weight (up to +41 / 39% & size (m/f), marked lobular pattern (f) Histopathology: N.I.	≥ 29/36 mg/kg bw/d: ↑liver weight (m, up to +8%), moderate liver hypertrophy with cytoplasmatic changes (rec: reversible in f, still slight hypertrophy at 123 mg/kg bw/d in 3/10 m) 123/161 mg/kg bw/d: ↑liver weight (m/f, up to 44 / 38%)	After 1 year: ≥ 25/34 mg/kg bw/d: liver hypertrophy (m/f), focal fat infiltration (m) After 2 years: ≥2.5 mg/kg bw/d: liver hypertrophy & cytoplasmatic change 52 mg/kg bw/d: ↑ liver weight (m, up to +31%)
Thyroid findings	120 mg/kg bw/d: slightly ↑ no. of mitoses in 1/3 m & 1/3 f Thyroid weight: N.I.	≥ 49/50 mg/kg bw/d: ↑mitotic index (m) 188/187 mg/kg bw/d: thyroid follicular cell hypertrophy (5/5 m slight, 1/5 f minimal) Thyroid weight: N.I.	≥ 37 (m) mg/kg bw/d: ↑incidences of min. to slight thyroid follicular cell hypertrophy (5/10 m), at 145/191 mg/kg bw/d in 8/10 m & 5/10 f Thyroid weight: no ↑ in weight ↑ treatment related effect	123 mg/kg bw/d: slightly ↑ thyroid weight (m), Thyroid histopathology: N.E.	After 1 year: ≥ 25 (m)/69(f) mg/kg bw/d: min. to slight thyroid follicular cell hypertrophy & colloid clumping 52 mg/kg bw/d: thyroid follicular cell adenoma (m: 1/10) After 2 years: ≥ 2.5 mg/kg bw/d: min. to slight thyroid follicular cell hypertrophy & colloid alteration (m) ≥25/34 mg/kg bw/d: min. to slight thyroid follicular cell hypertrophy & colloid alterartion (f), ↑ incidences of thyroid follicular cell adenoma (m)

N.E. = No effect N.I. = Not investigated m = male(s) f = female(s) rec = recovery min = minimal bw = body weight d = day
2-s or 3-s range of HCD = 2 sigma or 3 sigma range of historical control data

Table 5.8.3-2: Thyroid follicular cell adenoma observed in a 2-year dietary study on thiacloprid in rats (M-003817-02-1)

Dose level [mg/kg bw/day]	Dose [ppm]	Species/sex	Tumour	Tumour incidence [%]	BW in comparison to controls [%]	Food intake	Other findings regarding liver, thyroid, adrenals, sexual organs
0	0	Rat, male	Thyroid follicular cell adenoma	0 (0/50)	nad	nad	nad
1.2	25	Rat, male	Thyroid follicular cell adenoma	0 (0/50)	nad	nad	nad
2.5	50	Rat, male	Thyroid follicular cell adenoma	2 (1/50)	nad	nad	Liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy, cytoplasmatic changes, eosinophilic foci, hypertrophy of the thyroid follicular epithelium
25.2	500	Rat, male	Thyroid follicular cell adenoma	10 (5/50)	Up to -7% in weeks 1-10	Very slight ↓	Liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy, cytoplasmatic changes, eosinophilic foci, hypertrophy of the thyroid follicular epithelium
51.7	1000	Rat, male	Thyroid follicular cell adenoma	16.3 (8/49)	-12% week 1&2 + 1 year: up to -9%	Very slight ↓	Liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy, cytoplasmatic changes, eosinophilic foci, hypertrophy of the thyroid follicular epithelium

Thyroids: 1000 ppm after 1 year: 1/10 males: thyroid follicular cellular adenoma
Historical controls: in males up to 5.1% (Bayer internal data), up to 14.7% (RITA database)
Hypertrophy of thyroid follicular epithelium: in 500 + 1000 ppm males & 1000 ppm females after 1 + 2 years (minimal to slight) and 50 ppm in males after 2 years; not observed after 1600 ppm for 90 days
BW: body weight nad: nothing adverse detected M: male(s) F: female(s) ↑: increase(d)

Thyroid effects in dogs:

Re-evaluation of the thyroid hormone (T3, T4) and thyroxine binding capacity (TBC) data from all three dog studies on thiacloprid in the context of the historical control data of the years of study conduct revealed that there are no treatment related changes of thyroid hormones and thyroxine binding capacity. In addition, also thyroid weights and thyroid histopathology did not give any indication for an effect of thiacloprid. **Based on these data, and in contrast to the evaluations made in the study reports and at Annex I inclusion, it is concluded that thiacloprid has no effect on thyroid parameters in dogs.**

Conclusion on effects on the thyroid:
In summary, thiacloprid treatment leads to effects on the thyroid in rats, but not in dogs or mice. The mode of action in rats is secondary to liver enzyme induction, which leads to an increased metabolisation and excretion of thyroid hormones and, as a consequence, to an activation of the hypothalamic-pituitary-thyroid axis. Permanent stimulation of the thyroid follicular cells by TSH causes thyroid follicular cell hypertrophy and, after long-term treatment in males, a slightly increased incidence of thyroid follicular cell adenoma. Overall, the changes observed under thiacloprid treatment in rats are mostly minimal to slight and the body is able to maintain an euthyroid state up to highdoses approaching the MTD.
This mode of action for the development of thyroid follicular cell adenoma in rats is well known. Due to species specific differences between rat and man it is generally accepted as a mode of action, which is non-relevant for humans.

Effects on steroidogenesis:

Findings, which are or could be linked to effects on steroidogenesis have been observed in toxicological studies with thiacloprid in rats, mice and dogs as well as in in vitro studies.

In vitro studies:

Thiacloprid showed an effect on steroidogenesis in the H295R assay. Increased progesterone secretion was observed at 100 µM, the NOAEC for this finding was 50 µM (equivalent to unbound concentrations of about 25 or 12.5 mg/L thiacloprid, respectively). However, unbound plasma concentrations of up to 11.5 mg/L in female rats corresponding to dietary exposure with 1000 ppm thiacloprid (high dose of the 2-year rat study, clearly exceeding the MTD, and of several one-generation studies investigating dystocia) are still in the range of the NOAEC of 12.5 mg/L for progesterone increase in this assay ([redacted], 2014, M-498558-01-1).
Incubation of rat preantral follicles with thiacloprid led to increased estradiol and progesterone secretion at 500 µM or about 126 mg/L thiacloprid, indicating that preantral follicles are a cellular target for thiacloprid treatment. The NOAEC was 100 µM or about 25 mg/L, indicating again that hormone changes, tumour development and dystocia in rats occurred at unbound plasma concentrations in the range of the NOAEC of this assay. Since the effects on sex steroid hormones as well as tumour development and dystocia in rodents occurred always at dose levels showing

pronounced enzyme induction as well as increased expression of genes associated with sex steroid hormone biosynthesis, these effects are considered to be secondary to enzyme induction.

In vivo findings related or possibly related to effects on steroidogenesis in rats

In the 2-year rat study on thiacloprid (M-003817-02-1) increased incidences of uterine adenocarcinoma occurred. Incidences were 12 - 6 – 6 – 28 – 36% after 0 – 1.6 - 3.3 – 33.5 – 69.1 mg/kg bw/day, respectively. The Bayer internal historical control data for this type of spontaneously occurring malign tumor include incidences up to 24%, the peer reviewed historical control data from the RITA database contain incidences up to 28%. This shows that the incidence of 28 % at 33.5 mg/kg bw/day is rather a borderline effect. It has to be noted that with body weight decreases of -10% to -15% and -21% the affected dose levels of 33.5 and 69.1 mg/kg bw reach or exceed the MTD, respectively. The tumor data are presented in Table 5.8.3-3 below.

The proposed mode of action for the uterine adenocarcinoma is as follows (described in the position paper by [redacted], 2010, M-362441-01-1, KCA 5.8.1 with slight differences): In female rats thiacloprid affects steroid sex hormone secretion leading to changes in circulating levels of progesterone and estradiol. An overview on hormone data is given in Table 5.8.3-4 below. The changes were marginal to slight. The increases of progesterone levels gained statistical significance at 60 mg/kg bw/day, increases in estradiol levels were statistically significant at doses of 75 mg/kg bw and above. A statistically significant increase of FSH was also observed at 108 mg/kg bw/day. The hormone changes were accompanied by changes in gene expression associated with the regulation of steroid hormone synthesis (also observed at doses of 60 mg/kg bw/day and above) indicating overall an induction of enzymes of the steroid hormone biosynthesis (not including aromatase). In the young adult rat these hormone changes have no adverse effect: there was no influence on the estrous cycle (investigated in the two-generation study over 3 weeks ([redacted] & [redacted], 1997, M-001304-01-1, KCS 5.6.1)) or on other sex hormone sensitive tissues and organs like the ovaries, the mammary gland, the uterus or the pituitary. Only in aged, 1-2 years old females these hormone changes eventually lead to uterine changes (increased incidence of uterine glandular hyperplasia) including effects on the estrous cycle (i.e. fewer thiacloprid treated females in pseudopregnancy and more in the ambiguous phase, lower level of vaginal mucification, marginal increases of plasma estradiol), and finally tumors as the alterations in hormone secretion generally associated with the aging process are exacerbated following treatment with high doses of thiacloprid. Further findings with a possible endocrine background in old female rats after 2 years of treatment with high doses were an increased incidence of ovary cysts and decreased incidences of lacteal cysts and galactocele in the mammary gland, which are *per se* not adverse.

In conclusion, the increased incidences of uterine adenocarcinoma occurring only in aged female rats after high oral doses of thiacloprid at the MTD or higher are considered to be secondary to enzyme induction including enzymes involved in steroid biosynthesis. Since enzyme induction is less pronounced in humans and also the normal range of female sex hormones shows a much broader variation than in rodents (for details please refer to [redacted], 2007, M-293264-01-1), this mode of action is considered to be less relevant in humans.

Dystocia was another effect in female rats, which was noted in several 1 or 2-generation studies in Sprague-Dawley rats from the breeder Sasco at doses of 22 mg/kg bw and above. Besides an

unspecific mode of action secondary to stress, dystocia also seemed to be linked to perturbations of sex hormone levels. Increased estradiol levels, slightly increased progesterone and LH levels as well as increased corticosterone levels in plasma during premating, gestation and lactation shortly after parturition were observed in a previously conducted study ([redacted], 1998, M-004253-01-1, KCA 5.8.2). This was confirmed by the latest special one-generation study ([redacted]-[redacted], 2011 M-403763-01-1, KCA 5.8.2), in which the levels of progesterone (slightly increased mean value at GD 20, absence of normal decrease prior to parturition in one rat with dystocia) and estradiol (increased mean values at GD 21 and 22) and the respective balance between these hormones in the days before and during parturition were affected in thiacloprid treated animals. However, the regulation of birth in humans is different, i.e. a missing decrease in progesterone level, which is mandatory for normal parturition in rat, is not seen in humans, in which progesterone levels in plasma stay high during parturition. Therefore, this mode of action is considered to be rat specific and not relevant for humans. Since also the normal range of progesterone and estradiol levels in humans is much wider than in rat, the observed, rather slight changes of these plasma levels in rat are considered to pose no risk for dystocia in pregnant women (for more information, please refer to [redacted], 2007, M-293264-01-1).

In male rats, a slightly increased incidence of Leydig cell hypertrophy (mostly graded as minimal to slight) was noted in the testes after 2 years of treatment with 25 mg/kg bw/day and higher doses. However, no other effects on male reproductive organs or male fertility were observed in this species *in vivo*.
In the H295R steroidogenesis assay an inhibition of testosterone secretion was noted commencing at the lowest investigated concentration of 50 µM or 12.5 mg/L thiacloprid, repectively (being statistically significant after incubation for 48 h, but not for 24 h). However, unbound plasma concentrations of thiacloprid in male Wistar rats *in vivo* at the high dose of the 2-year rat study of 1000 ppm (equivalent to 52 mg/kg bw/day) with 5.7 – 6.9 mg/L were lower.

<u>Findings related or possibly related to effects on steroidogenesis in mice</u>
In the 13-week mechanistic study (M-003764-01-1) changes of steroid sex hormone levels in plasma were noted in females. These included slightly decreased estradiol levels and increased progesterone levels, which led to a decrease of the estradiol/progesterone ratio at dose levels of 1101 mg/kg bw/day and higher. Since a first, slight change of the estradiol level observed at 139 mg/kg bw/day had no effect on the estradiol/progesterone ratio, 139 mg/kg bw/day is considered to be the NOAEL for hormonal changes. The hormone changes were accompanied by reduced cholesterol levels. However, similar to the situation in rats, these hormonal perturbations had no influence on the estrous cycle in young adult animals (see 14-week dietary study in mice, M-000697-02-1). Details are given in Table 5.8.3-7.
Furthermore, female mice displayed enlargement, hypertrophy and more prominent vacuolisation of the adrenal X-zone commencing at 27 mg/kg bw and, at higher doses, also increases in adrenal weight (see Table 5.8.3-5). The adrenal X-zone of female mice is a species-specific zone not seen in other laboratory animals or man. X-zone vacuolation is regarded to be a physiological finding in the context

of age-related X-zone degeneration (Greaves 1990[5]). The X-zone involutes in male mice at puberty and persists in females until the first pregnancy. In non-pregnant females, it degenerates during adulthood depending on the genetic background of the animals (Shire and Beamer 1983[6]). The significance of the dose-related increase of X-zone vacuolation seen in the thiacloprid studies is unclear but probably related to hormonal imbalances since development and disappearance of this zone is under hormonal influence (Deacon et al. 1986[7]). However, these findings are not considered to be adverse effects, but biomarkers for an endocrine effect. The NOEL for these effects was 18 mg/kg bw.

In the 14-week dietary study (M-000697-02-1) histopathological investigation of the ovaries revealed a decrease of advanced corpora lutea with eosinophilic cells after 704 mg/kg bw/day and above and the interstitial glands of the ovaries appeared to be activated. These glands are derived mostly from atretic follicles and respond to gonadotropin stimulation but their functional significance is unknown (Yuan, Y.1991[8]). However, there were no effects on the mammary gland and, in the anterior pituitary, immunohistochemical demonstration of prolactin revealed also no evidence of any changes related to dosing with the test-compound.

In the oncogenicity study in mice (M-003819-02-1) increased incidences of ovarian luteoma, a benign tumor, were noted after two years of treatment (for details see Table 5.8.3-6). In contrast, no adverse findings in the ovaries were reported after one year of treatment. Incidences of ovarian luteoma after two years were 0 - 2 - 6 - 13% after 0 - 10.9 - 475.3 - 872.5 mg/kg bw/day. The Bayer internal historical control data for the period of time when the study was conducted showed incidences of up to 6%. At the mid and high dose luteoma were accompanied by a slightly increased incidence of eosinophilic, luteinized cells in the ovarian stroma (5/49 and 8/47 in comparison to 3/47 in the controls). Due to the large dose spacing between mid and low dose in this study the NOAEL for ovarian luteoma was 10.9 mg/kg bw/day.

In male mice, there were no effects on male reproductive organs in any study.

Findings related or possibly related to effects on steroidogenesis in dogs

In the subacute dog study (M-003816-02-1) a slight increase in prostate weight without any histopathological correlate was noted after administration of the high dose of 2500 ppm (equivalent to 80 mg/kg bw/day, administered for up to 10 weeks and to 66 mg/kg bw/day for 4 weeks). The finding was more pronounced in dogs treated with 80 mg/kg bw/day for 10 weeks. There were no findings in

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5 Greaves, P.: Histopathology of preclinical toxicity studies. Chapter XII endocrine glands. Elsevier Amsterdam, New York, Oxford. 677-755; 1990.

6 Shire, J.G.M. and W.G. Beamer: Adrenal Changes in Genetically Hypothyroid Mice. Journal of Endocrinology 102, 277-280; 1983.

7 Deacon, C.F., Mosley, W., Jones, J.C.: The X-zone of the mouse adrenal cortex of the Swiss albino strain. Gen. Comp. Endocrinol. 61, 87-99; 1986.

8 Yuan, Y.: Female Reproductive System, in: Handbook of Toxicologic Pathology, eds. Haschek, W.M. and C. G. Rousseaux, Academic Press Inc., San Diego U.S.A.; 891-935; 1991.

the testes and epididymides in this study. In the 15-week dietary study (M-003814-01-1) at 68 mg/kg bw/day there were slightly more prominent Leydig cells in the testes (3/4; controls: 1/4) as well as a slightly increased no. of degenerated spermatocytes (2/4). The second finding was also observed in the epididymides (4/4, controls: 1/4). Such changes are known to show a wide variation with respect to severity and incidence in young mature dogs. Prostate weights were increased at ≥ 35 mg/kg bw/day, exceeding slightly the historical control data, while very low absolute and relative weights ocurred in the concurrent control and low-dose groups. Increased prostate weights were accompanied by slight to moderate glandular hypertrophy (4/4 at both dose levels).
Since it is difficult to distinguish treatment related effects on these male reproductive organs from the normal, wide variability in young dogs, additional male dosage groups for an interim sacrifice after 26 week of treatment as well as sonographic investigations of the prostate were included in the study design of the 1-year study (M-003818-01-1). In this study no changes regarding testes or epididymides were reported. Prostate weight was also unchanged after 26 weeks of treatment, but was slightly increased at the high dose of 34 mg/kg bw/day after 1 year. Sonography of the prostate after 17, 26 and 52 weeks of treatment as well as histopathology revealed no treatment related effects.

Female dogs showed no effects on female reproductive organs in any of the studies.

Conclusion on effects on steroidogenesis:

***In vitro* assays showed that thiacloprid has a direct effect on steroidogenesis at high concentrations, which exceeded the unbound plasma concentrations of thiacloprid *in vivo* at the high dose of the 2-year rat study of 1000 ppm. The dose levels *in vivo* with findings like hormonal changes, uterine tumors and dystocia were always associated with enzyme induction including enzymes involved in steroid biosynthesis (with the exception of aromatase). The observed hormone changes and resulting toxicological findings are therefore considered to be secondary to enzyme induction.**

In female rats thiacloprid treatment caused changes in steroid sex hormone levels, which had no further consequences in young adult females. However, in old, acyclic rats these hormonal disturbances led to changes of the uterus and, eventually, to an increased incidence of uterine tumours, since alterations in hormone secretion generally associated with the aging process are exacerbated following treatment with high thiacloprid doses. Since enzyme induction is less pronounced in humans and also the range of female sex hormones shows a much broader variation than in rodents, this mode of action is considered to be less relevant in humans. The lowest dose level with a borderline increased tumor incidence was 33.5 mg/kg bw/day. Dystocia was another finding observed in female rats commencing at 22 mg/kg bw/day. Besides an unspecific mode of action secondary to stress, dystocia also seemed to be linked to perturbations of female sex hormone levels, especially to a missing progesterone decrease before parturition. This decrease is mandatory for normal birth in rats, but not in humans, in which regulation of birth is different. Therefore, this mode of action is considered to be rat specific and not relevant for humans. Since the range of progesterone and estradiol levels in humans is also

during pregnancy and birth much wider than in rat, the observed, rather slight changes of these plasma levels in rat are not considered to pose a risk for dystocia in pregnant women.
In male rats, only a slightly increased incidence of minimal to slight Leydig cell hypertrophy was noted in testes after 2 years of treatment with doses of 25 mg/kg bw and above. This is considered to be a biomarker for an endocrine effect, but no adverse effect.

In female mice, changes of steroid sex hormone levels, findings in the adrenal glands (increased weight and enlargement, hypertrophy and more prominent vacuolization) and in the ovaries (a decrease of advanced corpora lutea with eosinophilic cells and an activation of the interstitial as well as an increased incidence of ovarian luteoma) could be linked to effects on steroidogenesis. The adrenal findings are mouse specific, not relevant for humans and, as the slight hormone changes, considered at most as a biomarker for an endocrine effect. The only adverse finding, the ovary changes with the luteoma, was seen only at very high doses of 475 mg/kg bw/day and higher at the end of the 2-year study.

In male dogs findings with a possible endocrine background were not seen consistently. Increased prostate weights were noted repeatedly in studies up to 1 year, but not seen in every case. Only in the 15-week study they were accompanied by slight to moderate glandular hypertrophy of the prostate as well as by slightly more prominent Leydig cells in the testes and a slightly increased no. of degenerated spermatocytes in testes and epididymides. All of these findings can also be found in untreated young dogs during their normal development. In conclusion, there are at most transient possible endocrine effects in male dogs. A slightly higher prostate weight without morphological correlate is not seen as an adverse effect, but at most as a biomarker for an endocrine effect.

Assessment of the endocrine potential:

Taking all results together thiacloprid treatment leads to endocrine effects in toxicological animal studies. However, many of the effects were no adverse effects, but biomarkers for a (possible) endocrine effect, or they were species-specific and not relevant for humans (like the observed thyroid findings or dystocia in rat and adrenal findings in female mice). The only adverse findings with a possible, although less pronounced, relevance for humans are the increased incidences of uterine adenocarcinoma in female rats, commencing at 33.5 mg/kg bw/day, as well as the ovarian luteoma in female mice, which were observed at 475 mg/kg bw/day and higher doses. According to the Joint DE-UK position "Regulatory Definition of an Endocrine Disrupter in Relation to Potential Threat to Human Health" of March 2011 the uterine adenocarcinoma with its borderline increased incidence at 33.5 mg/kg bw/day observed in the combined chronic toxicity and carcinogenicitiy study in rats would fall into the STOT-RE Cat 2 guidance values of the CLP regulation (> 5 and ≤ 50 mg/kg bw for chronic / long-term studies). As such, thiacloprid would not be deemed an ED of regulatory concern and the standard risk assessment could be applied.

Table 5.8.3-3: Findings possibly linked to effects on steroidogenesis observed in a 2-year dietary study on thiacloprid in rats (M-003817-02-1)

Dose level [mg/kg bw/day]	Dose [ppm]	Species/sex	Tumour	Tumour incidence [%]	BW in comparison to controls [%]	Food intake	Other findings regarding liver, thyroid, adrenals, sexual organs
0	0	Rat, female	Uterine adenocarcinoma	12 (6/50)	nad	nad	nad
1.6	25	Rat, female	Uterine adenocarcinoma	6 (3/50)	nad	nad	nad
3.3	50	Rat, female	Uterine adenocarcinoma	6 (3/50)	nad	nad	nad
33.5	500	Rat, female	Uterine adenocarcinoma	28 (14/50)	↓ from week 8 on, -10 up to -15%	Very slight ↓	Liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy, cytoplasmatic changes, eosinophilic foci, hypertrophy of the thyroid follicular epithelium, ↑ incidences of ovarian cysts
69.1	1000	Rat, female	Uterine adenocarcinoma	36 (18/50)	↓ from week 3 on, decreased up to -21%	Very slight ↓	Liver enzyme induction, ↑ liver weight, hepatocellular hypertrophy, cytoplasmatic changes, eosinophilic foci, hypertrophy of the thyroid follicular epithelium, slight increase in follicular cell adenoma, ↑ incidences of ovarian cysts

Uterus: increased incidence of glandular hyperplasia at 500 and 1000 ppm after 1 year
Historical controls: up to 24% (Bayer internal data), up to 28% (RITA database)
BW: body weight nad: nothing adverse detected M: male(s) F: female(s) ↑: increase(d) ↓: decrease(d)

Table 5.8.3-4: Effects on sex hormones and steroidogenesis in toxicological studies on thiacloprid in rats

Parameter	**Single dose gavage study (females) M-359235-01-1**	**Repeated dose gavage study (females) M-360362-01-1**	**Repeated dose gavage study (females) M-360349-01-1**	**4-week dietary study in females M-360757-02-1**	**4-week dietary study in aged females M-359926-01-1**
Doses [ppm]				0-100-1000-1600 ppm	0-1000
[mg/kg bw/day]	0-60	4 x 0-60	4 x 0-60	0-8-75-108 in F	0-32 in F
Sampling:	2, 8, 24 h after dosing	2, 8 h after the last dose	24 h after the last dose		
Progesterone	***Significant ↑ 8 & 24 h after dosing***	***Significant ↑ 8 & 24 h after dosing***	***Significant ↑ 24 h after dosing***	Marginal ↑ ≥ 8 mg/kg bw/day, but statistically not significant	Large interanimal variability (difficult to interpret)
Estradiol	No relevant changes	Not detected (technical problems?)	Marginal ↑, but statistically not significant	***Statistically significant ↑ ≥ 75 mg/kg bw/day***	Marginal ↑, but statistically not significant
Testosterone	No relevant changes	No relevant changes	Detected more readily in thiacloprid animals (9/13, controls: 2/13)	No relevant changes	Not detected
FSH	Not investigated	No relevant changes	Slight ↑, but statistically not significant	***Slight ↑ at 108 mg/kg bw/day***	Not investigated
Gene expression: steroid hormone synthesis	***Liver: statistically significant ↑*** Ovary: no change	Not investigated	Liver, ovary, adrenals: tendency for an ↑	***Liver, ovary: ↑ at ≥ 75 mg/kg bw/day***	Not investigated

BW: body weight F: female(s) ↑: increase(d) ↓: decrease(d)

MOUSE

Table 5.8.3-5: Adrenal findings possibly linked to effects on steroidogenesis in toxicological studies on thiacloprid in mice

Adrenal findings	2- & 3-week dietary studies M-000821-01-1 & M-000688-01-1	13-week mechanistic dietary study in female mice M-003764-01-1	14-week dietary study M-000697-02-1	Oncogenicity study M-003819-02-1
Doses [ppm]	2-week: 0-50-200-2000-10000 3-week: 0-100-1000-10000	0-10-30-250-2500-2500+mecamylamine*	0-50-250-1250-6250	0-30-1250-2500
[mg/kg bw/day]	2-week:0-21.6/29.8-84.3/113.2-765.1/1201.2-4143.2/5449.8 3-week: 0-30.1/63.9-368/559-4141/5785 in M/F	0-6-18-139-1101-1244 in F	0-19.9/27.2-102.6/139.1-542.4/704.3-2819.9/3351 in M/F	0-5.7/10.9-234/475-546/873 in M/F
Organ weight	Not investigated	No effect up to 1244 mg/kg bw/day	↑ (statistically not significant) in females ≥ 704 mg/kg bw/day	↑ in females ≥ 873 mg/kg bw/day at interim sacrifice
Histology	Not investigated	Vacuolisation of the adrenal x-zone ≥ 139 mg/kg bw/day (NOEL: 18 mg/kg bw/day), hypertrophy ≥ 1101 mg/kg bw/day	Enlargement, more prominent vacuolisation of the adrenal x-zone in females ≥ 27 mg/kg bw/day	Vacuolisation of the adrenal x-zone in females at 873 mg/kg bw/day

BW: body weight M: male(s) F: female(s) ↑: increase(d) ↓: decrease(d) *: mecamylamine: nicotinic blocker

Table 5.8.3-6: Findings possibly linked to steroidogenesis observed in an oncogenicity study on thiacloprid in mice (M-003819-02-1)

Dose level [mg/kg bw/day]	Dose [ppm]	Species/sex	Tumour	Tumour incidence [%]	BW in comparison to controls [%]	Food intake	Other findings regarding liver, thyroid, adrenals and sexual organs
0	0	Mouse, female	Ovarian luteoma	0 (0/50)	nad	nad	↑no. of eosinophilic, luteinized cells in the ovarian stroma (3/47)
10.9	30	Mouse, female	Ovarian luteoma	2 (1/50)	nad	nad	nad
475.3	1250	Mouse, female	Ovarian luteoma	6 (3/50)	nad	nad	↑no. of eosinophilic, luteinized cells in the ovarian stroma (5/49), ↑ liver weight, hepatocellular hypertrophy, adrenal x-zone: hypertrophy & vacuolation
872.5	2500	Mouse, female	Ovarian luteoma	13 (6/47)	nad	nad	↑no. of eosinophilic, luteinized cells in the ovarian stroma (8/47), ↑ liver weight, hepatocellular hypertrophy, hepatocellular necrosis adrenal x-zone: hypertrophy & vacuolation

Ovaries: nothing adverse detected at 2500 ppm after 1 year
Historical controls: up to 2-6% (Bayer internal data)
BW: body weight nad: nothing adverse detected M: male(s) F: female(s) ↑: increase(d) ↓: decrease(d)

Table 5.8.3-7: Substrate & hormone changes in toxicological studies on thiacloprid in mice

Substrate & hormone changes	**2- & 3-week dietary studies M-000821-01-1 & M-000688-01-1**	**13-week mechanistic dietary study in female mice, M-003764-01-1**	**14-week dietary study M-000697-02-1**	**Oncogenicity study M-003819-02-1**
Doses [ppm]	2-week: 0-50-200-2000-10000 3-week: 0-100-1000-10000	0-10-30-250-2500-2500+mecamylamine	0-50-250-1250-6250	0-30-1250-2500
[mg/kg bw/day]	2-week:0-21.6/29.8-84.3/113.2-765.1/1201.2-4143.2/5449.8 3-week: 0-30.1/63.9-368/559-4141/5785 in M/F	0-6-18-139-1101-1244 in F	0-19.9/27.2-102-6/139.1-542.4/704.3-2819.9/3351 in M/F	0-5.7/10.9-234/475-546/873 in M/F
Cholesterol	↓ at 4143 mg/kg bw/day in males (2-week study) Not investigated (3-week study)	Not investigated	↓ ≥ 139 mg/kg bw/day in females, at 3551 mg/kg bw/day in males	Not investigated
Progesterone	Not investigated	↑ ≥ 1101 mg/kg bw/day	Not investigated	Not investigated
Estradiol	Not investigated	Slightly ↓ ≥ 139 mg/kg bw/day*	Not investigated	Not investigated
Estradiol/Progesterone ratio	Not investigated	↓ ≥ 1101 mg/kg bw/day	Not investigated	Not investigated

BW: body weight male: male(s) F: female(s) ↑: increase(d) ↓: decrease(d)

*: A first, slight effect at 139 mg7kg bw/day did not lead to changes of the estradiol/progesterone ratio. Therefore, the NOAEL for hormonal changes in female mice is 139 mg/kg bw/day.

DOG

Table 5.8.3-8: Findings possibly linked to endocrine effects on testes, prostrate and epididymides in toxicological studies on thiacloprid in dogs

Organ	10-week dietary study M-003816-02-1	15-week dietary study M-003814-01-1	1-year study M-003818-01-1
Doses [ppm]	0-100-300-1000* (1250-1600- 2500)-2500 (4 weeks)	0-250-1000-(4000-0)** 2000 ppm	0-40-100-250-1000 ppm (52 weeks) 0-100-1000 ppm (26 weeks, males only, investigation of prostata effects)
[mg/kg bw/day]	M+ F combined: 0 – 3.3 – 9.6 – 80.0 (10 weeks) – 65.7 (4 weeks)	M: 0 - 8.5 - 34.9 - 68.0	M: 0-1.43-3.60-8.88-34.42 (week 4-52) 0- 3.23 - 32.40 (26 weeks)
Testes	Weight: no treatment-related effect (at 1000 ppm slightly reduced weight) Histology: no treatment-related effects	Weight: no effect Histology: at 68 mg/kg bw/day: Leydig cells slightly more prominent (3/4; controls: 1/4), slightly ↑ no. of degenerated spermatocytes (2/4)	Weight: no effect Histology: no effects up to 34 mg/kg bw/day
Prostrate	Weight: ↑ at 80 / 66 mg/kg bw/day (more pronounced in 80 mg/kg bw/day group, possibly due to longer treatment duration/higher feed intake) Histology: no effect	Weight: ↑ ≥ 35 mg/kg bw/day (slightly higher than HCD; very low absolute & relative weights in control & low-dose groups) Histology: ≥ 35 mg/kg bw/day slight to moderate glandular hypertrophy (4/4 at both dose levels)	Weight: no effect at 26 weeks, slightly ↑ at 34 mg/kg bw/day after 1 year Sonographic investigations in weeks 17, 26 & 52: no treatment related effects Histology: no effect after 26 and 52 weeks
Epididymides	Weight: not investigated Histology: no treatment-related findings	Weight: not investigated Histology: at 68 mg/kg bw/day slightly ↑ no. of degenerated spermatocytes (4/4, controls: 1/4)	Weight: not investigated Histology: no effect

*: Since no toxic signs were observed at 1000 ppm the high-dose was increased to 1250 from day 19 onwards, to 1600 ppm from day 26 onwards, and to 2500 ppm from day 38 onwards. An additional 2500 ppm treatment group was added from day 38 to day 66.

**: Due to vomitus, slight tremor, feed refusal and reduced bodyweights the high-dose of 4000 ppm was set to 0 ppm from day 5 to 14 and then to 2000 ppm from day 15 throughout the study.

bw: body weight M: male(s) F: female(s) ↑: increase(d)

CA 5.9 Medical data

Medical surveillance data of manufacturing plant personal exposed to thiacloprid are available from the years 2004 to 2014. The annually conducted medical routine investigations included assessment of medical history, a full physical examination, laboratory investigations (PBC, liver enzymes, creatinine, urine stick), and technical examinations (vision testing, audiometry, lung function testing as needed for specific job tasks). None of these investigations revealed any unwanted effects in the workers. In addition, there were no accidents involving thiacloprid during the production period since 2005.
Further data on humans are only available from one publication. However, no clear relation to thiacloprid could be derived. The reported symptoms were typical for neonicotinoyl insecticides, and were considered to be due to prolonged high intake of treated fruits and teas. In addition, product stewardship surveillance has revealed some cases of nicotinergic symptoms from overexposures due to accidental ingestion and in suicide attempts with combination products (pyrethroids plus ethanol). No different symptoms were reported.

Direct observations in humans, as well as epidemiological studies with thiaclopird are not available.

Diagnosis of poisoning is mostly due to determination of compound-specific clinical signs, i.e. nicotine-like cholinergic symptoms like nausea, vomiting, abdominal pain, diarrhea, increased salivation, headache, dizziness, and central nervous system effects like agitation or confusion, as well as severe symptoms as coma, tachy- or bradycardia, hypotension, respiratory failure. For an exact diagnosis analytical determination of thiaclopird or its metabolites in blood, urine or gastrointestinal contents would be required.
A specific antidote therapy is not known.
For first aid measures removal of ingested compound by gastric lavage, application of activated charcoal and sodium sulphate is recommended. Further treatment has to be symptomatic and supportive with a focus on respiratory function, if needed mechanical ventilation.
Contaminated skin should be washed immediately with plenty of water and soap. Exposed eyes should be flushed with lukewarm water.

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Report:	[redacted]; 2014; M-486694-01-1
Title:	Occupational medical experience with Thiacloprid
Report No.:	M-486694-01-1
Document No.:	M-486694-01-1
Guidelines:	Not applicable Deviation(s): not applicable
GLP	**Not applicable**

In-company experience

Chemical name:	
IUPAC:	{(2Z)-3-[(6-Chloropyridin-3-yl)methyl]-1,3-thiazolidin-2-ylidene}cyanamide
CAS:	111988-49-9, [3-[(6-Chloro-3-pyridinyl)methyl]-2-thiazolidinylidene] cyanamide
Physical state:	light brown powder
Processing plant:	[redacted], Germany
Number of employees handling product:	40
Production period:	2005 -2014
Amount produced:	166-851 tons per year
Personal safety measures:	Helmet, safety glasses, safety shoes Tyvek type suit and FFP3 masks for some tasks

In-company experience:

No unusual occurrences or complaints.

Occupational Medical Experiences

No. of workers exposed:	40
Medical examinations:	History and full physical examination
Commenced in:	2005
Examination intervals:	annually
Laboratory examinations:	FBC, liver enzymes, creatinine, urine stick
Technical examinations:	Vision testing, audiometry, lung function testing as needed for specific job tasks
Other technical details:	n/a

Medical assessment:

Occupational medical surveillance of workers exposed to Thiacloprid performed since 2005 annually on a routine basis, not directly related to exposures, did not reveal any unwanted effects in the workers. The examinations included the above laboratory parameters and clinical and technical examinations.

During the production period since 2005 no accidents with Thiacloprid occurred in the workers. No further consultations of the Medical Department due to work or contact with Thiacloprid were required.

CA 5.9.2 Data collected on humans

Report:	[redacted]u; [redacted]; 2014; M-486526-01-1
Title:	KCA 5.9 Medical data for thiacloprid
Report No:	M-486526-01-1
Document No:	M-486526-01-1
Guidelines:	**EU Regulation 1107/2009 & EU Regulation 283/2013** **SANCO 10181/2013;** **Deviation(s): not specified**
GLP/GEP:	**Not applicable**

Any significant clinical findings related to exposure

One publication in 11 patients (6 to 52 years old) in Japan reported detection of the metabolite (6-chloronicotinic acid, not specific and a metabolite also of other neonicotinyl insecticides) in 6 patients. These 6 patients had symptoms of headache, general fatigue, finger tremor, and short time memory disturbance, fever, cough, palpitation, chest pain, stomachache, myalgia/muscle spasm/muscle weakness, heart rate abnormality (sinus tachycardia, sinus bradycardia, or intermittent WPW syndrome). The symptoms were assumed to be due to prolonged high intake of treated fruits and tea. [Taira K et al: Chudoku Kenkyu, 2011, 24(3), 222-30 – abstract only in English].

Product stewardship surveillance has revealed some cases of nicotinergic symptoms from overexposures with abdominal pain, nausea, dizziness and vomiting. Irritations of skin and mucous membranes have been described. In 2 cases of accidental ingestion and in suicide attempts with combination products (pyrethroids plus ethanol) no different symptoms were reported.

CA 5.9.3 Direct observations

Report:	[redacted]u; [redacted]; 2014; M-486526-01-1
Title:	KCA 5.9 Medical data for thiacloprid
Report No:	M-486526-01-1
Document No:	M-486526-01-1
Guidelines:	**EU Regulation 1107/2009 & EU Regulation 283/2013** **SANCO 10181/2013;** **Deviation(s): not specified**
GLP/GEP:	**Not applicable**

None available.

CA 5.9.4 Epidemiological studies

Report:	[redacted]t; [redacted]; 2014; M-486526-01-1
Title:	KCA 5.9 Medical data for thiacloprid
Report No:	M-486526-01-1
Document No:	M-486526-01-1
Guidelines:	**EU Regulation 1107/2009 & EU Regulation 283/2013 SANCO 10181/2013; Deviation(s): not specified**
GLP/GEP:	**Not applicable**

None available.

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

Report:	[redacted]t; [redacted]; 2014; M-486526-01-1
Title:	KCA 5.9 Medical data for thiacloprid
Report No:	M-486526-01-1
Document No:	M-486526-01-1
Guidelines:	EU Regulation 1107/2009 & EU Regulation 283/2013 SANCO 10181/2013; Deviation(s): not specified
GLP/GEP:	**Not applicable**

As for all chloronicotinyl insecticides nicotinc-like cholinergic symptoms can occur like nausea, vomiting, abdominal pain, diarrhea, increased salivation, headache, dizziness, and central nervous system effects like agitation or confusion.
Severe symptoms may be coma, tachy- or bradycardia, hypotension, respiratory failure.
While most chloronicotinyl poisonings seem to have a mild course, there are reports of fatalities in literature for other chloronicotinyls than thiacloprid.

In high dose animal experiments with other chloronicotinyl insecticides disturbances of breathing and movement, tremor and cramps, impaired papillary function and hypothermia have been observed. In total the symptoms were similar to nicotine poisoning.

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

Report:	[redacted]i; [redacted]; 2014; M-486526-01-1
Title:	KCA 5.9 Medical data for thiacloprid
Report No:	M-486526-01-1
Document No:	M-486526-01-1
Guidelines:	EU Regulation 1107/2009 & EU Regulation 283/2013 SANCO 10181/2013; Deviation(s): not specified
GLP/GEP:	**Not applicable**

First Aid:

- Remove patient from exposure/terminate exposure
- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylenglykol 300 followed by water.
  Note: Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethyleneglykol 300 is not required.
- Flushing of the eyes with luke warm water for 15 minutes.
- Induction of vomiting should only be considered if a large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.
- Induced vomiting can remove maximum 50% of the ingested substance.
  Note: Induction of vomiting is forbidden, if a formulation containing organic solvents has been ingested!

Treatment:

- Gastric lavage should be considered in cases of significant ingestions within the first (2) hour(s)
- The application of activated charcoal and sodium sulphate (or other carthartic) can be considered in significant ingestions.
- Treatment has to be symptomatic and supportive with a focus on respiratory function, if needed mechanical ventilation.
- Some experts recommended - for other neonicotinyls - to consider judicious use of atropine in cases with life-threatening muscarinic symptoms like bronchorrhea with airway compromise. However, the symptoms will rather be nicotinic than muscarinic.

CA 5.9.7 Expected effects of poisoning

Report:	[redacted]t; [redacted]; 2014; M-486526-01-1
Title:	KCA 5.9 Medical data for thiacloprid
Report No:	M-486526-01-1
Document No:	M-486526-01-1
Guidelines:	EU Regulation 1107/2009 & EU Regulation 283/2013 SANCO 10181/2013; Deviation(s): not specified
GLP/GEP:	**Not applicable**

No persisting effects are known or expected.

CA 5.10 Overall conclusions

Summary of absorption, distribution, metabolism and excretion

In the *in vivo* rat metabolism studies (reported in the baseline dossier) thiacloprid is intensively metabolised. On average, only ca. 6% of the administered radioactivity was identified as unchanged parent compound, while more than one third of the given dose consisted of 6-chloronicotinic acid and its glycine conjugate. Altogether 13 metabolites were identified, but most of them at amounts below 5% of the administered radioactivity. These metabolites were either formed by ring-opening of the thiazolidine ring and/or by conjugation, i.e. by a phase II reaction. This may be the reason that they were not found in the in vitro microsomal system.

In the comparative in vitro metabolism study conducted in human and rat liver microsomes ^{14}C-thiacloprid was highly metabolically stable. The in vitro metabolism was comparable in human and rat liver microsomes, with no indication for the formation of a unique human metabolite. Only one metabolite was detected in very low amounts of the relative percentage (<1.7%) after ^{14}C-thiacloprid *in-vitro* incubations with both, rat and human liver microsomes. The results of the comparative *in vitro* metabolism study demonstrate that phase I metabolism plays a very moderate role in the biotransformation of thiacloprid in rat and human liver microsomes. In addition, no differences with respect to the metabolic pattern were found in both in-vitro test systems.

Acute toxicity

Thiacloprid displayed moderate acute oral toxicity in male and female Wistar rats, but was toxic after acute oral administration to Fisher 344 rats in a pilot study for the acute oral neurotoxicity study. The results obtained with fasted and non-fasted Wistar rats suggest that dietary status can influence the toxicity of thiacloprid. Thiacloprid was moderately toxic after inhalation and of low toxicity after dermal application. Sex differences are evident in rats when exposed *via* the oral or inhalative route, females appear to be more sensitive than males. Thiacloprid does not cause skin or eye irritation and is no skin sensitizer.
An in vitro 3T3 NRU phototoxicity test conducted with thiacloprid did not give any indication for a phototoxic potential of the compound.

Short-term toxicity

Short-term toxicity studies have been conducted in rats, mice and dogs.

The main target in rodents proved to be the liver. There was no evidence of accumulation in the short-term studies at dose levels that did not overload the metabolic capacity of the liver. A dose-dependent

liver enzyme induction occurred in rats and mice. This enzyme induction was associated with increased liver weight, centrilobular hypertrophy and changes in the cytoplasm of the hepatocytes.

In rats, the enzyme induction was believed to be responsible for the secondary effects observed in the thyroid glands (e.g. increased weight, increased mitotic rate and hypertrophy of follicular epithelium). A comparison of the enzyme induction seen at the top dose levels in the 14 day rat study and 13 week rat study indicate that some but not all of the enzyme levels increased with the duration of exposure. The cytochrome P-450 (males) and UDP-glucuronyl-transferase (males and females) levels appeared to increase with time. Liver enzyme induction and the related morphological changes were also observed in the inhalation and dermal studies. Following oral (13 weeks) and dermal administration (22 applications), the enzyme induction and increased liver weight were shown to be reversible or at least partly reversible. The thyroid follicular cell hypertrophy was also shown to be at least partly reversible following dermal administration and a 2-week recovery period. There was evidence of an effect on circulating thyroid hormone levels and biochemical parameters. Body weight and food intake effects were also observed in rats.

In mice, the liver effects also included an increase in the lipid content of the hepatocytes. A dose-related increase in fatty vacuolation and hypertrophy of the adrenal X-zone was also detected in female mice. A NOAEL was not established for this finding.

In dogs the liver was also a target, but the effects were less pronounced, and although an enzyme induction was observed, it was weaker than in rodents. Re-evaluation of the thyroid hormone data in dogs with the adequate historical control data of the year of study conduct revealed, that there is no effect on the thyroid in this species. The mean prostate weights were increased in the 10- and 15-week dog studies at dose levels > 1000 ppm. Microscopy revealed slight to moderate hypertrophy of the prostate at dose levels > 1000 ppm in the 15-week study only. In the 52-week dog study, there was no evidence of increased prostate weights at 26 weeks but the mean prostate weight was increased at 1000 ppm on termination. Microscopic and ultrasonographic investigations of the prostates did not detect any treatment-related effects at week 26 or week 52. Therefore, the report regarded the prostate effects seen at 1000 ppm as incidental and possibly related to high individual variation in growing dogs. It was noted that six treated dogs had individual prostate weights that were noticeably higher than the cited historical control data.
A toxicokinetic evaluation of thiacloprid concentrations in blood plasma of dogs from the subchronic dietary study demonstrated an efficient oral absorption of thiacloprid. The less pronounced toxicity observed in dogs as compared to rodents are therefore not due to low absorption of the test substance.

Genotoxicity testing

Guideline genotoxicity studies conducted with thiacloprid were consistently negative. They comprised point mutation assays in bacteria and mammalian cells, an in vitro cytogenetic study, an unscheduled DNA synthesis assay on primary rat hepatocytes as well as a micronucleus test in vivo.

Furthermore, an additionally available bacterial DNA-repair test (rec-assay) revealed no indication for a DNA-damaging effect of thiacloprid.
Three publications emerging from public literature between 2012 and 2013 described genotoxic effects of thiacloprid in different test designs in vitro and in vivo. All three of them were based on non-GLP studies, which according to different deficiencies were considered to be non reliable and, thus, not relevant.

Long-term toxicity and carcinogenicity

Long-term studies have been conducted in the rat and mouse.

In rats, body weight effects were observed in both sexes with more pronounced effects in females. The main target was the liver. Hepatic enzyme induction was evident in males at dose levels > 50 ppm and in females at dose levels > 500 ppm. Liver changes, probably caused by chronic induction of hepatic phase I and II enzymes, were increased weight, hepatocellular hypertrophy, altered hepatocellular foci and cytoplasmic changes in the hepatocytes. NOAELs for enzyme induction in males and females were 25 ppm (1.6 mg/kg bw/day) and 50 ppm (3.3 mg/kg bw/day), respectively.
Thyroid changes were observed including hypertrophy and hyperplasia of the follicular epithelium, colloid alteration and follicular cell adenoma. These changes were also considered to be a consequence of the liver enzyme induction. It has been proposed that the increased enzyme activities enhance the capacity of the liver to deactivate and excrete the circulating thyroid hormones. Alterations of the hormone levels trigger a compensatory increase in TSH, which induces the morphological changes in the thyroid gland. At 1000 ppm (69.1 / 51.7 mg/kg bw/day (males / females)), ***re-evaluation of the data with the appropriate historical control data revealed a trend for an increase in TSH in high dose females in weeks 26 and 105 (statistically significant increases, but values still in the 2-s range of historical controls).*** Although the expected decreases in circulating T3/T4 levels were not detected in this study, such decreases were seen in the short-term rat studies. It has been assumed that the expected decreases in T3/T4 levels are masked by the rapid compensatory reactions of the thyroid system.

There were increased incidences of uterine adenocarcinomas and ***reduced incidences of lacteal cysts and*** galactocele in the mammary glands, which were again considered to be secondary to the liver enzyme induction. ***Special mechanistic studies seemed to indicate an induction of aromatase resulting in increased estradiol levels and, via continuous stimulation of the uterine endometrium, after 2 years in an increased incidence of uterine adenocarcinoma. However, more recent investigations showed that the apparent aromatase induction was an artefact caused by the unspecificty of the assay used and that thiacloprid is not an aromatase inducer.***
An additional histopathological investigation of the uteri of females of all dose groups after 1 year of treatment revealed a slightly increased incidence of slight to moderate uterine glandular hyperplasia after 500 and 1000 ppm. This lesion is a spontaneous finding. While it occurred as a reactive change in some animals up to and including 500 ppm due to endometritis, or stromal polyp or both conditions, it could be treatment related in 2 or 4 out of 10 animals after 500 and 1000 ppm.

There were increased incidences of retinal atrophy (females), lens degeneration/opacity (females), radiculoneuropathy (females), sciatic nerve degeneration (both sexes) and skeletal muscle atrophy (females). These age-associated findings were mainly seen at the top dose level or were sex specific but are consistent with the neurotoxic mode of action of the test material.

In mice, there were effects on male body weight and food intake. Leukocyte counts were increased in males and in females at some sampling points. Liver effects including increased weight, hypertrophy, fat storage, necrosis and degeneration, were seen in males and females. In females, increased adrenal weight was associated with hypertrophy and vacuolisation of the cortical X-zone. The liver effects and the concomitant hormonal changes may have caused these adrenal changes by affecting the development of the hormone-dependent X-zone. The incidence of eosinophilic luteinised cells in the ovarian stroma or the surrounding adipose tissue and ovarian luteomas were increased. The report considered these effects to be secondary to the known liver enzyme induction and the subsequent hormone imbalance.

Reproductive Toxicity

In a rat two generation study, decreased food consumption and bodyweight gain were seen at the top dose level of 600 ppm. Clinical signs of toxicity were noted in dams at 300 and 600 ppm, the incidence of dystocia was also increased at these dose levels. Litter size and pup survival were significantly decreased at the top dose level, pup growth was significantly decreased at 300 ppm and 600 ppm in both generations. Increased thyroid, liver and gonad weights were seen in adults of both generations at ≥ 300 ppm. Histological correlates of hepatocyte and thyroid follicular hypertrophy were also reported.

In the rat developmental study, decreased bodyweights and food consumption were noted at the top dose level of 50 mg/kg bw/day. Effects on urine and faecal production were also seen. Forelimb malformations (bone dysplasia) were also seen in the presence of marked maternal toxicity and the incidence was within the historical control range. Post-implantation loss was increased in this group as a result of late resorption. The incidences of placental border necrosis and foetal renal pelvic dilatation were increased in treated groups, however values were within or close to the historical control ranges. Numerous skeletal findings indicative of delayed or reduced ossification were noted at the top dose level.

In the rabbit developmental study, increased abortion, decreased food consumption and bodyweight gain were seen at 10 mg/kg bw/day. Foetal skeletal effects indicative of reduced or delayed ossification were noted in the top dose group. A marginal increase in the incidence of supernumerary 13th ribs was also noted in this group. An increase in the number of foetal malformations in this group is largely attributable to the incidence of forelimb arthrogryposis. This effect is a common spontaneous malformation ***(nowadays termed "malposition of forelimb(s)" - ventral flexure in the***

region of the wrist)[9] in this strain of rabbit, the incidence is within the historical control range and there is no clear dose-response relationship.

Although forelimb malformations were seen in the rat and rabbit, these findings are not directly comparable. Both the bone dysplasia and arthrogryposis are common spontaneous findings and the incidences of these effects are within the relevant historical control data.

Neurotoxicity studies in rodents

As thiacloprid does not belong to the class of the organophosphates and has a different mode of action, i.e. action at the nicotinic receptor, testing for delayed neurotoxicity was not necessary. However, thiacloprid was tested in acute and subchronic neurotoxicity studies, as well as in a developmental neurotoxicity study.

Administration of single oral doses of thiacloprid to rats by gavage produced only transient clinical signs of toxicity. The overt signs included tremors, decreased activity, ataxia, repetitive chewing movements, dilated pupils, eyelid ptosis and clear lacrimation, oral and nasal staining and reduced body temperature. Brain weight was not affected by treatment. Histopathology did not reveal any lesions in the nervous system, eyes or skeletal muscle. The only treatment-related effects in the 13 week feeding study were reduced body weight and food consumption. The large differences between the NOELs determined for neurotoxicity in the acute and short-term feeding study may be due to bolus dosing or possibly adaptation.

For the registration of thiacloprid in the United States, a developmental neurotoxicity study was conducted. The study was already submitted for Annex I inclusion, but not discussed in the Monograph. In this study dietary exposure to thiacloprid did not cause any neurotoxic effects in parental as well as offspring animals. Treatment-related findings consisted of reduced maternal body weights and body weight gain during gestation and lactation, as well as reduced food consumption during gestation in the mid and high dose. Body weights and body weight gain were also reduced in mid- and high dose F1 males and females and absolute food consumption was also reduced in mid and high dose F1 males. Relative food consumption was increased in mid and high dose F1 rats of both sexes due to the reduced body weights. Terminal body weights were also decreased in mid and high dose males and high dose females of the F1 generation. F1 offspring of the mid- and high-dose groups exhibited also a delay in development (preputial separation in mid

[9] ***Malposition of forelimb(s): This finding is the most common spontaneous malformation in the strain of rabbits used and some companies regard this finding as variation only. It is most likely the consequence of restriction of fetal movements in the uterus, which results in a caudal flexure of the forelimb(s) in the region of the wrist (carpal joint). Except from the flexion of the limb, there are no further morphological changes involved in this joint region, otherwise it would not be regarded as a "common finding". Furthermore, arthrogryposis in rabbits is considered to be reversible after birth, since such a finding was never observed in the young by the breeder. Therefore, this finding in rabbits is completely different from the dysplasia of limb bones seen in the rat, where the bones are changed morphologically.***

and high dose males and vaginal patency in high dose females) which is considered to be secondary to body weight changes.

Delayed polyneuropathy studies

A publication on histopathological alterations in chicken after oral subacute treatment with thiacloprid was reviewed for its possible relevance for delayed neurotoxicity. Since investigations regarding delayed neurotoxicity were not included in the study design, the publication is not relevant for the endpoint delayed neurotoxicity.

Toxicity studies of metabolites

During the previous EU review, the toxicological properties of several plant and/or soil/ groundwater metabolites (YRC 2894-amide (M02), YRC 2894-sulfonic acid (M30), and YRC2894-sulfonic acid amide (M34)) had already been evaluated based on studies on acute oral toxicity in rats, genotoxicity and liver enzyme induction in rats.
In addition, new studies on 6-chloronicotinic acid (M03), YRC 2894-sulfonic acid (M30), sulfonic acid amide (M34) and thiacloprid-thiadiazine (Z5) on acute oral toxicity, genotoxicity, steroidogenesis in vitro or liver enzyme induction in rats are now available or were conducted, respectively. With regard to the in vitro steroidogenesis assays on the metabolites thiacloprid was tested in parallel again in order to be able to compare the results obtained with the metabolites with those of the parent compound.
The results of all available studies on the above mentioned metabolites are provided in the following paragraphs.

Thiacloprid-amide (M02)
The plant metabolite thiacloprid-amide (also a postulated intermediate in rat metabolism) displayed a LD_{50} > 2000 mg/kg bw in an acute oral toxicity study in rats showing that M02 is of less acute toxicity than thiacloprid. In addition, M02 was negative for point mutations in an Ames test.

YRC 2894-sulfonic acid (M30)
The groundwater metabolite YRC 2894-sulfonic acid (also a postulated intermediate in rat metabolism) also has a lower acute oral toxicity than thiacloprid (LD_{50} M30 > 2000 mg/kg bw). YRC 2894-sulfonic acid did not induce mutations *in vitro* in bacteria and mammalian cells and displayed no clastogenic potential in mammalian cells. Therefore, M30 is considered to be non-genotoxic. Furthermore, M30 did not induce liver enzymes in female rats after dietary exposure with 1000 ppm for one week. ***An in vitro H295R steroidogenesis assay did not give any indication for an effect on steroidogenesis.***

YRC 2894-sulfonic acid amide (M34)

The groundwater metabolite YRC 2894-sulfonic acid amide displayed an acute oral LD_{50} > 2000 mg/kg bw in rats indicating that it is less acutely toxic than thiacloprid. In addition, M34 is considered to be non-genotoxic based on negative results in *in vitro* assays for point mutation in bacteria and mammalian cells as well as for clastogenicity. YRC 2894-sulfonic acid amide caused no liver enzyme induction after dietary exposure of female rats with 1000 ppm for one week. ***An in vitro H295R steroidogenesis assay did not give any indication for an effect on steroidogenesis.***

6-Chloronicotinic acid (M03)

An acute oral toxicity study and an Ames test are available on the rat, plant and soil metabolite 6-chloronicotinic acid. LD_{50} in rats was > 5000 mg/kg bw indicating that 6-chloronicotininc acid is less acutely toxic than thiacloprid. Furthermore, 6-chloronicotininc acid was negative in the Ames test.

Thiacloprid-thiadiazine (Z5)

Investigations on the groundwater metabolite thiacloprid-thiadiazine (Z5) revealed a lower acute oral toxicity with a LD_{50} > 2000 mg/kg bw in comparison to thiacloprid. With negative results in a bacterial reverse mutation assay, a mammalian cell gene mutation test and a micronucleus test in human lymphocytes thiacloprid-thiadiazine is considered to be non-genotoxic. The metabolite did not lead to liver enzyme induction after dietary administration of 1000 ppm to male rats for one week and has no effect on steroidogenesis in the in vitro H295R steroidogenesis assay.

Additional steroidogenesis assay on thiacloprid

For the evaluation of possible effects of the metabolites on steroidogenesis in comparison with the parent compound, an additional H295R steroidogenesis assay was conducted in parallel with thiacloprid. Thiacloprid caused a slight, statistically significant effect on testosterone and estradiol secretion at the highest concentration of 100 µM.

Supplementary studies on the active substance

Supplementary studies on thiacloprid comprise previously and recently conducted studies on toxicokinetics, an immunotoxicity study as well as mechanistic studies investigating the modes of action of the observed tumors as well of dystocia.

Toxicokinetic studies

Toxicokinetic studies in rats revealed dose proportional increases in plasma concentrations in males at high doses of 1000 ppm, while increases were over-proportional in females indicating an overload of the metabolic capacity of the liver. A decrease of plasma concentrations over time due to enzyme induction and increased metabolisation of thiacloprid was not visible. Therefore, a possible inhibitory effect of thiacloprid on CYP450 dependent monooxigenases was investigated. Only a weak inhibitory effect on 7-ethoxycoumarin-deethylation in liver microsomes was noted, which is not very relevant *in vivo* because the necessary concentrations will not be reached. A study comparing toxicokinetics in

pregnant to non-pregnant rats revealed higher plasma concentrations of thiacloprid in pregnant animals. ***Protein binding of thiacloprid in plasma of humans and rats was low and of similar magnitude in both species (40.7% in human, 54.7% in rat plasma). Toxicokinetic determinations in plasma of dogs from the 15-week dietary toxicity study revealed a high oral absorption of thiacloprid. Insufficient oral absorption can therefore be excluded as a possible reason for the fact that toxicity of thiacloprid in dogs was not very pronounced.***

Immunotoxicity study
An immunotoxicity study in rats revealed that thiacloprid has no immunosuppressive potential.

Supplementary studies to elucidate the tumor mode of actions

Additional work on thyroid tumors: An *in vitro* study showed that thiacloprid and its metabolites are no inhibitors of the thyroid peroxidase (TPO). A 3-week dietary study was especially designed to investigate the effects of thiacloprid on the thyroid in rats. It was shown that thyroid findings (changes of thyroid hormones and TSH as well as thyroid follicular cell hypertrophy) occurred only at doses linked with marked liver enzyme induction including pronounced UDP-GT increases. This indicates that the mode of action of thyroid effects including thyroid follicular cell adenoma in male rats after long-term treatment with thiacloprid is secondary to liver enzyme induction. This mode of action is rat specific and not relevant to humans.

Additional work to elucidate the mode of action of tumors of the female reproductive tract (uterine adenocarcinoma in rat, ovarian luteoma in mice):
In the 1990ies studies in rats and mice have been conducted, in which aromatase (CYP19, catalysing the conversion from testosterone to estradiol or from androstenedione to estrone, respectively) levels have been determined mostly in liver tissue and ovaries. This was done in the framework of the mode of action work for the tumors of the female reproductive tract as well as for dystocia. An induction of aromatase could have been a possible reason for an increased estradiol/progesterone ratio which after long-term treatment would lead to the observed tumors or dystocia, respectively. However, due to the recent work by [redacted] (2009, M-360757-02-1) it is known that the observed increases of aromatase in the old studies were artifacts caused by the unspecificity of the tritiated water assay used and that thiacloprid is no inducer of aromatase (at high dietary thiacloprid doses ≥ 1000 ppm even a marginal inhibition of aromatase in the ovaries was seen).

In a 13-week dietary study in female mice for aromatase induction also slight changes of sex steroid hormones were observed. There were slight decreases of plasma estradiol and increases of plasma progesterone, which resulted in a sligthly decreased estradiol/progesterone ratio. The NOAEL for the hormone changes (based on the estradiol/ progesterone ratio) was 139 mg/kg bw/day. Further findings, besides changes in motility, reduced reactivity and increased liver weight, were increased vacuolization and hypertrophy of the adrenal X-zone. The overall NOAEL in this study was 18 mg/kg bw/day.

Several mode of action studies on development of uterine adenocarcinoma in rats have been conducted by [redacted] between 2007 and 2010 in order to improve the Q Star risk assessment in the US. In these studies it was shown that thiacloprid treatment leads to slight changes of estradiol or progesterone plasma levels. These are accompanied by a slightly increased expression of genes associated with sex steroid hormone biosynthesis in ovary, liver & adrenal gland. It is assumed that this translates into an increase of CYP450 enzymes involved in steroid biosynthesis with the magnitude of this effect not being known. However, also moderation of the steroidogenic effects was evident from the data due to an increased expression of genes associated with the metabolism of sex steroid hormones. While the observed slight hormonal changes in young adult rats do not lead to an effect on the estrous cycle or on other sex steroid hormone sensitive organs or tissues (see 2-generation study and short term toxicity studies in rats), effects on the estrous cycle were noted in 72-week old, aging female rats. In comparison to untreated controls fewer thiacloprid treated females were in pseudopregnancy and more in the ambiguous phase. In addition, thiacloprid treated rats displayed a lower level of vaginal mucification and marginal, non significant increases of plasma estradiol, which were more pronounced in females in pseudopregnancy in comparison to those in the ambiguous phase.
In depth mode of action work showed that thiacloprid has no direct estrogenic effect, since it was negative in an uterotrophic assay. However, it was positive for an effect on steroidogenesis in a H295R assay. Increased progesterone secretion was observed at 100 µM (equivalent to an unbound concentration of about 25 mg/L), while unbound plasma concentrations in female rats after dietary exposure with 1000 ppm (high dose of the 2-year rat study, clearly exceeding the MTD) with up to 11.5 mg/L are still in the range of the NOAEC of 50 µM or 12.5 mg/L for progesterone in this assay. Incubation of rat preantral follicles with thiacloprid led to increased estradiol and progesterone secretion at 500 µM, indicating that preantral follicles are a cellular target for thiacloprid treatment. The NOAEC was 100 µM, indicating again that hormone changes and tumour development in rats occurred at unbound plasma concentrations in the range of the NOAEC of this assay. However, the observed effects on sex steroid hormones as well as tumour development in rodents occurred always at dose levels showing pronounced enzyme induction as well as increased expression of genes associated with sex steroid hormone biosynthesis. Therefore, these effects are considered to be secondary to enzyme induction.

<u>Supplementary studies to elucidate the mode of action for dystocia</u>
In the 1990ies several special 1-generation and mechanistic studies have been conducted to elucidate the mode of action for dystocia in the Sasco Sprague-Dawley rat (used before in the two-generation study on thiacloprid). Dystocia occurred repeatedly, in incidences between 3.3 and 13.3%, after dietary intake of 300 to 1000 ppm thiacloprid for 10 weeks during premating and during gestation, while it was not seen in the concurrent controls.
In contrast, thiacloprid related dystocia was not seen after short-term treatment with oral gavage doses of 17.5 to 100 mg/kg bw on gestation days 18 to 21. In a further dietary study no effect of thiacloprid on direct birth functions could be shown. However, further data showed that thiacloprid treated animals had increased estradiol levels, slightly increased progesterone and LH levels as well as increased corticosterone levels in plasma during premating, gestation and lactation shortly after parturition.

In an additional special 1-generation study the mode of action of dystocia was further investigated using video-recording of parturition as well as sex homone determinations in plasma shortly before and after parturition. Already in two feasibility studies (conducted before the start of the main study to develop and optimize the procedure of video recording and blood sampling during parturition) several cases of dystocia were observed in untreated animals. This indicates that stress alone can cause dystocia in Sasco Sprague-Dawley rats. In the main study dystocia was noted in 3/28 dams treated with thiacloprid and in none of the controls. In one dam dystocia was due to a missing progesterone decrease, which in rodents is mandatory for a normal parturition. This is not the case in humans, in which progesterone withdrawal is regulated differently and plasma progesterone levels stay high during parturition. In a second dam dystocia there were no changes of hormone levels. Dystocia was obviously due to stress by blood sampling and the high general toxicity of thiacloprid together with the increased sensitivity of the Sasco Sprague-Daley rat towards such effects. A third dam with dystocia was found dead; blood sampling was not possible. Furthermore in thiacloprid treated animals the levels of progesterone (slightly increased mean value at GD 20, absence of normal decrease prior to parturition in one rat with dystocia) and estradiol (increased mean values at GD 21 and 22) and the respective balance between these hormones in the days before and during parturition were affected. There was no effect on onset and duration of parturition in all other treated rats. In addition, also the known effects of thiacloprid toxicity were present in treated rats, i.e. reduced body weight and food consumption, increased liver and thyroid weights, hepatocellular hypertrophy and thyroid follicular cell hypertrophy.

In conclusion, dystocia in rats is considered to be either due to hormonal perturbations (a missing progesterone decrease before start of parturition, which is mandatory for normal birth in rat, but not in humans) or due to stress by blood sampling and thiacloprid toxicity together with the relatively high sensitivity of the Sasco Sprague Dawley rat towards stress. The first mode of action is rat-specific and not relevant for humans, the second mode of action is unspecific and secondary to stress.

Publications:

In addition, three articles on thiacloprid or thiacloprid containing formulations were published in 2011 and 2012.

One publication, considered to be non-reliable, described changes of thyroid hormone levels in rat serum after treatment with thiacloprid containing formulations. This is a well investigated phenomenon and the reported results do not change existing endpoints.

In the second publication (considered as reliable with restrictions), thiacloprid was reported to induce human CYP 1A1 and 1A2 in HepG2 cells. The determined enzyme induction was not pronounced and observed at a concentration exceeding the maximal unbound plasma concentrations of thiacloprid in vivo. Therefore, the finding was considered to be non-relevant for human safety.

The third publication (also assessed as reliable with restrictions) provides supplemental information on oxidative stress in lymphoid organs, polymorphonuclear leucocytes and plasma of rats after treatment with thiacloprid containing formulations. Also this publication has no influence on existing endpoints.

Endocrine disrupting properties

A review of the whole data base on thiacloprid was conducted to identify possible effects of thiacloprid on endocrine organs, tissues or parameters.
Thiacloprid treatment led to findings linked to the thyroid as well as to findings linked to steroidogenesis. However, the data give no indications for effects on the pancreas (including blood glucose, urinalysis data or histopathology), pituitary (organ weight, histopathology), thymus (histopathology) or parathyroid (based on blood calcium levels and histopathology) in rat, mouse or dog.

Effects on the thyroid

Thiacloprid treatment leads to effects on the thyroid in rats, but not in dogs or mice. The mode of action in rats is secondary to liver enzyme induction, which leads to an increased metabolisation and excretion of thyroid hormones and, as a consequence, to an activation of the hypothalamic-pituitary-thyroid axis. Permanent stimulation of the thyroid follicular cells by TSH causes thyroid follicular cell hypertrophy and, after long-term treatment in males, a slightly increased incidence of thyroid follicular cell adenoma. Overall, the changes observed under thiacloprid treatment in rats are mostly minimal to slight and the body is able to maintain an euthyroid state up to very high doses approaching the MTD.
This mode of action for the development of thyroid follicular cell adenoma in rats is well known. Due to species specific differences between rat and man it is generally accepted as a mode of action, which is non-relevant for humans.

Effects on steroidogenesis

In vitro assays showed that thiacloprid has a direct effect on steroidogenesis at high concentrations, which exceeded the unbound plasma concentrations of thiacloprid in vivo. The dose levels in vivo with findings like hormonal changes, uterine tumors and dystocia were always associated with enzyme induction including enzymes involved in steroid biosynthesis (with the exception of aromatase). The observed hormone changes and resulting toxicological findings are therefore considered to be secondary to enzyme induction.
In female rats thiacloprid treatment caused changes in steroid sex hormone levels, which had no further consequences in young adult females. However, in old, acyclic rats these hormonal disturbances led to changes of the uterus and, eventually, to an increased incidence of uterine tumours, since alterations in hormone secretion generally associated with the aging process are exacerbated following treatment with high thiacloprid doses. Since enzyme induction is less pronounced in humans and also the range of female sex hormones shows a much broader variation than in rodents, this mode of action is considered to be less relevant in humans. The lowest dose level with a borderline increased tumor incidence was 33.5 mg/kg bw/day.
Dystocia was another finding observed in female rats commencing at 22 mg/kg bw/day. Besides an unspecific mode of action secondary to stress, dystocia also seemed to be linked to perturbations of female sex hormone levels,especially to a missing progesterone decrease before parturition. This decrease is mandatory for normal birth in rats, but not in humans, in which regulation of birth is different. Therefore, this mode of action is considered to be rat specific and not relevant for humans. Since the range of progesterone and estradiol levels in humans is also during pregnancy

and birth much wider than in rat, the observed, rather slight changes of these plasma levels in rat are not considered to pose a risk for dystocia in pregnant women.
In male rats, only a slightly increased incidence of minimal to slight Leydig cell hypertrophy was noted in testes after 2 years of treatment with doses of 25 mg/kg bw and above. This is considered to be a biomarker for an endocrine effect, but no adverse effect.

In female mice, changes of steroid sex hormone levels, findings in the adrenal glands (increased weight and enlargement, hypertrophy and more prominent vacuolization)and in the ovaries (a decrease of advanced corpora lutea with eosinophilic cells and an activation of the interstitial as well as an increased incidence of ovarian luteoma) could be linked to effects on steroidogenesis. The adrenal findings are mouse specific, not relevant for humans and, as the slight hormone changes, considered at most as a biomarker for an endocrine effect. The only adverse finding, the ovary changes with the luteoma, was seen only at very high doses of 475 mg/kg bw/day and higher at the end of the 2-year study.

In male dogs findings with a possible endocrine background were not seen consistently. Increased prostate weights were noted repeatedly in studies up to 1 year, but not seen in every case. Only in the 15-week study they were accompanied by slight to moderate glandular hypertrophy of the prostate as well as by slightly more prominent Leydig cells in the testes and a slightly increased no. of degenerated spermatocytes in testes and epididymides. All of these findings can also be found in untreated young dogs during their normal development. In conclusion, there are at most transient possible endocrine effects in male dogs. A slightly higher prostate weight without morphological correlate is not seen as an adverse effect, but at most as a biomarker for an endocrine effect.

<u>Assessment of the endocrine potential</u>
Taking all results together thiacloprid treatment leads to endocrine effects in toxicological animal studies. However, many of the effects were no adverse effects, but biomarkers for a (possible) endocrine effect, or they were species-specific and not relevant for humans (like the observed thyroid findings or dystocia in rat and adrenal findings in female mice). The only adverse findings with a possible, although less pronounced, relevance for humans are the increased incidences of uterine adenocarcinoma in female rats, commencing at 33.5 mg/kg bw/day, as well as the ovarian luteoma in female mice, which were observed at 475 mg/kg bw/day and higher doses. According to the Joint DE-UK position "Regulatory Definition of an Endocrine Disrupter in Relation to Potential Threat to Human Health" of March 2011 the uterine adenocarcinoma with its borderline increased incidence of 33.5 mg/kg bw/day observed in the combined chronic toxicity and carcinogenicity study in rats would fall into the STOT-RE Cat 2 guidance values of the CLP regulation (> 5 and < 50 mg/kg bw for chronic / long-term studies). As such, thiacloprid would not be deemed an ED of regulatory concern and the standard risk assessment could be applied.

Calculation of the acceptable daily intake (ADI)

During the EU review process for the Annex I listing of thiacloprid an ADI was already established. In general, the ADI is based on the lowest NOAEL in the most sensitive species observed in chronic feeding studies in rats, mice and dogs.

For thiacloprid the rat proved to be the most sensitive species. Therefore, the NOAEL of 1.23 mg/kg bw/day observed in males in the chronic toxicity / carcinogenicity study in rats, was selected for ADI derivation at that time and is still regarded as valid. Thus, by applying a safety factor of 100, the resulting **ADI** is **0.01 mg/kg bw/day**.

Acceptable Operator Exposure Level (AOEL)

Also an AOEL was already derived during the EU review process for the Annex I listing of thiacloprid.
The AOEL should be based on the lowest relevant NOAEL obtained in the subacute and subchronic toxicity studies in the most sensitive species.
The lowest relevant NOAEL of 2 mg/kg bw/day was found in the developmental rabbit study.
Considering the whole toxicological database of thiacloprid this NOAEL is still regarded as appropriate for the derivation of a systemic AOEL for thiacloprid. Thus, by applying safety factor of 100, the resulting **AOEL** is **0.02 mg/kg bw/day**.

Acute Reference Dose (ARfD)

During the EU review process for the Annex I listing of thiacloprid also an ARfD was set.
The appropriate basis for the derivation of an ARfD is the the lowest NOAEL after acute exposure.
Considering the whole toxicological database of thiacloprid, the NOAEL of the acute neurotoxicity study in rats was considered to be the most appropiate value for deriving an ArfD and this is still valid at the time being.
The NOAEL in the acute neurotoxicity study was 3 mg/kg bw/day. Applying a default safety factor of 100 this results in an **ARfD** of **0.03 mg/kg bw/day**.