Bayer CropScience

Document Title

Summary of the toxicological and metabolism studies for Prothioconazole

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

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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

A dossier on prothioconazole (CAS No. 178928-70-6) was submitted February 2002 by Bayer CropScience to the EU RMS United Kingdom for agricultural use as a fungicide. Prothioconazole was included into Annex I of the Council Directive 91/414/EEC by the Commission Directive 2008/44/EC published 4 April 2008, with an entry into force by 1 August 2008.

This Supplemental Dossier contains only detailed study summaries of additional studies which were not part of the dossier during the first Annex I inclusion of prothioconazole and were, therefore, not evaluated during the first EU review of this compound. The summaries on the relevant endpoints were taken from the DAR for the first Annex I inclusion and supplemented with additional information (additional studies, additional references, further comments).
In order to facilitate discrimination between additional and original information, the additional information is written in black letters whereas grey letters describe the original information.
All studies which have been already submitted by Bayer CropScience for the first Annex I inclusion are content of the DAR for the first Annex I inclusion and are included in the Baseline Dossier provided by Bayer CropScience.

Synonymous names for prothioconazole used at several locations in this Supplementary Dossier are JAU 6476 and PTZ.

The following table provides an overview on the batches of prothioconazole used in all toxicological studies on this compound. Studies evaluated during the first EU review are written in grey letters and studies not evaluated during the first EU review are written in black letters. Details on the toxicological justification of the technical specification are described in the confidential documents M-252977-02-1 and M-291556-01-1.

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

Batch Number	Purity (%)*	Study type	Reference (DART No.)
NLL6096-4	99.5	Subacute oral rat	M-012358-01-1
NLL6096-4	99.5-99.8	Developmental oral rat	M-012279-01-1
NLL6096-4	99.5	Ames test	M-012254-01-1
NLL6096-9.1	99.8	Skin irritation rabbit	M-009890-02-1
NLL6096-9.1	99.8	Eye irritation rabbit	M-009893-02-1
NLL6096-9.1	99.8	Sensitisation MK	M-009898-03-1
NLL6096-9.1	99.8-99.9	Clastogenicity in vitro	M-012277-01-1
NLL6096-9.1	99.8	Mammalian cell mutation assay (HPRT (1/79))	M-012278-01-1
NLL6096-9.1	99.9	Micronucleus test in vivo mouse bone marrow	M-012265-01-1
NLL6096-12	99.8	Acute oral rat	M-012312-01-1
NLL6096-12	99.5	Subacute oral rat (comparing different modes of administration)	M-012415-01-1
NLL6096-12	99.7	Developmental rabbit, dose toleration	M-012332-01-1
NLL6096-12	99.5-99.7	Developmental rabbit	M-012237-01-1
NLL6096-12	99.7	Rat liver UDS assay (in vitro)	M-012317-01-1
NLL6096-12	99.5-99.7	Rat liver UDS assay (in vivo)	M-007155-01-1
06233/0031	98.8	Acute dermal rat	M-009688-01-1
06233/0031	98.8	Acute inhalation rat	M-008846-01-1
06233/0031	98.5	Subacute dermal rat	M-044301-01-1
06233/0031	98.1-98.8	Subchronic oral dog	M-035825-01-1
06233/0031	98.4-98.8	Chronic oral dog	M-035967-01-1
06233/0031	98.5-98.8	Oncogenicity mouse	M-085068-01-1
06233/0031	98.1-98.8	2-generation rat, pilot	M-018760-01-1
06233/0031	98.1-98.8	2-generation rat	M-036206-01-1
06233/0031	98.1-98.8	Developmental dermal rat	M-035764-01-1
06233/0031	97.8-98.8	Developmental rat, supplemental	M-067839-01-1
06233/0031 898803005	98.1-98.8 97.6-98.8	Subchronic neurotoxicity rat	M-053225-01-1
898803005	97.6	Subchronic rat	M-011757-01-1
898803005	97.6	Subchronic mouse	M-012244-01-1
898803005	97.6-98.8	Acute neurotoxicity rat	M-023861-01-1
06023/0046	97.7	Micronucleus test in vivo mouse bone marrow	M-102790-01-1
06233/0044	98.3-99.8	Chronic rat (1yr)	M-030441-01-1
06233/0044	98.6 (98.5-98.7)	Carcinogenicity rat (2yrs)	M-084962-01-1
2007-000236	97.2	Sensitization LLNA	M-291490-01-1
HEC 21597-1-1	96.7	Phototoxicity assay in vitro with BALB/c 3T3 cells	M-498655-01-1

* Purity as stated in the study report

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

Studies to address the data requirements for ADME for both, prothioconazole and prothioconazole-desthio were presented in the dossier submitted for first inclusion in Annex I of Directive 91/414/EEC (March 2002) and were deemed acceptable following evaluation and peer review at EU level (2007). The conclusions from each of these studies are presented in Table 5.1.1-1 below.

Two new *in vitro* comparative metabolism studies have been conducted with prothioconazole using rat and human liver microsomes as well as freshly prepared rat and human hepatocytes. These studies were conducted to demonstrate that the "mechanism of detoxification" of prothioconazole is identical in rats and humans. These studies are summarised in chapter CA 5.1.2.

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

Table 5.1.1- 1: Summary of absorption, distribution, metabolism and excretion data

Parameter	Conclusion	Reference
Rate and extent of oral absorption	Prothioconazole: Rapid and nearly complete (peak plasma levels less than 1 h after dosing > 90 % of dose absorbed within 48 h after dosing Prothioconazole-desthio (M04): Rapid and nearly complete (peak plasma levels at 1-1.5 h after dosing > 90 % of dose absorbed within 48 h after dosing	[redacted] (2001) / MR-251/01 / M-034280-01-1 [redacted] (2001) / MR-056/01 / M-032318-01-1
Distribution	Prothioconazole: Broad distribution, but primarily to liver and kidney Prothioconazole-desthio (M04): Limited distribution to peripheral tissues, mainly to liver and renal cortex, intensive enterohepatic re-circulation	[redacted] (2001) / MR-251/01 / M-034280-01-1 [redacted] (2001) / MR-437/01 / M-034660-01-1 [redacted] (1990) / BF-3554 / M-008524-01-1 [redacted] (2001) / MR-056/01 / M-032318-01-1 [redacted] (2001) / MR-514/00 / M-034777-01-1
Rate and extent of excretion	Prothioconazole: The excretion of radioactivity is almost complete within 48 hours of oral administration of [triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole. Approximately 90 - 100 % of orally administered doses was excreted with urine, faeces or bile within 7 days of treatment, and less than 5.82 % of the administered dose remained in the body. At sacrifice, 78 -96 % of the administered dose had been excreted with the faeces and 4 -16 % in the urine. Prothioconazole-desthio (M04): Between 68 and 74 % was excreted with the faeces and between 10 and 11 % in urine.	[redacted] (2001) / MR-251/01 / M-034280-01-1 [redacted] (2001) / MR-056/01 / M-032318-01-1
Metabolism	Prothioconazole: Prothioconazole is extensively metabolised to 18 metabolites, with the major metabolic reactions being S-conjugation with glucuronic acid, oxidative hydroxylation of the phenyl moiety, and desulfuration (almost exlusively in the faeces). Two major metabolites are found: prothioconazole-S-glucuronide (JAU6476-S-glucuronide, M06) was the main (approx. 50% of the administered dose) systemic (urine + bile) metabolite, whereas prothioconazole-desthio (JAU6476-desthio, M04) was almost exlusively found in the faeces (max. 17.7% of the administered dose) and only to a minor proportion systemically (urine: 0.07%, bile: 0.45% of the administered dose). Parent compound was found between 1 and 22% of the administered dose. 1,2,4-triazole (M13) was found in urine in amounts of up to 2.3% of the administered dose. Prothioconazole-desthio (M04): Metabolism proceeds via oxidation reactions on the phenyl moiety only with subsequent glucuronidation and methylation of the oxidation products. No metabolic reactions at the cyclopropyl- and triazole rings were observed.	[redacted] (2001) / MR-251/01 / M-034280-01-1 [redacted] (2001) / MR-056/01 / M-032318-01-1

Potential for accumulation	None	[redacted] (2001) / MR-251/01 / M-034280-01-1. [redacted] (2001) / MR-056/01 / M-032318-01-1

Prothioconazole was almost completely absorbed via the intestinal tract. At least ca. 90% of the administered dose was absorbed at the low dose level (2 mg/kg) by male rats, as was calculated from the bile-duct cannulation experiment using the triazole-labelled compound. The rate and extent of absorption of the total radioactivity was essentially independent of sex and labelling position.

The radioactivity administered with prothioconazole was widely distributed over various organs (plasma t_{max}= 0.1 – 0.7 h), with rapid decrease of residues to levels close to or at the LOQ after 72 h for most organs and tissues. The radioactivity was at least partially subjected to an enterohepatic circulation, as was concluded from the oscillating plasma concentration curves. The excretion of radioactivity was almost complete already 48 h after oral administration of triazole- or phenyl-labelled prothioconazole. In almost all tests, between ca. 90 and 100% of the administered dose was excreted with urine, faeces, or bile at the time of sacrifice, i. e. 48 h or 168 h after dosing. About 78 - 96% of the administered dose was excreted with the faeces and only ca. 4 – 16% renally. The residues in the body at sacrifice ranged from ca. 0.1 to 1.5% of the administered dose for the animals sacrificed after 168 h and from 1 to 6% for the animals sacrificed 48 h following administration. By far the greatest amounts of radioactivity were found in the organs responsible for the absorption, degradation, and excretion, i. e. in the gastrointestinal tract, liver, and kidney. Consequently, only less than 0.5% of the administered dose was found in all remaining organs and tissues. There was no evidence of accumulation.

Prothioconazole was intensively metabolised in the rat. Eighteen metabolites, including the parent compound, were identified in urine, faeces, and bile. The overall most abundant metabolite was prothioconazole-S-glucuronide (JAU6476- S-glucuronide, ca. 46% of the administered dose in the bile and up to 7.7% in urine), followed by the unchanged parent compound (ca. 1 – 22%) and prothioconazole-desthio (JAU6476-desthio), ca. 0.4 – 18%). Prothioconazole-desthio was found almost exclusively in the faeces and only to a minor extent systemically (urine: 0.07%, bile: 0.45%) since the sulfur moiety is protected against cleavage following the S-glucuronidation. All metabolites present in the excreta at amounts ≥ 5% and many other metabolites accounting for less than 5% of the administered dose were identified. The major systemic formation (and excretion) of prothioconazole-S-glucuronide (JAU6476-S-glucuronide) in conjunction with the negligible systemic formation of prothioconazole-desthio (JAU6476-desthio) is interpreted as the "mechanism of detoxification" of prothioconazole in the rat.

The proposed metabolic pathway of prothioconazole in the rat is shown in Figure 5.1.1- 1.

Report:	KCA 5.1.1/01 [redacted]Y; 2001; M-034280-01-1
Title:	[14C]JAU6476: Rat metabolism - Part 1 of 2: Investigation of the biokinetic behaviour and the metabolism (ADME) in the rat with [triazole-UL-14C]- and [phenyl-UL-14C]JAU6476
Report No.:	MR-251/01
Document No.:	M-034280-01-1
Guideline(s):	US EPA OPPTS 870.7485; Canadian PMRA Ref.: DACO 4.5.9; OECD 417; Japanese MAFF 59 NohSan 4200
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Executive Summary

The absorption, distribution, excretion and metabolism were investigated with two forms of radiolabelled prothioconazole, ^{14}C-labelled in the triazole ring (triazole-UL-^{14}C) and in the phenyl ring (triazole-UL-^{14}C), in male and female Wistar rats.

[triazole-UL-^{14}C]Prothioconazole was administered orally to four groups of five male or female rats at a low dose of 2 mg/kg bw or at a high dose of 150 mg/kg bw. In addition, [triazole-UL-^{14}C]prothioconazole was administered intraduodenally to one group of eight bile-duct cannulated male rats at a low dose of 2 mg/kg bw.

[phenyl-UL-^{14}C]Prothioconazole was administered orally to one group of five male rats at a low dose of 5 mg/kg bw. Two groups of five rats received 14 (male) or 15 (female) daily doses of non-labelled prothioconazole at 2 mg/kg bw, and then one dose of [phenyl-UL-^{14}C]prothioconazole at 2 mg/kg bw (multiple dose tests). In addition, [phenyl-UL-^{14}C]prothioconazole was administered orally to one group of 20 bile-duct cannulated male rats at a low dose of 2 mg/kg bw. In pilot test, [phenyl-UL-^{14}C]prothioconazole was administered orally to one group of five male rats at a low dose of 2 mg/kg bw to investigate the expiration of ^{14}C-carbon dioxide and other ^{14}C-labelled volatile compounds.

Urine and faeces were collected from all rats at several intervals over the time period from dosing until sacrifice. Additionally, bile was collected from the bile-duct cannulated rats and plasma micro samples from the intact rats. In the pilot test expired air was collected in addition to urine and faeces. Intact rats were sacrificed 48 hours after dosing of [phenyl-UL-^{14}C]prothioconazole and 7 days after dosing of [triazole-UL-^{14}C]prothioconazole and blood, tissues and organs were collected, except for the pilot test where only GIT, skin and carcass were collected at sacrifice. Bile-duct cannulated rats were sacrificed 6 hours after dosing of [phenyl-UL-^{14}C]prothioconazole and 48 hours after dosing of [triazole-UL-^{14}C]prothioconazole and GIT, skin and carcass were collected. The radioactivity was determined in the collected samples. The metabolism was investigated in urine, faeces and bile.

At least approx. 94% of the administered radioactivity was recovered in all tests, except for the pilot expired air experiment with a recovery of approx. 90%. The absorption of [triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole was rapid and almost complete during the test periods. Short half-lives of absorption ($t_{1/2\ abs}$ < 0.3 h) were calculated. Plasma peak levels were observed within 0.1 - 0.7 hours after dosing. The oral absorption was at least approx. 90% of the administered dose at the low dose level of 2 mg/kg bw, as was calculated from the bile-duct cannulation experiment with the [triazole-UL-^{14}C] label and estimated from the bile-duct cannulation experiment with the [phenyl-UL-^{14}C] label. The rate and extent of absorption of the total radioactivity was essentially independent of sex and ^{14}C-labelling position.

From peak levels, the time course of radioactivity in plasma showed a decline and efficient elimination of prothioconazole and its metabolites from the body. The calculated first elimination half-lives [$t_{1/2}$ e(1)] ranged from 0.4 - 0.9 hours and the second elimination half-lives [$t_{1/2}$e(2)] ranged from 8 - 19 hours. An enterohepatic circulation was observed.

The excretion of radioactivity was almost complete within 48 hours of oral administration of [triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole. At sacrifice 48 or 168 hours post-treatment, approximately 90 - 100% of the administered dose had been excreted with urine, faeces, or bile, and less than 6% of the administered dose remained in the body, including the gastrointestinal tract. At sacrifice, the intact rats had excreted 78 - 96% of the administered dose with the faeces and 4 -16% with the urine. Renal excretion was slightly higher in female animals (10 - 16%) than in males (4 - 10%). The bile-duct cannulated rats excreted 90.2% and 82.2% of the administered dose with the bile at 48 and 64 hours post-treatment, respectively. Only 0.06% of the administered dose was expired as $^{14}CO_2$ and other ^{14}C-labelled volatile compounds. At sacrifice, most of the residual radioactivity was detected in the gastrointestinal tract, liver, and kidneys. Less than 0.5% of the administered dose was found in all other organs and tissues.

[Triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole were intensively metabolised. Eighteen metabolites, including the parent compound, were identified in urine, faeces, and bile. The proposed biotransformation pathway of prothioconazole in the rat is shown in Figure 5.1.1- 1. The unchanged parent compound was found at ca. 1 – 22% of the administered dose. The major metabolic reaction was conjugation with glucuronic acid at the sulfur atom of prothioconazole. Thus, the overall most abundant metabolite was JAU6476-S-glucuronide (prothioconazole-S-glucuronide, M06), which was found at ca. 46% of the administered dose in the bile and up to 7.7% in the urine. Another major metabolite was JAU6476-desthio (prothioconazole-desthio, M04) found at ca. 0.4 – 18% of the administered dose. JAU6476-desthio was found almost exclusively in the faeces and only to a very minor extent systemically, up to 0.07% in the urine and 0.35% - 0.45% in the bile. Several hydroxylated metabolites were identified, some of which were conjugated with glucuronic acid. Solely detectable by the [triazole-UL-^{14}C] label, 1,2,4-triazole (M13) was found in urine at up to 2.3% of the administered dose. A metabolite without the triazole moiety was not identified in the experiments with [phenyl-UL-^{14}C]prothioconazole. All metabolites present in the total excreta at ≥ 5% of the administered dose and many other metabolites representing < 5% of the administered dose were identified.

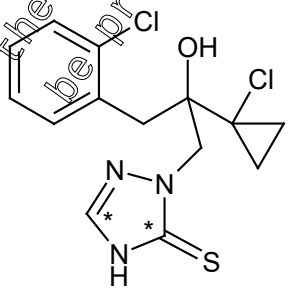
In summary, after absorption the main biotransformation route of prothioconazole is conjugation with glucuronic acid at the sulfur atom. The formed metabolite JAU6476-S-glucuronide is excreted rapidly with the bile, and thus the systemic cleavage of the sulfur atom, i.e. the systemic formation of JAU6476-desthio is inhibited. The major systemic formation and excretion of the metabolite JAU6476-S-glucuronide in conjunction with the negligible systemic formation of JAU6476-desthio is interpreted as the "mechanism of detoxification" of prothioconazole in the rat.

I. Materials and methods

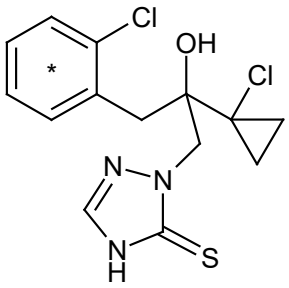
A. Materials

1. Test material: [triazole-UL-^{14}C]Prothioconazole [phenyl-UL-^{14}C]Prothioconazole

Chemical structure:



* labelling position



* labelling position

Description: solid, dried in vacuo solid, dried in vacuo

Batch no.:	10708/1	11403/1, 12268/1 and 14015/1
Specific radioactivity:	1.94 MBq/mg (18.1 Ci/mol); for the high dose tests (tests 3 and 16) radiodilution with non-labelled prothioconazole: 0.026 MBq/mg (0.24 Ci/mol)	2.97 MBq/mg (27.6 Ci/mol), 3.66 MBq/mg (34.1 Ci/mol)* and 4.68 MBq/mg (43.6 Ci/mol); *for the single low dose test at 5 mg/kg (test 9) and bile-duct cannulation test (test 11); radiodilution with non-labelled prothioconazole: 1.46 MBq/mg (13.6 Ci/mol) and 1.79 MBq/mg (16.7 Ci/mol)
Radiochemical purity:	> 98%/99% (HPLC/radiodetection)	> 99% (HPLC/radiodetection)
Chemical purity:	> 99% (HPLC/UV)	> 98%/99% (HPLC/UV)
CAS no.:	178928-70-6 (non-labelled prothioconazole)	
Stability of test compound:	The administration suspensions of representative tests were analysed by HPLC. The radiochemical purity of the test compound was at least 98% for all analyses, demonstrating the stability of the test compound under the conditions of storage and handling prior to administration.	

2. Vehicle: 0.5 % aqueous Tragacanth solution

3. Test animals:

Species:	Rat
Strain:	Albino male and female rats derived from *Rattus norvegicus* Strain: Wistar Hsd Cpb:Wu
Age:	7 – 12 weeks
Weight at dosing:	Approximately 200 g and 400 g for the single low dose test at 5 mg/kg b.w. with the [phenyl-UL-^{14}C]-label (test 9)
Source:	[redacted], Germany
Acclimation period:	The animals were acclimated to the laboratory conditions in Makrolon® type III cages on wood shavings for at least one week prior to the start of each experiment
Identification:	Cage cards with study number, substance name, and animal number as well as coloured spots on the tail
Diet:	For the multiple dose tests (test 12 and 18) and the high dose female test (test 16): rat-mice maintenance long life diet (no. 9439), Fa. [redacted] CH-[redacted], Switzerland For all other tests: Altromin 1324 standard food, [redacted], Germany The animals were fed with ca. 20 g per day and animal. The animals were fed the last time ca. 14 – 16 h prior to administration of the dose and again ca. 6 h after administration of the dose.
Water:	Tap water, *ad libitum*
Housing:	During the excretion studies the animals were kept in special Makrolon® metabolism cages, which allowed for a separate and quantitative sampling of the excreta. During the period of pre-treatment with non-

labelled prothioconazole, the rats were housed as single animals in Makrolon® type II cages.

Environmental conditions

Temperature:	17 – 25°C
Humidity:	32 – 92%
Air changes:	10 – 15 fold air change per hour
Photoperiod:	12 hours light/dark-cycle

4. Preparation of dosing solutions

Upon receipt the solid radiolabelled test compound was dissolved in acetonitrile. For the preparation of each administration suspension an adequate portion of the stock solution was pipetted and evaporated to dryness under a gentle stream of nitrogen. The dry residue was suspended in 5 – 27.5 mL of a 0.5% aqueous Tragacanth solution by ultrasonication in a water bath at 50°C for 15 – 30 min. The suspension was stirred overnight at room temperature and an adequate volume of the test suspension was administered to each rat.

B. Study design and methods

1. Dose regimen and design of tests

Test no.	Administered single dose of ^{14}C-Prothioconazole, route (experiment)	^{14}C-label	Number of rats and sex	Collection of samples during the test and at sacrifice	Duration
1	2 mg/kg bw, oral (single low dose)	triazole-UL	5 male	urine, faeces, plasma, organs, GIT, skin, carcass	7 days
2	2 mg/kg bw, oral (single low dose)	triazole-UL	5 female	urine, faeces, plasma, organs, GIT, skin, carcass	7 days
3	150 mg/kg bw, oral (single high dose)	triazole-UL	5 male	urine, faeces, plasma, organs, GIT, skin, carcass	7 days
4	2 mg/kg bw, intraduodenal (bile-duct cannulation)	triazole-UL	8 male	bile, urine, faeces, GIT, skin, carcass	48 hours
8	2 mg/kg bw, oral (expired air test)	phenyl-UL	5 male	expired air, urine, faeces, GIT, skin, carcass	48 hours
9	5 mg/kg bw, oral (single low dose)	phenyl-UL	5 male	urine, faeces, plasma, organs, GIT, skin, carcass	48 hours
11	2 mg/kg bw, oral (bile-duct cannulation)	phenyl-UL	20 male	bile, urine, faeces, GIT, skin, carcass	6 hours
12	2 mg/kg bw, oral, after 14 daily non-labelled doses at 2 mg/kg bw (multiple low dose)	phenyl-UL	5 male	urine, faeces, plasma, organs, GIT, skin, carcass	48 hours
16	150 mg/kg bw, oral (single high dose)	triazole-UL	5 female	urine, faeces, plasma, organs, GIT, skin, carcass	7 days
18	2 mg/kg bw, oral, after 15 daily non-labelled doses at 2 mg/kg bw (multiple low dose)	phenyl-UL	5 female	urine, faeces, plasma, organs, GIT, skin, carcass	48 hours

The rats of all test groups were given a single dose of radiolabelled prothioconazole, either labelled in the triazole ring (triazole-UL-^{14}C) or in the phenyl ring (triazole-UL-^{14}C).

[triazole-UL-^{14}C]Prothioconazole was administered orally to four groups of five male or female rats at a low dose of 2 mg/kg bw or at a high dose of 150 mg/kg bw (tests 1, 2, 3 and 16). Urine, faeces and plasma samples were collected for seven days until sacrifice, and organs, GIT, skin and carcass were collected. In addition, [triazole-UL-^{14}C]prothioconazole was administered intraduodenally to one group of eight bile-duct cannulated male rats at a low dose of 2 mg/kg bw (test 4). Bile, urine and faeces were collected for 48 hours until sacrifice, and GIT, skin and carcass were collected.

[phenyl-UL-^{14}C]Prothioconazole was administered orally to one group of five male rats at a low dose of 5 mg/kg bw (test 9). Two groups of five rats received 14 (male) or 15 (female) daily doses of non-labelled prothioconazole at 2 mg/kg bw, and then one dose of [phenyl-UL-^{14}C]Prothioconazole at 2 mg/kg bw (tests 12 and 18). In these three tests, urine, faeces and plasma samples were collected for 48 hours until sacrifice, and organs, GIT, skin and carcass were collected. In addition, [phenyl-UL-^{14}C]prothioconazole was administered orally to one group of 20 bile-duct cannulated male rats at a low dose of 2 mg/kg bw (test 11). Bile, urine and faeces were collected for 6 hours until sacrifice, and GIT, skin and carcass were collected. In another test, [phenyl-UL-^{14}C]prothioconazole was administered orally to one group of five male rats at a low dose of 2 mg/kg bw, and expired air, urine and faeces were collected for 48 hours until sacrifice, when GIT, skin and carcass were collected (test 8).

2. Cholangiostomy

The animals of the bile-duct cannulation tests were anaesthetised for the cholangiostomy surgery with Narcoren ([redacted], Germany) mixed with Atropin ([redacted], Germany) ad 2.0 mL with physiological NaCl solution. After shaving the stomach area below the rib cage, a small incision was made. The portion of the small intestine with the pancreatic tissue containing the bile duct was pulled out carefully. Surgical thread was pulled through prior to puncturing the bile duct. Afterwards, biliary and duodenal cannulae were implanted for collection of the bile and donation of rat/ox bile. The operated zone was relocated into the body by lifting the skin. A small incision was made on the back of the animal to pull the tubings through. The muscle layer was closed by careful sewing and the use of surgical clamps. The incisions were sealed with all-purpose glue. The rats were placed onto warming pads while regaining consciousness and were kept individually in specially designed cages to restrict movement. While in the cages, the animals had unrestricted access to food and water. Ox bile was infused to the duodenum with a peristaltic pump at a flow rate of ca. 1 mL/h.

3. Dosing

Adequate volumes of the suspensions of the test compound in 0.5% aqueous Tragacanth were administered to each rat, 0.4 mL intraduodenally or 1 mL orally to the bile-duct cannulated rats and 2 mL to the rats of all other tests. Oral dosing was performed using a syringe attached to an animal-feeding knob cannula. The concentrations of the test compound in the suspensions were calculated to give the dose rates of 2, 5 and 150 mg/kg body weight (bw) using an average animal weight of approx. 200 g or, for test 9, 420 g. As the animal weights varied slightly, the actual doses varied slightly with the body weight.

The concentration of the active substance in the administration suspensions was determined by radioassay. The results of these measurements served as a basis for the calculation of the total radioactivity in the biological samples. The stability of the active substance in the administration suspensions was checked for some representative tests by HPLC, showing a radiochemical purity of at least 98%.

4. Collection of excreta

After the administration of the radiolabelled test substance, the rats were kept individually in Makrolon® metabolism cages (tests 1 – 3, 8 – 9, 12, 16, and 18), which allowed for separate and quantitative collection of expired air, urine, and faeces. In the case of the bile-duct cannulation experiment (tests 4

and 11), the rats were kept in individual restraining cages to allow the collection of bile, urine, and faeces.

Urine was collected separately for each rat in a cryogenic trap cooled with dry ice after administration of the radiolabelled dose in intervals of
- 4, 8, 24, 48, 72, 96, 120, 144 and 168 hours in tests 1,2, 3 and 16,
- 4, 8, 24 and 48 hours in tests 8, 9, 12 and 18,
- 1, 2, 3, 4, 6, 8, 24, 32 hours and 48 h in test 4 and
- 3 and 6 hours in test 11.

The funnels for urine collection were rinsed with demineralised water at the end of each sampling period. The rinse solutions were added to the corresponding urine fraction. The radioactivity was determined by LSC.

Faeces was collected separately for each rat every 24 h after administration of the radiolabelled dose, except for faeces from test 11 which was collected 6 h after administration. The faeces samples were lyophilised (freeze-dried), weighed, and homogenised. The radioactivity was determined by combustion/LSC.

Bile was collected separately for each cannulated rat after administration at intervals of
- 1, 2, 3, 4, 6, 8, 24, 32 and 48 hours in test 4 and
- 3 and 6 hours in test 11.

The radioactivity was determined by LSC.

5. Expired air

^{14}C-carbon dioxide and other volatiles from expired air were collected separately for each rat of test 8 after administration at intervals of 8 h, 24 h and 48 h. The metabolism cages were attached to a high velocity air pump and ventilated with ca. 2 L of air per minute and cage. The outcoming air was passed through a trapping system of two gas-washing bottles containing about 250 mL of a 1:1-mixture of ethanolamine/ethanol each. At each sampling the exact volume was determined, and an aliquot was taken for the determination of radioactivity by LSC. The trapping system was refilled after each sampling.

6. Plasma micro samples

Blood samples were collected in heparinised capillaries separately for each rat by puncture of the tail veins. The samples were taken after administration of the radiolabelled dose at intervals of
- 0.08, 0.16, 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8, 24, 32, 48, 72, 96, 120, 144 and 168 hours in tests 1, 2, 3 and 16 and
- 0.08, 0.16, 0.33, 0.66, 1, 1.5, 2, 3, 4, 6, 8, 24, 32 and 48 hours in tests 9, 12 and 18.

The wound was closed with adhesive tape. The capillaries were centrifuged at ca. 12000 g for 10 minutes using a haematocrit centrifuge to separate plasma from erythrocytes. After centrifugation the capillary was broken at the border between plasma and erythrocytes and the plasma (ca. 30 µL) was pressed onto a small metal dish for weighing. This dish was then placed into a scintillation vial for radioactivity measurement. For pharmacokinetic calculations, the average plasma value of the rats of the group was used. By this method, it was possible to collect blood samples at the different time points from the same animals and thus generating plasma curves from single animals. The results show lower variability compared to curves that were calculated from whole blood samples of different animals.

7. Sacrifice

The rats were sacrificed in carbon dioxide anaesthesia by transection of the cervical vessels and exsanguinated.

8. Blood, tissues and organs at sacrifice

At sacrifice, the blood was collected and separated into plasma and erythrocytes by centrifugation. The following organs and tissues were collected:

- spleen, gastrointestinal tract (GIT), liver, kidney, perirenal fat, testis/uterus & ovaries, skeletal muscle, bone femur, heart, lung, brain, skin, adrenal gland, thyroid gland and carcass from rats of tests 1,2, 3, 9, 12, 16 and 18, and
- skin, gastrointestinal tract (GIT) and carcass from rats of tests 4, 8 and 11.

The organs and tissues prepared at the end of the experiments were weighed immediately after the dissection and again after lyophilisation. Finally, they were homogenised prior to taking aliquots for the determination of radioactivity by combustion /LSC. For the small organs and tissues (adrenal glands, thyroid, ovaries, renal fat and uterus), only the wet weight was determined, and they were solubilised in BTS 450 (Beckman Tissue Solubiliser) and radioassayed by LSC.

9. Sample handling and storage

All freeze dried samples like faeces or organs were stored in plastic vials at room temperature or at ca. +4°C in a refrigerator. All other liquid samples were kept frozen at ca. –20°C at all times except during aliquotation for analysis. During the analytical work (see below), the samples were stored either at ca. +4°C in a refrigerator or at ca. –20°C in a freezer.

10. Measurement of radioactivity

The measurement of the radioactivity in liquid samples was carried out by liquid scintillation counting (LSC). All solid samples were combusted in an oxygen atmosphere using an oxidizer. The released $^{14}CO_2$ was trapped in an alkaline scintillation cocktail and the radioactivity was determined by LSC.

11. Toxicokinetic analysis

In this study, the software TOPFIT (version 2.0) was used to calculate the pharmacokinetic parameters by plasma concentration-time curve analysis. The standard 3-compartment model was applied for curve fitting computations. The calculations are based on the mean equivalent concentration of the total radioactivity in the plasma sampled from the rats of the test group. For comparability, all analyses were performed for the plasma concentrations of the period 0 – 48 hours after administration of the radiolabelled dose.

12. Preparation of urine, faeces and bile for analysis

Generally, the excreta samples of the rats were combined to representative pools of each test group.

Pools of urine samples were prepared to represent the following excretion intervals:
0 – 72 hours for tests 1, 2 and 3,
0 – 48 hours for tests 12, 16 and 18 and
0 – 4, 4 – 8, 8 – 24 and 24 – 48 hours for test 9.
Urine pools of tests 1 – 3 were concentrated with a rotary evaporator. Urine pool samples were injected directly into the HPLC device without any sample preparation.

Pools of faeces samples were prepared to represent the following excretion intervals:
0 – 72 hours for tests 1 and 2,
0 – 48 hours for tests 3, 12, 16 and 18 and
0 – 24 and 24 – 48 hours for test 9.
For tests 1, 2, 3 and 16, samples of these pools were successively extracted with acetonitrile/water and acetonitrile/water/acetic acid mixtures. For each of these tests, the individual extracts were combined to an acetonitrile/water extract representing the first extraction steps, and a second acetonitrile/water extract (contained also HOAc) representing the subsequent and more polar extraction steps. For tests 9, 12, and 18, samples of the pools were successively extracted with acetonitrile/water mixtures of increasing polarity, which were then combined and partitioned against hexane. The faeces extracts were

concentrated with a rotary evaporator and aliquots were analysed by HPLC. The radioactivity in the extracts was determined by LSC and in the remaining solids by combustion/LSC.

Pools of bile samples were prepared to represent the excretion intervals of 0 – 3 h and 3 – 6 h. For test 4 the bile samples of only 3 rats and for test 11 the bile samples of only 15 rats were pooled. Samples of the other rats were not considered representative and were not used for pooling. Some rats died before scheduled termination of the test, probably as a consequence of the surgery. The courses of excretion of other rats showed distinctly higher radioactivity values in the urine or low total radioactivity values, therefore the values were regarded as outliers. Bile pool samples were analysed by HPLC.

13. Analytical methods
Urine, faeces and bile were analysed for parent compound and metabolites by HPLC with radioactivity detection, TLC & radioluminography, and with LC-MS and NMR methods.

14. High performance liquid chromatography (HPLC)
High performance liquid chromatography (HPLC) was used for recording of the metabolic profiles and identification with reference compounds. HPLC analysis was performed on Hewlett Packard modular LC systems with radiometric (Raytest radioactivity) supported by UV detection. The separation was mainly carried out on a reversed phase column using an acidic or a neutral water / acetonitrile gradient. All solvents were of HPLC-quality. The chromatograms were recorded electronically and were quantitatively evaluated using the software package GINA® (Raytest, Straubenhardt, Germany). A radioactive peak is regarded as relevant having a signal to noise ratio at least 2.5 (LOD). Each radiochromatogram was divided into regions of interest (ROI), corresponding to the separated radioactive components and areas in between. The average radioactivity levels in the background regions were proportionately subtracted from all other regions for calculation of the percentage of total net radioactivity in each region. In order to check the completeness of the chromatographic elution, representative samples were injected, re-collected, and radioassayed by LSC. The chromatographic recoveries were equal to or greater than 95%, as shown with bile and faeces extract samples.

For co-chromatography, the sample was mixed with the reference compound before injection. The detection was carried out either by UV-absorbance of the non-radiolabelled or by ^{14}C-detection of the radiolabelled reference compound. Chromatographic matching with the non-radiolabelled reference compound was assessed by comparison of the UV-trace and the associated ^{14}C-trace, taking into account the time delay between the radioactivity and absorbance detectors. Chromatographic matching with the radiolabelled reference compound was assessed by comparison of the ^{14}C-chromatogram of the mixture with the ^{14}C-chromatogram of the sample without the reference compound.

15. Thin layer chromatography (TLC)
Thin layer chromatography (TLC) was used for identification with reference compounds. For one-dimensional TLC, pre-layered, glass-backed plates from Merck (Germany) with a size of 20 x 10 cm or 20 x 20 cm were used regularly. The absorbent was silica 60F_{254} (normal phase) with a layer thickness of 0.25 mm. The plates were pre-treated by flushing with air bubbled through a 5% aqueous ammonia solution and then developed using either an instrument for automatic multiple development ([redacted] Switzerland) with methanol and methylene chloride as eluents or a standard TLC-chamber without chamber saturation with a alkaline methanol/methylene chloride mixture as eluent. For the AMD method, the start zone was pre-treated with a acetonitrile/water solution of cysteine hydrochloride and non-radiolabelled Prothioconazole to reduce oxidative decomposition of active compound in the samples. For both methods, the samples were applied on the plates using a Linomat IV instrument ([redacted], Germany). The TLC bands or spots were visualised under an UV-light (254 nm). The radioactive zones were detected by radioluminography, using a Fujibas® 2000 bio imaging system (Fuji, Japan & Raytest, Germany). Fujibas® imaging plates were exposed to the TLC-plates. The imaging data were transferred with Basreader software (version 2.13e, Raytest, Germany) to an

appropriate computer. Evaluation and visualisation of recorded data was performed with TINA software (version 2.09g, Raytest, Germany).

For co-chromatography, a solution of the reference substance was applied to the plate as a 2 cm-wide band. The sample solution was also applied as a 2 cm-wide band, part of which overlapping with the band of the reference substance. After development of the TLC plate, chromatographic correspondence was assessed either by visual inspection of the plate under UV light and its associated radioluminogram or by analysis of only the radioluminogram in cases where radiolabelled reference compounds were used.

16. Mass spectroscopy (MS)
The electro-spray ionisation MS spectra (ESI) were obtained with a TSQ 7000 instrument by Finnigan (San Jose, CA, U.S.A.). For the MS/MS experiments, argon was used as the collision gas. Hewlett Packard modular LC systems were used for chromatography. The separation was carried out on a reversed phase column using an acidic water/acetonitrile gradient or on a normal phase column using an alkaline hexane/ethanol gradient.

17. Nuclear magnetic resonance spectroscopy (NMR)
NMR spectra were recorded on a BRUKER DPX 300 (300 MHz) instrument or on a BRUKER DMX 600 (600 MHz) instrument.

18. Enzymatic hydrolysis
Enzymatic treatment was performed to cleave conjugated metabolites. In a typical procedure, 500 µL sample solution, i. e. bile, was mixed with 500 µL water and 200 µL enzyme solution (mixture of ß-glucuronidase and arylsulfatase from Helix pomatia). The solution was incubated for 4 h at 50°C.

19. Derivatisation with dansyl chloride
An aqueous sample solution containing the metabolite 1,2,4-triazole isolated from urine was mixed with a solution of 0.5 g 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) in 150 mL acetone and the mixture was stirred for 1.5 h at room temperature. The acetone was evaporated and the concentrated aqueous solution was extracted with ethyl acetate. In the same way, a sample of synthetic [UL-^{14}C]-1,2,4-triazole was treated and then used as a reference compound for co-chromatography.

20. Identification, characterisation and quantification
Urine and bile samples and faeces extracts were analysed by HPLC using a reversed phase column (RP 18) with an acidic water/acetonitrile gradient and radioactivity detection. Major metabolites were isolated from the faeces extracts with semi-preparative HPLC. These metabolites were then identified by LC-MS/MS and ^{1}H-NMR to serve as reference compounds for the identification or confirmation in the urine and bile with HPLC co-chromatography. In urine, further metabolites were identified with LC-MS/MS. The metabolite 1,2,4-triazole was identified by co-chromatography after derivatisation with dansyl chloride. In bile, further metabolites were identified with LC-MS/MS, in some cases after isolation with semi-preparative HPLC. For some of the glucuronic acid conjugates in bile, the identification was confirmed by enzymatic hydrolysis. Reference compounds from synthesis or from isolation in livestock and plant metabolism studies conducted with prothioconazole or prothioconazole-desthio served for the identification or confirmation of metabolites in urine and faeces, using HPLC or TLC co-chromatography.

II. Results and discussion

A. Recovery

At least approx. 94% of the administered radioactivity was recovered in all tests, except for the pilot expired air experiment (test 8) with a recovery of approx. 90%. A summary of the radioactivity in percent of the administered dose found in excreta and organs and tissues at sacrifice are presented in Table 5.1.1-2.

Table 5.1.1- 2: Recovery of radioactivity in excreta, gastrointestinal tract and the body of rats following oral or intraduodenal dosing of [phenyl-UL-^{14}C]- and [triazole-UL-^{14}C]Prothioconazole, data presented as % of dose administered

Test no.	Test 1	Test 2	Test 3	Test 4	Test 8
Dose, route	2 mg/kg, p.o.	2 mg/kg, p.o.	150 mg/kg, p.o.	2 mg/kg, i. duo.	2 mg/kg, p.o.
Experiment	single low dose	single low dose	single high dose	bile-duct cannulation	expired air test
^{14}C-radiolabel	triazole-UL	triazole-UL	triazole-UL	triazole-UL	phenyl-UL
Duration, sex	7 days, male	7 days, female	7 days, male	48 h, male	48 h, male
Expired air	----	----	----	----	0.062
Urine	10.47	15.97	3.710	2.048	5.899
Bile	----	----	----	90.21	----
Faeces	84.49	78.40	95.88	1.280	80.59
Sum excreta	94.96	94.37	99.59	93.54	86.56
Body w/o GIT	1.414	0.321	0.090	0.840	2.236
GIT	0.127	0.071	0.019	0.062	1.335
Total body	1.541	0.392	0.109	0.902	3.571
Balance	96.50	94.76	99.70	94.14	90.13

Test no.	Test 9	Test 11	Test 12	Test 16	Test 18
Dose, route	5 mg/kg, p.o.	2 mg/kg, p.o.	2 mg/kg, p.o.*	150 mg/kg, p.o.	2 mg/kg, p.o.*
Experiment	single low dose	bile-duct cannulation	multiple low dose*	single high dose	multiple low dose*
^{14}C-radiolabel	phenyl-UL	phenyl-UL	phenyl-UL	triazole-UL	phenyl-UL
Duration, sex	48 h, male	6 h, male	48 h, male	7 days, female	48 h, female
Expired air	----	----	----	----	----
Urine	4.369	1.154	5.137	11.81	10.24
Bile	----	82.17	----	----	----
Faeces	85.37	1.524	93.22	87.76	86.80
Sum excreta	89.94	84.85	98.36	99.57	97.05
Body w/o GIT	2.675	3.198	2.899	0.094	0.367
GIT	3.143	19.62	0.924	0.017	0.462
Total body	5.818	22.81	3.824	0.111	0.829
Balance	95.76	107.70	102.20	99.68	97.88

p.o. = per os, orally, i.duo. = intraduodenal
* one radiolabelled dose after 14 (test 12) or 15 (test 18) daily non-labelled doses at 2 mg/kg bw

B. Absorption

The absorption of [triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole was rapid and almost complete during the test periods. The absorption commenced immediately after oral administration as shown by the time course of the plasma concentrations of radioactivity (Table 5.1.1- 3) and the pharmacokinetic calculations (The radiolabelled residues at sacrifice in the whole body were in the range 0.1 - 1.5% of administered dose for the animals sacrificed 168 hours after treatment with [triazole-UL-14C]prothioconazole (tests 1, 2, 3 and 16) and in the range 0.83 – 5.8% for the animals sacrificed 48 hours after treatment with [phenyl-UL-14C]Prothioconazole (tests 9, 12, and 18) (see Table 5.1.1- 6). Most of the residual radioactivity was detected in the gastrointestinal tract, liver, and kidneys. Consequently, < 0.5% of the administered dose occurred in all other organs and tissues. Dose normalised concentrations of < 0.02 were found in all organs and tissues, except in the organs involved in absorption, metabolism, and excretion, and the thyroid gland (Table 5.1.1- 7). The gastrointestinal tract, liver, and kidneys showed distinctly higher concentrations at sacrifice. They had values of up to 0.6 for liver at 48 hours post-treatment. Females showed lower concentrations of 0.03 in the liver than males. The thyroid had dose normalised concentrations at sacrifice below ca. 0.06, with the males showing lower values than females. In most tests, the amount of the radioactivity in the thyroid gland was below the limit of detection (0.01 - 0.06). These results are in good agreement with the results obtained by whole body autoradiography ([redacted], K.; 2001; M-034660-01-1).

Table 5.1.1- 5). In the pharmacokinetic model short lag times of absorption ($t_{lag\ abs}$ < 0.2 hours) and short half-lives of absorption ($t_{1/2\ abs}$ < 0.3 hours) were calculated. Plasma peak levels were observed within 0.1 - 0.7 hours after dosing.

The oral absorption was at least approx. 90% of the administered dose at the low dose level of 2 mg/kg bw. This was calculated from the results of the bile-duct cannulation experiment with male rats and [triazole-UL-^{14}C]prothioconazole (test 4) by adding the radioactivity excreted with the bile and urine and the radioactivity residues in the body without the gastrointestinal tract at 48 hours after dosing (Table 5.1.1- 2).

In the bile-duct cannulation experiment with [phenyl-UL-^{14}C]prothioconazole (test 11) 20 rats were dosed at the low dose level of 2 mg/kg b.w. This experiment was mainly conducted to collect large amounts of bile for the structure elucidation of bile metabolites and the rats were sacrificed already 6 h after administration, when the major part of the radioactivity was excreted with the bile. At this time the gastrointestinal tract of the rats still contained ca. 20% of the administered dose (Table 5.1.1- 2) and approx. 80% was excreted with the bile. However, it was estimated that if this experiment was conducted for 48 hours an oral absorption of at least ca. 90% of the administered dose would be observed. This extrapolation was based on the course of excretion observed in the bile-duct cannulation experiment with [triazole-UL-^{14}C]prothioconazole (see test 4 in Table 5.1.1- 8). This comparison of the two different radiolabelled experiments was justified, because by far the major part of the metabolites were detectable regardless of the radiolabel position (see below chapter E. Metabolism). It was also taken into account that the excretion in test 11 was slightly delayed likely due to the oral administration, while the rats of test 4 were dosed intraduodenally.

The rate and extent of absorption of the total radioactivity was essentially independent of sex and labelling position.

Table 5.1.1- 3: Time course of radioactivity in the plasma of male and female rats following an oral dose of [triazole-UL-^{14}C]- or [phenyl-UL-^{14}C]Prothioconazole expressed as parent equivalent concentration in µg/g

Test no.	Test 1	Test 2	Test 3	Test 9	Test 12	Test 16	Test 18
Dose, route	2 mg/kg, p.o.	2 mg/kg, p.o.	150 mg/kg, p.o.	5 mg/kg, p.o.	2 mg/kg, p.o.*	150 mg/kg, p.o.	2 mg/kg, p.o.*
Experiment	single low dose	single low dose	single high dose	single low dose	multiple low dose*	single high dose	multiple low dose*
^{14}C-label	triazole-UL	triazole-UL	triazole-UL	phenyl-UL	phenyl-UL	triazole-UL	phenyl-UL
Duration, sex	7 days, male	7 days, female	7 days, male	48 h, male	48 h, male	7 days, female	48 h, female
0.08 h	0.1461	1.9609	5.7059	n.d.	n.d.	7.0855	n.d.
0.16 h	0.2982	0.2633	24.9565	0.6508	0.4520	21.3078	0.3434
0.33 h	0.4204	0.6037	47.0277	0.5565	0.4115	38.3734	0.3361
0.66 h	0.4096	1.2025	71.9156	0.4674	0.2091	41.8074	0.3078
1.0 h	0.3333	0.3420	71.6825	0.4552	0.1300	39.7091	0.2907
1.5 h	0.2936	0.4794	52.8610	0.4587	0.0838	33.5262	0.2246
2.0 h	0.2780	0.2815	32.8290	0.4429	0.0746	21.6089	0.1490
3.0 h	0.2441	0.3181	16.3004	0.3972	0.0718	9.7292	0.0895
4.0 h	0.2039	0.1969	15.2931	0.3405	0.0837	11.1363	0.0770
6.0 h	0.1911	0.3603	16.5916	0.2664	0.0682	14.3737	0.0794
8.0 h	0.1993	0.1556	14.9439	0.2461	0.0685	13.4179	0.0718
24 h	0.0802	0.1234	2.6251	0.0738	0.0170	1.8899	0.0112
32 h	0.0753	0.0797	1.1664	0.0605	0.0123	1.3344	0.0085
48 h	0.0355	0.0451	0.7945	0.0364	0.0078	0.4273	0.0032
72 h	0.0208	0.0302	0.3921	----	----	0.2521	----
96 h	0.0153	0.0118	0.2877	----	----	0.1835	----
120 h	0.0094	0.0209	0.2053	----	----	0.1728	----
144 h	0.0069	0.0073	0.1420	----	----	0.1210	----
168 h	0.0047	0.0110	0.0991	----	----	0.1496	----

p.o. = per os, oral; n.d. = not determined (<LOD)
* one radiolabelled dose after 14 (test 12) or 15 (test 18) daily non-labelled doses at 2 mg/kg bw

Table 5.1.1- 4: Time course of radioactivity in the plasma of male and female rats following an oral dose of [triazole-UL-^{14}C]- or [phenyl-UL-^{14}C]Prothioconazole expressed as dose normalised concentration

Test no. Dose, route Experiment ^{14}C-label Duration, sex	Test 1 2 mg/kg, p.o. single low dose triazole-UL 7 days, male	Test 2 2 mg/kg, p.o. single low dose triazole-UL 7 days, female	Test 3 150 mg/kg, p.o. single high dose triazole-UL 7 days, male	Test 9 5 mg/kg, p.o. single low dose phenyl-UL 48 h, male	Test 12 2 mg/kg, p.o.* multiple low dose* phenyl-UL 48 h, male	Test 16 150 mg/kg, p.o. single high dose triazole-UL 7 days, female	Test 18 2 mg/kg, p.o.* multiple low dose* phenyl-UL 48 h, female
0.08 h	0.0754	0.9623	0.0390	n.d.	n.d.	0.0503	n.d.
0.16 h	0.1538	0.1294	0.1708	0.1235	0.2904	0.1395	0.2030
0.33 h	0.2163	0.2956	0.3219	0.1074	0.2644	0.2520	0.1984
0.66 h	0.2103	0.5961	0.4920	0.0903	0.1344	0.2743	0.1831
1.0 h	0.1714	0.1678	0.4903	0.0880	0.0836	0.2606	0.1735
1.5 h	0.1512	0.2360	0.3623	0.0888	0.0533	0.2197	0.1346
2.0 h	0.1432	0.1385	0.2257	0.0856	0.0475	0.1416	0.0894
3.0 h	0.1257	0.1565	0.1124	0.0768	0.0457	0.0639	0.0535
4.0 h	0.1050	0.0972	0.1055	0.0658	0.0536	0.0729	0.0456
6.0 h	0.0982	0.1768	0.1140	0.0515	0.0436	0.0940	0.0472
8.0 h	0.1024	0.0762	0.1029	0.0476	0.0438	0.0881	0.0426
24 h	0.0412	0.0607	0.0181	0.0143	0.0109	0.0124	0.0066
32 h	0.0387	0.0393	0.0149	0.0117	0.0079	0.0087	0.0050
48 h	0.0182	0.0222	0.0055	0.0070	0.0050	0.0028	0.0019
72 h	0.0107	0.0149	0.0027	----	----	0.0017	----
96 h	0.0079	0.0058	0.0020	----	----	0.0012	----
120 h	0.0048	0.0103	0.0014	----	----	0.0011	----
144 h	0.0036	0.0036	0.0010	----	----	0.0008	----
168 h	0.0024	0.0054	0.0007	----	----	0.0010	----

p.o. = per os, oral; n.d. = not determined (<LOD)
* one radiolabelled dose after 14 (test 12) or 15 (test 18) daily non-labelled doses at 2 mg/kg bw

C. Distribution

The time course of dose normalised concentrations of radioactivity in plasma was comparable for the tested treatment regimes (Table 5.1.1- 4). Although some minor differences between the time courses were evident from 24 hours after administration, they were of minor relevance because the major part of radioactivity had already been eliminated at that time. The radioactivity was partially subjected to enterohepatic circulation, as shown by oscillations in the plasma radioactivity concentrations. The effect was more pronounced in female animals. Plasma concentrations of radioactivity reached peak values 0.1 – 0.7 hours after oral administration, as shown by the experimental plasma concentrations of radioactivity and by calculated tmax values in the range 0.2 - 0.7 hours (The radiolabelled residues at sacrifice in the whole body were in the range 0.1 - 1.5% of administered dose for the animals sacrificed 168 hours after treatment with [triazole-UL-^{14}C]prothioconazole (tests 1, 2, 3 and 16) and in the range 0.83 – 5.8% for the animals sacrificed 48 hours after treatment with [phenyl-UL-^{14}C]Prothioconazole (tests 9, 12, and 18) (see Table 5.1.1- 6). Most of the residual radioactivity was detected in the gastrointestinal tract, liver, and kidneys. Consequently, < 0.5% of the administered dose occurred in all other organs and tissues. Dose normalised concentrations of < 0.02 were found in all organs and tissues, except in the organs involved in absorption, metabolism, and excretion, and the thyroid gland (Table 5.1.1- 7). The gastrointestinal tract, liver, and kidneys showed distinctly higher concentrations at sacrifice. They had values of up to 0.6 for liver at 48 hours post-treatment. Females showed lower concentrations of 0.03 in the liver than males. The thyroid had dose normalised concentrations at

sacrifice below ca. 0.06, with the males showing lower values than females. In most tests, the amount of the radioactivity in the thyroid gland was below the limit of detection (0.01 - 0.06). These results are in good agreement with the results obtained by whole body autoradiography ([redacted], K.; 2001; M-034660-01-1).

Table 5.1.1- 5). Maximum measured plasma concentrations were in the range 0.34 - 72 µg/mL (Table 5.1.1- 3), calculated plasma Cmax values were in the range 0.35 - 70 µg/mL. Maximum dose normalised concentrations were in the range of 0.1 – 1, this large variation is due to the occurrence of enterohepatic re-circulation. Comparison of AUC values for single 2 mg/kg doses (6.31 and 8.43 µg/mL*h), the single 5 mg/kg dose (5.84 µg/mL*h), single 150 mg/kg doses (358 and 249 µg/mL*h) and repeated 2 mg/kg doses (1.77 and 1.67 µg/mL*h) did not indicate a treatment regimen-related change in biokinetic behaviour. The mean residence times (MRT) were short for the tested treatment regimes, and ranged from 10 – 25 hours and clearance values (CL) ranged from 4 - 20 mL/min/kg. The 3-compartment model yielded two elimination half-lives for each treatment regimen, the first elimination half-lives [$t_{1/2}$ e(1)] ranged from 0.4 - 0.9 hours and the second ones [$t_{1/2e}$(2)] ranged from 8 - 19 hours. Thus, there was efficient elimination of radioactivity.

The radiolabelled residues at sacrifice in the whole body were in the range 0.1 - 1.5% of administered dose for the animals sacrificed 168 hours after treatment with [triazole-UL-^{14}C]prothioconazole (tests 1, 2, 3 and 16) and in the range 0.83 – 3.8% for the animals sacrificed 48 hours after treatment with [phenyl-UL-^{14}C]Prothioconazole (tests 9, 12, and 18) (see Table 5.1.1- 6). Most of the residual radioactivity was detected in the gastrointestinal tract, liver, and kidneys. Consequently, < 0.5% of the administered dose occurred in all other organs and tissues. Dose normalised concentrations of < 0.02 were found in all organs and tissues, except in the organs involved in absorption, metabolism, and excretion, and the thyroid gland (Table 5.1.1- 7). The gastrointestinal tract, liver, and kidneys showed distinctly higher concentrations at sacrifice. They had values of up to 0.6 for liver at 48 hours post-treatment. Females showed lower concentrations of 0.03 in the liver than males. The thyroid had dose normalised concentrations at sacrifice below ca. 0.06, with the males showing lower values than females. In most tests, the amount of the radioactivity in the thyroid gland was below the limit of detection (0.01 - 0.06). These results are in good agreement with the results obtained by whole body autoradiography ([redacted]; 2001; M-034660-01-1).

Table 5.1.1- 5: Pharmacokinetic parameters of [triazole-UL-^{14}C]- or [phenyl-UL-^{14}C]-Prothioconazole after oral administration to male and female rats, derived from plasma curve analysis

Test no. **Dose, route** **Experiment** **^{14}C-label** **Duration, sex**	**Test 1** **2 mg/kg, p.o.** **single low dose** **triazole-UL** **7 days, male**	**Test 2** **2 mg/kg, p.o.** **single low dose** **triazole-UL** **7 days, female**	**Test 3** **150 mg/kg, p.o.** **single high dose** **triazole-UL** **7 days, male**	**Test 9** **5 mg/kg, p.o.** **single low dose** **phenyl-UL** **48 h, male**	**Test 12** **2 mg/kg, p.o.*** **multiple low dose*** **phenyl-UL** **48 h, male**	**Test 16** **150 mg/kg, p.o.** **single high dose** **triazole-UL** **7 days, female**	**Test 18** **2 mg/kg, p.o.*** **multiple low dose*** **phenyl-UL** **48 h, female**
AUC [µg/mL*h]	6.31	8.43	358	5.84	1.77	249	1.67
$t_{1/2\ abs}$ [h]	0.172	0.233	0.180	0.056	0.0784	0.082	0.011
$t_{1/2\ elim(1)}$ [h]	0.926	0.499	0.404	0.446	0.597	0.350	0.424
$t_{1/2\ elim(2)}$ [h]	16.8	18.7	9.83	8.08	11.9	9.16	8.91
$t_{lag\ abs}$ [h]	0.034	0.133	0.065	0.052	0.025	0.046	0.001
CL [mL/min/kg bw]	5.28	3.96	6.99	14.30	18.83	10.00	19.90
CL_R [mL/min/kg bw]	0.57	0.67	0.26	0.68	0.95	1.19	2.08
C_{max} (calc.) [µg/mL]	0.43	0.92	69.80	0.65	0.47	45.00	0.35
t_{max} (calc.) [h]	0.43	0.52	0.71	0.18	0.21	0.63	0.38

C_{max} (exp.) [µg/mL]	0.42	1.96	71.92	0.65	0.45	41.80	0.34
t_{max} (exp.) [h]	0.33	0.08	0.66	0.16	0.16	0.66	0.16
MRT [h]	23.5	25.3	11.3	11.7	15.3	11.3	10.4
MRT_{abs} [h]	0.28	0.47	0.65	0.13	0.31	0.55	0.61
MRT_{disp} [h]	23.2	24.9	10.7	11.5	15.0	10.7	9.5

CL_R = CL x renal excretion

Table 5.1.1- 6: Radioactive residues in organs and tissues at sacrifice expressed as % dose administered

Test no.	Test 1	Test 2	Test 3	Test 9	Test 12	Test 16	Test 18
Dose, route	2 mg/kg, p.o.	2 mg/kg, p.o.	150 mg/kg, p.o.	5 mg/kg, p.o.	2 mg/kg, p.o.*	150 mg/kg, p.o.	2 mg/kg, p.o.*
Experiment	single low dose	single low dose	single high dose	single low dose	multiple low dose*	single high dose	multiple low dose*
^{14}C-label	triazole-UL	triazole-UL	triazole-UL	phenyl-UL	phenyl-UL	triazole-UL	phenyl-UL
Duration, sex	7 days, male	7 days, female	7 days, male	48 h, male	48 h, male	7 days, female	48 h, female
Erythrocytes	0.0253+	0.0094+	0.0085+	0.0208+	0.0206+	0.0025+	0.0055+
Plasma	0.0018+	0.0028+	0.0007+	0.0067+	0.0048+	0.0004+	0.0014+
Spleen	0.0010	0.0013	0.0004	0.0013	0.0013	0.0004	0.0007
GIT	0.1268	0.0709	0.0188	3.1430	0.9244	0.0166	0.4622
Liver	1.1340	0.0486	0.0751	2.2290	2.6200	0.0121	0.1145
Kidney	0.0154	0.0138	0.0028	0.0307	0.0334	0.0023	0.0119
Perirenal fat	0.0005+	0.0004+	<LOD	0.0016+	0.0010+	0.0011+	0.0014+
Testis	0.0022	----	0.0007	0.0018	0.0020	----	----
Uterus	----	0.0013	----	----	----	0.0012	0.0011
Skel. Muscle	0.0012+	0.0012+	<LOD	0.0010+	0.0013+	0.0012	0.0010
Bone femur	0.0009+	<LOD+	<LOD	0.0005+	0.0005+	<LOD	0.0003
Heart	0.0013	0.0015	0.0006	0.0012	0.0010	<LOD	0.0008
Lung	0.0058	0.0106	0.0015	0.0055	0.0064	0.0021	0.0033
Brain	0.0008	0.0009	<LOD	0.0005	0.0008	<LOD	0.0006
Skin	0.0746	0.0624	<LOD	0.0485	0.0491	<LOD	0.0226
Carcass	0.1495	0.1790	<LOD	0.3260	0.1570	0.1179	0.2062
Adrenal gland	<LOD	<LOD	<LOD	0.0001	<LOD	0.0003	0.0002
Thyroid gland	<LOD	<LOD	<LOD	0.0001	<LOD	0.0002	0.0003
Ovaries	----	<LOD	----	----	----	0.0003	0.0003
GIT	0.1268	0.0709	0.0188	3.1430	0.9244	0.0166	0.4622
Body w/o GIT	1.4140	0.3207	0.0897	2.6750	2.8990	0.0943	0.3671
Total Body	1.5410	0.3917	0.1085	5.8180	3.8240	0.1109	0.8293

p.o. = per os, oral; n.d. = not determined (<LOD)

* one radiolabelled dose after 14 (test 12) or 15 (test 18) daily non-labelled doses at 2 mg/kg bw

+ Of these organs/tissues only a part was sampled at sacrifice. % of dose administered is relating to the part of organ/tissue sampled and analysed. The contribution of the part not sampled is included in the value for the carcass.

Table 5.1.1- 7: Radioactive residues in organs and tissues at sacrifice expressed as dose normalised concentration

Test no.	Test 1	Test 2	Test 3	Test 9	Test 12	Test 16	Test 18
Dose, route	2 mg/kg, p.o.	2 mg/kg, p.o.	150 mg/kg, p.o.	5 mg/kg, p.o.	2 mg/kg, p.o.*	150 mg/kg, p.o.	2 mg/kg, p.o.*
Experiment	single low dose	single low dose	single high dose	single low dose	multiple low dose*	single high dose	multiple low dose*
^{14}C-label	triazole-UL	triazole-UL	triazole-UL	phenyl-UL	phenyl-UL	triazole-UL	phenyl-UL
Duration, sex	7 days, male	7 days, female	7 days, male	48 h, male	48 h, male	7 days, female	48 h, female
Erythrocytes	0.0126	0.0072	0.0054	0.0122	0.0136	0.0020	0.0042
Plasma	0.0022	0.0033	0.0007	0.0069	0.0050	0.0004	0.0016
Spleen	0.0043	0.0054	0.0015	0.0063	0.0056	0.0015	0.0029
GIT	0.0126	0.0067	0.0016	0.4247	0.0763	0.0021	0.0427
Liver	0.2476	0.0133	0.0169	0.2960	0.6048	0.0037	0.0295
Kidney	0.0197	0.0204	0.0037	0.0503	0.0475	0.0031	0.0178
Perirenal fat	0.0016	0.0012	<LOD	0.0043	0.0030	0.0052	0.0054
Testis	0.0016	----	0.0005	0.0020	0.0017	----	----
Uterus	----	0.0033	----	----	----	0.0029	0.0039
Skel. Muscle	0.0021	0.0017	<LOD	0.0014	0.0016	0.0015	0.0012
Bone femur	0.0029	<LOD	<LOD	0.0019	0.0020	<LOD	0.0010
Heart	0.0036	0.0028	0.0015	0.0039	0.0034	<LOD	0.0023
Lung	0.0089	0.0067	0.0024	0.0119	0.0147	0.0035	0.0060
Brain	0.0012	0.0012	<LOD	0.0012	0.0012	<LOD	0.0008
Skin	0.0035	0.0031	<LOD	0.0023	0.0025	<LOD	0.0011
Carcass	0.0028	0.0033	<LOD	0.0056	0.0029	0.0020	0.0037
Adrenal gland	<LOD	<LOD	<LOD	0.0083	<LOD	0.0075	0.0065
Thyroid gland	<LOD	<LOD	<LOD	0.0248	<LOD	0.0567	0.0571
Ovaries	----	<LOD	----	----	----	0.0040	0.0044
GIT	0.0126	0.0067	0.0016	0.4247	0.0763	0.0021	0.0427
Body w/o GIT	0.0157	0.0036	0.0010	0.0289	0.0330	0.0010	0.0041
Total Body	0.0154	0.0039	0.0011	0.0582	0.0382	0.0011	0.0083

p.o. = per os, oral; n.d. = not determined (<LOD)
* one radiolabelled dose after 14 (test 12) or 15 (test 18) daily non-labelled doses at 2 mg/kg bw

D. Excretion

The expiration of ^{14}C-carbon dioxide and other ^{14}C-labelled volatile compounds amounted to 0.06% of the administered dose in the pilot experiment (test 8, expired air test) during the 48 hours following a single oral administration of 2 mg/kg [phenyl-UL-^{14}C]prothioconazole (Table 5.1.1- 2). This demonstrates the high stability of the phenyl labelling position for possible formation of volatile products.

The excretion of radioactivity was almost complete within 48 hours of oral administration of [triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole (Table 5.1.1- 8). Approximately 90 - 100% of the administered dose had been excreted with urine, faeces, or bile at the time of sacrifice, 48 or 168 hours post treatment, and less than 6% of the administered dose remained in the body, including the gastrointestinal tract (Table 5.1.1- 2). At sacrifice 6 hours post-treatment, the total excretion in the bile-duct cannulation test with [phenyl-UL-^{14}C]prothioconazole was slightly lower at approximately 85% (The other bile-duct cannulation test with [triazole-UL-^{14}C]prothioconazole showed 94% excretion at sacrifice 48 hours post-treatment). The course of excretion for all treatment regimens was similar (Table 5.1.1- 8) with the exception of the bile-duct cannulation experiments where excretion was fastest. At sacrifice, in almost all tests, 78 - 96% of the administered dose had been excreted with the faeces and only 4 -16% in the urine. Renal excretion was slightly higher in female animals (10 - 16%) than in males (4 - 10%). The rats of the bile-duct cannulation tests 4 and 11 excreted 90.2 and 82.2% of the administered dose with the bile at 48 and 6 hours post treatment, respectively with urinary and faecal excretion accounting for 1.5 - 3.3% of the administered dose. Comparison of the bile-duct cannulation tests 4 and 11 showed a slightly accelerated excretion following an intraduodenal administration of 2 mg/kg [phenyl-UL-^{14}C]prothioconazole in test 4, probably due to intraduodenal administration, in contrast to oral dosage in test 11.

Table 5.1.1- 8: Cumulative excretion of radioactivity at time intervals expressed as % dose administered

Test no. Dose, route Experiment ^{14}C-radiolabel Duration, sex	Test 1 2 mg/kg, p.o. single low dose triazole-UL 7 days, male	Test 2 2 mg/kg, p.o. single low dose triazole-UL 7 days, female	Test 3 150 mg/kg, p.o. single high dose triazole-UL 7 days, male	Test 4 2 mg/kg, i. duo. bile-duct cannulation triazole-UL 48 h, male	Test 8 2 mg/kg, p.o. expired air test phenyl-UL 48 h, male
Expired air					
8	----	----	----	----	0.04
24	----	----	----	----	0.06
48	----	----	----	----	0.06
Urine					
1	----	----	----	0.38	----
2	----	----	----	0.42	----
3	----	----	----	0.50	----
4	0.99	2.81	0.39	1.22	1.18
6	----	----	----	1.62	----
8	2.66	4.82	0.75	1.69	2.55
24	7.23	12.72	2.43	1.93	5.29
32	----	----	----	2.00	----
48	9.17	14.92	3.22	2.05	5.90
72	9.79	15.47	3.49	----	----
96	10.06	15.70	3.59	----	----
120	10.24	15.81	3.64	----	----
144	10.36	15.90	3.67	----	----
168	10.47	15.97	3.71	----	----
Bile					
1	----	----	----	74.49	----
2	----	----	----	81.83	----
3	----	----	----	84.88	----
4	----	----	----	86.27	----
6	----	----	----	87.63	----
8	----	----	----	88.22	----
24	----	----	----	89.60	----
32	----	----	----	89.87	----
48	----	----	----	90.21	----
Faeces					
6	----	----	----	----	----
24	71.65	53.53	80.12	1.22	73.33
48	81.09	73.28	93.89	1.28	80.59
72	82.42	75.80	94.82	----	----
96	82.88	76.31	94.97	----	----
120	83.30	76.46	95.03	----	----
144	83.50	76.54	95.06	----	----
168	84.49	78.40	95.88	----	----
Sum	94.96	94.37	99.59	93.54	86.56

Table 5.1.1- 8: Cumulative excretion of radioactivity at time intervals expressed as % dose administered (continued)

Test no.	Test 9	Test 11	Test 12	Test 16	Test 18
Dose, route	5 mg/kg, p.o.	2 mg/kg, p.o.	2 mg/kg, p.o.*	150 mg/kg, p.o.	2 mg/kg, p.o.*
Experiment	single low dose	bile-duct cannulation	multiple low dose*	single high dose	multiple low dose*
^{14}C-radiolabel	phenyl-UL	phenyl-UL	phenyl-UL	triazole-UL	phenyl-UL
Duration, sex	48 h, male	6 h, male	48 h, male	7 days, female	48 h, female
Expired air					
8	----	----	----	----	----
24	----	----	----	----	----
48	----	----	----	----	----
Urine					
1	----	----	----	----	----
2	----	----	----	----	----
3	----	0.27	----	----	----
4	0.51	----	1.62	2.11	2.61
6	----	1.45	----	----	----
8	1.17	----	2.33	3.69	3.47
24	3.53	----	3.83	10.05	9.38
32	----	----	----	----	----
48	4.57	----	5.14	11.45	10.24
72	----	----	----	11.66	----
96	----	----	----	11.74	----
120	----	----	----	11.77	----
144	----	----	----	11.79	----
168	----	----	----	11.81	----
Bile					
1	----	----	----	----	----
2	----	----	----	----	----
3	----	68.24	----	----	----
4	----	----	----	----	----
6	----	82.17	----	----	----
8	----	----	----	----	----
24	----	----	----	----	----
32	----	----	----	----	----
48	----	----	----	----	----
Faeces					
6	----	2.52	----	----	----
24	67.95	----	81.28	71.60	75.89
48	85.37	----	93.22	87.13	86.80
72	----	----	----	87.56	----
96	----	----	----	87.66	----
120	----	----	----	87.71	----
144	----	----	----	87.74	----
168	----	----	----	87.76	----
Sum	89.94	84.85	98.36	99.57	97.05

* one radiolabelled dose after 14 (test 12) or 15 (test 18) daily non-labelled doses at 2 mg/kg bw

E. Metabolism

[Triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]prothioconazole were intensively metabolised. Eighteen metabolites, including the parent compound, were identified in urine, faeces, and bile (Table 5.1.1- 9). The total identification rate in most tests was between ca. 47 and 63% of the administered dose, but in 3 tests the identification rate was between ca. 26 and 39% of the administered dose. A major peak group of unidentified metabolites in the faeces extract could not be resolved. Based on the comparison with retention times of reference compounds and other metabolites, these metabolites were characterised as a group of hydroxylated and/or conjugated faecal metabolites.

The proposed biotransformation pathway of prothioconazole in the rat is shown in Figure 5.1.1- 1. The unchanged parent compound was found at ca. 1 – 22% of the administered dose. The major metabolic reaction was conjugation with glucuronic acid at the sulfur atom of prothioconazole. Thus, the overall most abundant metabolite was JAU6476-S-glucuronide (prothioconazole-S-glucuronide, M06), which was found at ca. 46% of the administered dose in the bile and up to 7.7% in the urine.

In the urine of the female rats, JAU 6476-S-glucuronide (M06) was the most abundant metabolite at 3.9 - 7.7% of the administered dose, but was it found only in very minor amounts of ca. 0.1% in the urine of the males. Thus, JAU 6476-S-glucuronide represented the only metabolite with a sex-dependent occurrence in urine. However, it occurred in the bile of the male rats.

In this ADME rat study, the glucuronide of prothioconazole was reported as an S- or O-glucuronide, the position of the conjugation could not unambiguously be determined. The same metabolite was found in the goat metabolism studies. This was demonstrated in the concurrently conducted goat metabolism study with [phenyl-UL-^{14}C]prothioconazole by co-chromatography experiments with samples from the rat study ([redacted], H.; [redacted] K.; 2004; M-034900-01-1). In the later conducted goat metabolism study with [triazole-UL-^{14}C]prothioconazole the exact structure was determined as the JAU6476-S-glucuronide by 2D-NMR-spectroscopic experiments ([redacted] E.; [redacted] H.; [redacted], K.; 2005; M-116219-02-1). Thus it was demonstrated that in the goat and also in the rat the glucuronidation took place at the sulfur atom of the molecule.

Another major metabolite was JAU6476-desthio (prothioconazole-desthio, M04) found at ca. 0.4 – 18% of the administered dose. JAU6476-desthio was found almost exclusively in the faeces and only to a very minor extent systemically, up to 0.07% in the urine and 0.35% - 0.45% in the bile.

Several hydroxylated metabolites were identified, some of which were conjugated with glucuronic acid. Solely detectable by the [triazole-UL-^{14}C] label, 1,2,4-triazole (M13) was found in urine at up to 2.3% of the administered dose. This was the only distinct difference between the tests conducted with the [triazole-UL-^{14}C] and [phenyl-UL-^{14}C]-labelled prothioconazole. A metabolite without the triazole moiety was not identified in the experiments with [phenyl-UL-^{14}C]prothioconazole. All metabolites present in the total excreta at ≥ 5% of the administered dose and many other metabolites representing < 5% of the administered dose were identified.

In summary, after absorption the main biotransformation route of prothioconazole is conjugation with glucuronic acid at the sulfur atom. The formed metabolite JAU6476-S-glucuronide is excreted rapidly with the bile, and thus the systemic cleavage of the sulfur atom, i.e. the systemic formation of JAU6476-desthio is inhibited. The major systemic formation and excretion of the metabolite JAU6476-S-glucuronide in conjunction with the negligible systemic formation of JAU6476-desthio is interpreted as the "mechanism of detoxification" of prothioconazole in the rat.

Table 5.1.1- 9: Balance of [triazole-UL-^{14}C]- and [phenyl-UL-^{14}C]-Prothioconazole and metabolites excreted expressed as % dose administered

Test no. Dose, route Experiment ^{14}C-radiolabel Duration, sex	Test 1 2 mg/kg, p.o. single low dose triazole-UL 7 days, male			Test 2 2 mg/kg, p.o. single low dose triazole-UL 7 days, female			Test 3 150 mg/kg, p.o. single high dose triazole-UL 7 days, male		
Excreta	**Urine**	**Faeces**	**Total**	**Urine**	**Faeces**	**Total**	**Urine**	**Faeces**	**Total**
Prothioconazole (JAU 6476)	-	1.35	1.35	0.47	21.10	21.56	0.04	22.33	22.37
JAU 6476-desthio-3-hydroxy (M14)	-	1.44	1.44	-	1.06	1.06	-	1.76	1.76
JAU 6476-desthio-4,5-dihydroxy (M35)	-	5.03	5.03	-	1.08	1.08	-	1.15	1.15
JAU6476-4-hydroxy (M08)	-	0.44	0.44	-	1.48	1.48	-	2.57	2.57
JAU 6476-desthio-4-hydroxy (M15) [+]	-	5.34	5.34	-	2.61	2.61	-	2.39	2.39
JAU6476-desthio (M04)	-	3.45	3.45	0.07	13.20	13.27	0.02	13.50	13.52
JAU6476-desthio-dihydroxy-diene (M32/38)	0.76	-	0.76	1.36	-	1.36	0.25	-	0.25
JAU6476-desthio-dihydroxy-dienyl-glucuronide (M73)	0.81	-	0.81	0.20	-	0.20	0.22	-	0.22
JAU6476-desthio-dihydroxy (M34)	-	3.57	3.57	-	1.24	1.24	-	0.84	0.84
JAU6476-desthio-hydroxy-glucuronide (M75), JAU6476-desthio-dihydroxy-glucuronide (M72) and JAU6476-desthio-hydroxy-methoxy-glucuronide (M49)	-	-	-	-	-	-	-	-	-
JAU6476-N-glucuronide (M05)	-	1.33	1.33	-	4.44	4.44	-	7.72	7.72
JAU6476-S-methyl (M01)	-	-	-	-	1.63	1.63	-	0.35	0.35
JAU6476-S-glucuronide (M06)	-	-	-	4.49	-	4.49	0.09	-	0.09
JAU6476-triazolinone (M03)	-	0.33	0.33	-	0.56	0.56	-	0.65	0.65
1,2,4-triazole (M13)	2.29	-	2.29	0.80	-	0.80	0.87	-	0.87
Total identified	3.85	22.29	26.14	7.40	48.39	55.79	1.49	53.27	54.75
Sum of unknowns (largest unknown)	4.09 (0.85)	4.37 (1.59)	8.46 (1.59)	6.80 (1.60)	6.17 (3.07)	12.96 (3.07)	0.37 (0.14)	0.93 (0.48)	1.29 (0.48)
Hydroxylated and/or conjugated faecal metabolites	-	34.47	34.47	-	3.50	3.50	-	12.83	12.83
Total characterised	4.09	38.84	42.93	6.80	9.67	16.46	0.37	13.76	14.13
Total identified and characterised	7.95	61.13	69.07	14.19	58.06	72.25	1.85	67.02	68.88
Total radioactivity excreted	10.47	84.49	94.96	15.97	78.40	94.37	3.71	95.88	99.59

[+] The metabolite JAU6476-desthio-hydroxy-methoxy (M28) was identified as a minor component of the fraction containing JAU6476-4-hydroxy-desthio (M15).

Table 5.1.1- 9: Balance of [triazole-UL-14C]- and [phenyl-UL-14C]-Prothioconazole and metabolites excreted expressed as % dose administered (continued)

Test no.	Test 9			Test 12			Test 16		
Dose, route	5 mg/kg, p.o.			2 mg/kg, p.o.*			150 mg/kg, p.o.		
Experiment	single low dose			multiple low dose*			single high dose		
^{14}C-radiolabel	phenyl-UL			phenyl-UL			triazole-UL		
Duration, sex	48 h, male			48 h, male			7 days, female		
Excreta	**Urine**	**Faeces**	**Total**	**Urine**	**Faeces**	**Total**	**Urine**	**Faeces**	**Total**
Prothioconazole (JAU 6476)	-	10.59	10.59	-	13.14	13.14	1.04	19.43	20.47
JAU 6476-desthio-3-hydroxy (M14)	-	1.07	1.07	-	2.19	2.19	-	1.84	1.84
JAU 6476-desthio-4,5-dihydroxy (M35)	-	2.93	2.93	-	5.08	5.08	-	0.92	0.92
JAU6476-4-hydroxy (M08)	-	0.67	0.67	-	1.01	1.01	-	2.72	2.72
JAU 6476-desthio-4-hydroxy (M15)$^{+}$	-	2.31	2.31	-	5.49	5.49	-	2.04	2.04
JAU6476-desthio (M04)	-	6.74	6.74	-	3.68	3.68	-	17.71	17.71
JAU6476-desthio-dihydroxy-diene (M32/38)	0.71	-	0.71	0.51	-	0.51	0.58	-	0.58
JAU6476-desthio-dihydroxy-dienyl-glucuronide (M73)	0.47	-	0.47	0.18	-	0.18	-	-	-
JAU6476-desthio-dihydroxy (M34)	-	1.99	1.99	-	2.72	2.72	-	1.17	1.17
JAU6476-desthio-hydroxy-glucuronide (M75); JAU6476-desthio-dihydroxy-glucuronide (M72) and JAU6476-desthio-hydroxy-methoxy-glucuronide (M49)	-	-	-	-	-	-	-	-	-
JAU6476-N-glucuronide (M05)	-	2.01	2.01	-	3.03	3.03	-	8.16	8.16
JAU6476-S-methyl (M01)	-	0.70	0.70	-	1.00	1.00	-	-	-
JAU6476-S-glucuronide (M06)	-	-	-	-	-	-	7.73	-	7.73
JAU6476-triazolinone (M03)	-	0.41	0.41	-	0.53	0.53	-	-	-
1,2,4-triazole (M13)	-	-	-	-	-	-	-	-	-
Total identified	1.18	29.42	30.61	0.70	37.89	38.59	9.35	53.99	63.34
Sum of unknowns (largest unknown)	0.48 (0.39)	2.31 (0.81)	2.78 (0.81)	-	1.93 (0.86)	1.93 (0.86)	1.23 (0.50)	7.34 (2.90)	8.57 (2.90)
Hydroxylated and/or conjugated faecal metabolites	-	30.53	30.53	-	26.05	26.05	-	-	-
Total characterised	0.48	32.84	33.31	-	27.98	27.98	1.23	7.34	8.57
Total identified and characterised	1.66	62.26	63.92	0.70	65.87	66.57	10.59	61.32	71.91
Total radioactivity excreted	4.57	85.37	89.94	5.14	93.22	98.36	11.81	87.76	99.57

* one radiolabelled dose after 14 daily non-labelled doses at 2 mg/kg bw

$^{+}$ The metabolite JAU6476-desthio-hydroxy-methoxy (M28) was identified as a minor component of the fraction containing JAU6476-4-hydroxy-desthio (M15).

Table 5.1.1- 9: Balance of [triazole-UL-14C]- and [phenyl-UL-14C]-Prothioconazole and metabolites excreted expressed as % dose administered (continued)

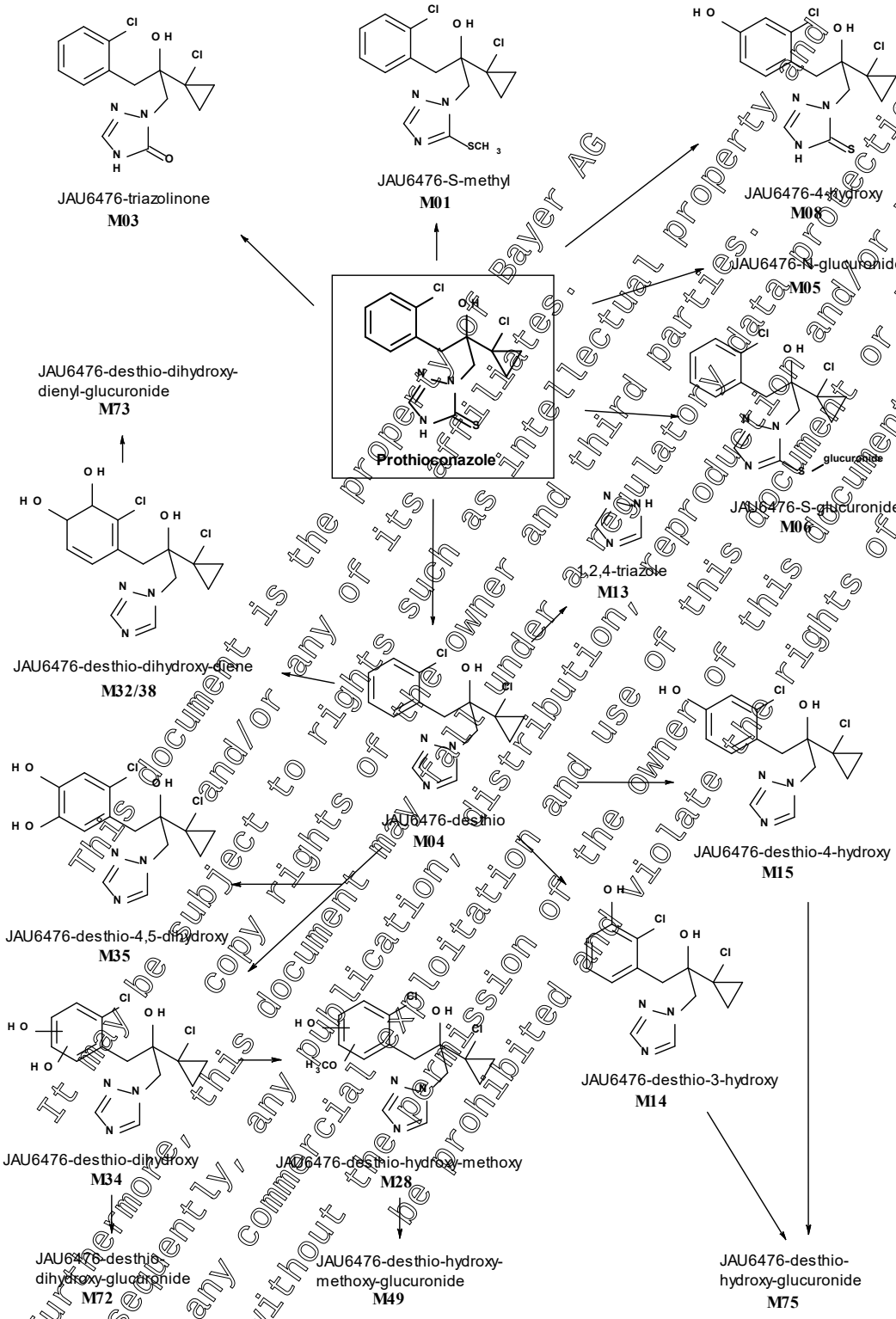
Test no. Dose, route Experiment ^{14}C-radiolabel Duration, sex	Test 18 2 mg/kg, p.o.* multiple low dose* phenyl-UL 48 h, female			Test 4 2 mg/kg, i. duo. bile-duct cannulation triazole-UL 48 h, male	Test 11 2 mg/kg, p.o. bile-duct cannulation phenyl-UL 6 h, male
Excreta	**Urine**	**Faeces**	**Total**	**Bile**	**Bile**
Prothioconazole (JAU 6476)	0.86	9.92	10.78	4.63	3.42
JAU 6476-desthio-3-hydroxy (M14)	-	1.41	1.41	-	-
JAU 6476-desthio-4,5-dihydroxy (M35)	-	4.47	4.47	-	-
JAU6476-4-hydroxy (M08)	-	0.99	0.99	-	-
JAU 6476-desthio-4-hydroxy (M15)[+]	-	3.55	3.55	-	-
JAU6476-desthio (M04)	-	15.53	15.53	0.35	0.45
JAU6476-desthio-dihydroxy-diene (M32/38)	1.00		1.00		-
JAU6476-desthio-dihydroxy-dienyl-glucuronide (M73)	-	-	-	-	-
JAU6476-desthio-dihydroxy (M34)	-	1.84	1.84	-	-
JAU6476-desthio-hydroxy-glucuronide (M75), JAU6476-desthio-dihydroxy-glucuronide (M72) and JAU6476-desthio-hydroxy-methoxy-glucuronide (M49)	-		-	9.50	7.89
JAU6476-N-glucuronide (M05)	-	2.97	2.97	1.86	2.22
JAU6476-S-methyl (M01)	-			-	-
JAU6476-S-glucuronide (M06)	3.87	-	3.87	45.50	46.59
JAU6476-triazolinone (M03)	-	0.57	0.57	-	-
1,2,4-triazole (M13)	-			-	-
Total identified	5.73	41.26	46.99	61.83	60.57
Sum of unknowns (largest unknown)	1.85 (0.61)	6.01 (0.89)	7.86 (0.89)	18.98 (14.54)[#]	14.51 (10.30)[#]
Hydroxylated and/or conjugated faecal metabolites		10.65	10.65	-	-
Total characterised	1.85	16.66	18.51	18.98	14.51
Total identified and characterised	7.58	57.92	65.51	80.81	75.08
Total radioactivity excreted	10.24	86.80	97.05	90.21	82.17

***** one radiolabelled dose after 15 daily non-labelled doses at 2 mg/kg bw

[#] group of several minor metabolites, each < 5% of the administered dose

[+] The metabolite JAU6476-desthio-hydroxy-methoxy (M28) was identified as a minor component of the fraction containing JAU6476-4-hydroxy-desthio (M15).

Figure 5.1.1- 1: Proposed metabolic pathway of prothioconazole in the rat



III. Conclusions

The kinetic and metabolic behaviour of [phenyl-UL-^{14}C]- and [triazole-UL-^{14}C]prothioconazole in male and female rats after low and high oral (or intraduodenal) dosage can be characterised by the following observations:

- The absorption of prothioconazole was rapid and almost complete. Half-lives of absorption were short ($t_{1/2}$ abs < 0.3 h). The oral absorption was at least approx. 90% of the administered dose. The rate and extent of absorption of the total radioactivity was essentially independent of sex and labelling position. Plasma peak levels were observed within 0.1 - 0.7 hours after dosing.
- From peak levels, the radioactivity concentrations in plasma declined. Prothioconazole and its metabolites were efficiently eliminated of from the body. The calculated first elimination half-lives [t1/2 e(1)] ranged from 0.4 - 0.9 hours and the second elimination half-lives [t1/2e(2)] ranged from 8 - 19 hours. An enterohepatic circulation was observed.
- The excretion of radioactivity was almost complete within 48 hours of oral administration. Approximately 90 - 100% of the administered doses were excreted with urine, faeces, or bile within 7 days of treatment, and less than 6% of the administered dose remained in the body. At sacrifice, 78 - 96% of the administered dose had been excreted with the faeces and 4 - 16% with the urine. Renal excretion was slightly higher in female animals. The biliary excretion was 90.2% of the administered dose at 48 hours post-treatment. Only 0.06% of the administered dose was expired as $^{14}CO_2$ and other ^{14}C-labelled volatile compounds.
- At sacrifice, most of the residual radioactivity was detected in the gastrointestinal tract, liver, and kidneys. Less than 0.5% of the administered dose was found in all other organs and tissues.
- Prothioconazole was intensively metabolised. Eighteen metabolites, including the parent compound, were identified in urine, faeces, and bile. All metabolites present in the total excreta at greater than 5% of the administered dose and many other metabolites representing less than 5% of the administered dose were identified.
- The unchanged parent compound was found at ca. 1 – 22% of the administered dose.
- The major metabolic reaction was conjugation with glucuronic acid at the sulfur atom of prothioconazole. Thus, the overall most abundant metabolite was JAU6476-S-glucuronide (prothioconazole-S-glucuronide, M06), which was found at ca. 46% of the administered dose in the bile and up to 7.7% in the urine.
- Another major metabolite was JAU6476-desthio (prothioconazole-desthio, M04) found at ca. 0.4 – 18% of the administered dose. JAU6476-desthio was found almost exclusively in the faeces and only to a very minor extent systemically, up to 0.07% in the urine and 0.35% - 0.45% in the bile.
- Several hydroxylated metabolites were identified, some of which were conjugated with glucuronic acid. 1,2,4-triazole (M13) was found as minor metabolite in urine.
- In summary, after absorption the main biotransformation route of prothioconazole is conjugation with glucuronic acid at the sulfur atom. The formed metabolite JAU6476-S-glucuronide is excreted rapidly with the bile, and thus the systemic cleavage of the sulfur atom, i.e. the systemic formation of JAU6476-desthio is inhibited. The major systemic formation and excretion of the metabolite JAU6476-S-glucuronide in conjunction with the negligible systemic formation of JAU6476-desthio is interpreted as the "mechanism of detoxification" of prothioconazole in the rat.

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

There is no concern for toxicity following dermal exposure compared with that following oral exposure. The vapour pressure of prothioconazole is << $4x10^{-7}$ Pascal at 20°C. Therefore, studies on prothioconazole by the dermal or inhalation routes are not required.

The two new *in vitro* comparative metabolism studies, using rat and human liver microsomes as well as freshly prepared rat and human hepatocytes, are summarised below.

Report:	KCA 5.1.2/02[redacted]R; 2014; M-505499-01-1
Title:	[Phenyl-UL-14C]prothioconazole: Metabolic stability and profiling in liver microsomes from rats and humans for inter-species comparison
Report No.:	S48308
Document No.:	M-505499-01-1
Guideline(s):	Regulation (EC) No 1107/2009 amended by the Commission Regulation (EU) No. 283/2013 (Europe) US EPA OCSPP not applicable
Guideline deviation(s):	none
GLP/GEP:	**yes**

Executive Summary

The comparative metabolism of [phenyl-UL-^{14}C]prothioconazole was investigated in animal *in vitro* systems by incubating the test substance with liver microsomes from male Wistar rats and humans in the presence of NADPH cofactor. The concentration was 10 µM and the protein concentration 1 mg/mL. This concentration was chosen in order to create enough material for possible identification of metabolites by chromatographic or spectroscopic methods. The sampling times were 0, 0.5 and 1 hour after test start. The test durations of 0.5 and 1 hour were considered reasonable because positive results were obtained from the enzymatic reaction of testosterone to hydroxytestosterone already after 5 minutes. Samples were analysed following protein precipitation by reversed phase HPLC. The metabolic activity of the microsomes was demonstrated by determining 6β-hydroxytestosterone that was formed from testosterone by testosterone 6β-hydroxylase.

The recovery of radioactivity in both microsome incubations amounted to ≥ 97.1% for the 0.5 and 1 hour samples. The results of the tests with ^{14}C-prothioconazole demonstrated that the test substance was stable after incubation with buffer (pH 7.4) at 37 ± 1 °C.

The *in vitro* metabolite profile of ^{14}C-prothioconazole when incubated with liver microsomes was found to be slightly different between rats and humans.

In rat liver microsomes, 61.4% and 56.5% of the initial ^{14}C-prothioconazole remained unchanged after 0.5 h and 1 h incubation, respectively. In this species, ^{14}C-prothioconazole was metabolised towards a high number of metabolites. A total of eleven metabolites were detected; three of them were above 5% of the relative percentage after 1 hour incubation.

In human liver microsomes, a total of seven metabolites were found. The amount of ^{14}C-prothioconazole remaining after 0.5 h and 1 h incubation was considerably higher as compared to the rat liver microsomal system indicating a slower metabolism rate of ^{14}C-prothioconazole in human liver microsomes. From the seven detectable metabolites formed by human liver microsomes, one (Pr-7) was dominating because of its high relative percentage value (from 3.5% to 6.3%). Metabolite Pr-7 was also detected as major metabolite in incubations with rat liver microsomes.

In summary, the metabolic pattern in rat and human liver microsomes was qualitatively comparable and no unique human metabolite was detected.

Materials and Methods

Test Substances

[Phenyl-UL-^{14}C]prothioconazole was used as a test substance. The radiochemical purity was > 98%, the chemical purity was > 99%, both determined by HPLC. The specific radioactivity was 4.75 MBq/mg (128.4 µCi/mg). Nonlabelled prothioconazole with a chemical purity of 99.8% was used as a reference compound. 6β-hydroxytestosterone (chemical purity 99.8%) was used as a metabolite standard in positive metabolism control tests. In these tests, dexamethasone (chemical purity 99.5%) was used as an internal standard.

Test System

Pooled liver microsomes from male Wistar rats (batch 1310003, pool of 200 individuals) and humans (batch 1110189, pool of 50 donors from both genders) were purchased from Xenotech, LLC (USA). Liver microsomes were stored frozen at -80 ± 10°C for not longer than 5 years. Each microsome batch was at least characterised by batch number, protein concentration, total cytochrome P450 content and drug metabolising enzyme activity.

Experimental Procedures

A stock solution of ^{14}C-prothioconazole was prepared at 0.5 mCi/mL by dissolving 21.45 MBq of ^{14}C-prothioconazole with 1.7 mL of acetonitrile. The stock solution was stirred until complete dissolution and stored at - 80 ± 10°C in 5 aliquots of approximately 350 µL in Eppendorf tubes. A molecular weight of 344.26 g/mol was considered for calculation of ^{14}C-prothioconazole dilutions. A different aliquot was used each experimental day. This stock solution was further diluted for the determination of linearity and the lower limit of quantification (LLOQ) as described in detail in the report.

Microsome incubations with ^{14}C-prothioconazole

30 µL of ^{14}C-prothioconazole stock solution were diluted with 648 µL of acetonitrile. This solution was freshly prepared and kept at room temperature until use. A 1mM solution of non-radiolabelled prothioconazole was freshly prepared and kept at room temperature until use.

^{14}C-prothioconazole was incubated separately with rat liver microsomes and human liver microsomes (n=3) at 37 ± 1°C in 100 mM sodium phosphate buffer (pH 7.4, final volume 500 µL). The incubations were performed in a thermomixer device with shaking at 1000 rpm. Microsomes were thawed at room temperature and kept in a tray with ice until use.

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 acetonitrile at room temperature. Final concentrations of the incubates were: 5 mM $MgCl_2$, 1 mg/mL microsome protein; 10 µM ^{14}C-prothioconazole (0.221 µCi/incubate); 1 mM reduced NADPH. Triplicate samples at T=0 were prepared by adding the same components as test samples but in different order (acetonitrile was added prior to NADPH and ^{14}C-prothioconazole was the last compound added).

After incubation of the microsome samples, an aliquot of approximately 50 µL of each incubate was analysed for total radioactivity by liquid scintillation counting. The microsomal incubates were centrifuged at 16000 x g for 15 minutes at 20°C. After centrifugation, 100 µL of each supernatant were diluted with 400 µL of 50 mM ammonium acetate (pH 5.0). The samples were directly analysed by HPLC without any further extraction or purification procedure.

Microsomal Metabolism: Positive Control

The metabolic activity of the microsomes was determined by measurement of 6β-hydroxytestosterone that was formed from testosterone by testosterone 6β-hydroxylase. This biochemical reaction is well-known for CYP3A microsomal enzyme. Testosterone was incubated separately with rat liver and human liver microsomes (n=3) at 37 ± 1°C in sodium phosphate buffer (pH 7.4). The reactions were started by the addition of 10 mM NADPH solution and were quenched after 5 minutes incubation at 37 ± 1°C with 120 µL acetonitrile. The final concentrations were 5 mM $MgCl_2$, 0.15 mg/mL microsome protein, 150 µM testosterone and 1 mM reduced NADPH. After incubation, 20 µL of dexamethasone solution (0.5 µg/mL) and methanol was added to each incubate. The samples were maintained at room temperature until the end of the experiment and were further stored at - 80°C ± 10°C until analysis. 6β-hydroxytestosterone was determined by LC-MS/MS.

Results and Discussion

Positive Metabolism Controls

Formation of 6β-hydroxytestosterone from testosterone demonstrated sufficient metabolic capability of the rat and human liver microsome batches used in the study. Testosterone 6 β-hydroxylase activities were found to be 2193.9 pmol/mg/minute in rat liver microsomes and 3797.8 pmol/mg/minute in pooled human liver microsomes.

Metabolite Profile of ^{14}C-prothioconazole

^{14}C-prothioconazole 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 h.

Incubation with Rat Liver Microsomes

When incubated with rat liver microsomes for 0.5 h and 1 h, 61.4% and 56.5% of ^{14}C-prothioconazole remained unchanged, respectively. In total, 11 metabolites (Pr-1, Pr-2, Pr-3, Pr-4, Pr-5, Pr-6, Pr-7, Pr-8, Pr-9, Pr-10 and Pr-11) were detected. However, it should be noted that not all peaks in the radioactivity detector could be baseline separated. After 0.5 h incubation, two metabolites representing more than 5% of the relative percentage were detected: Pr-7 (5.9%) and Pr-9 (30.2%) and one metabolite (Pr-1) with less than 5% (2.4%). The remaining metabolites were found to be below the LLOQ. Metabolites Pr-4 and Pr-5 were not detected after 0.5 h incubation.

After 1 h incubation a total of ten metabolites were detected. Three of them showed relative percentages above 5%: Pr-1 (6.0%), Pr-7 (6.6%) and Pr-9 (29.6%) and one (Pr-6) less than 5% (1.3%). The remaining metabolites were found to be below the LLOQ.

Incubation with Human Liver Microsomes

When incubated with human liver microsomes for 0.5 h and 1 h, 94.5% and 89.6% of ^{14}C-prothioconazole remained unchanged, respectively. These percentages were remarkably higher as compared with the ones in the samples incubated with rat liver microsomes. In total, seven metabolites (Pr-2, Pr-3, Pr-6, Pr-7, Pr-8, Pr-9 and Pr-11) were detected.

After 0.5 h incubation, two metabolites (Pr-7 and Pr-9) showed relative percentages of 3.5% and 1.9%, respectively and the remaining metabolites were found to be below the LLOQ.

After 1 h incubation, only metabolite Pr-7 represented more than 5% of the relative percentage (6.3%) and two (Pr-2 and Pr-9) less than 5% (0.7% and 3.4%). All remaining metabolites were found to be below the LLOQ.

With the exception of metabolite Pr-1 that was detected in the 1 h incubation with rat liver microsomes in a slightly higher amount than 5% the main metabolites (Pr-7 and Pr-9) were qualitatively identical in rat and human liver microsomes.

All other qualitative differences in the metabolic patterns were insignificant because of the very small quantities of the individual components.

The HPLC chromatograms of the rat and human liver microsomal incubations are shown below in Figures 5.1.2-1 and 5.1.2-2, respectively.

Figure 5.1.2-1: HPLC-radioactivity profiles of ^{14}C-prothioconazole metabolism in rat liver microsomes.

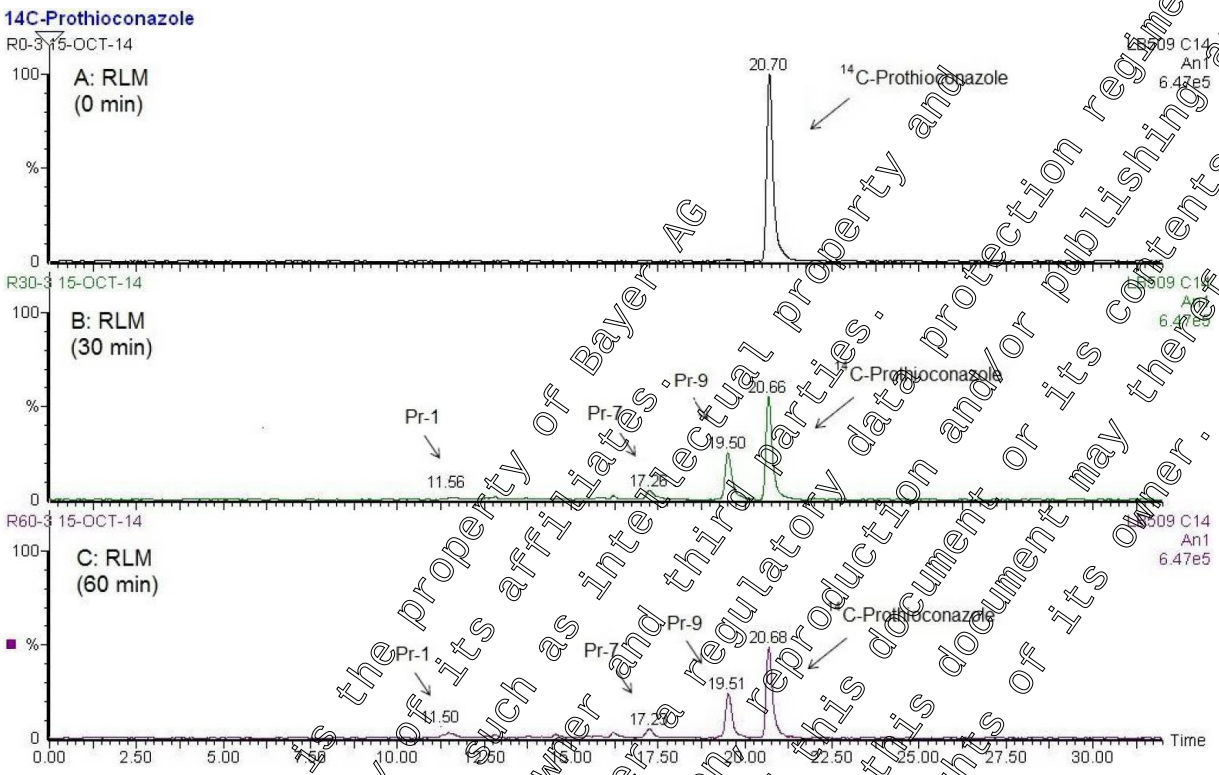
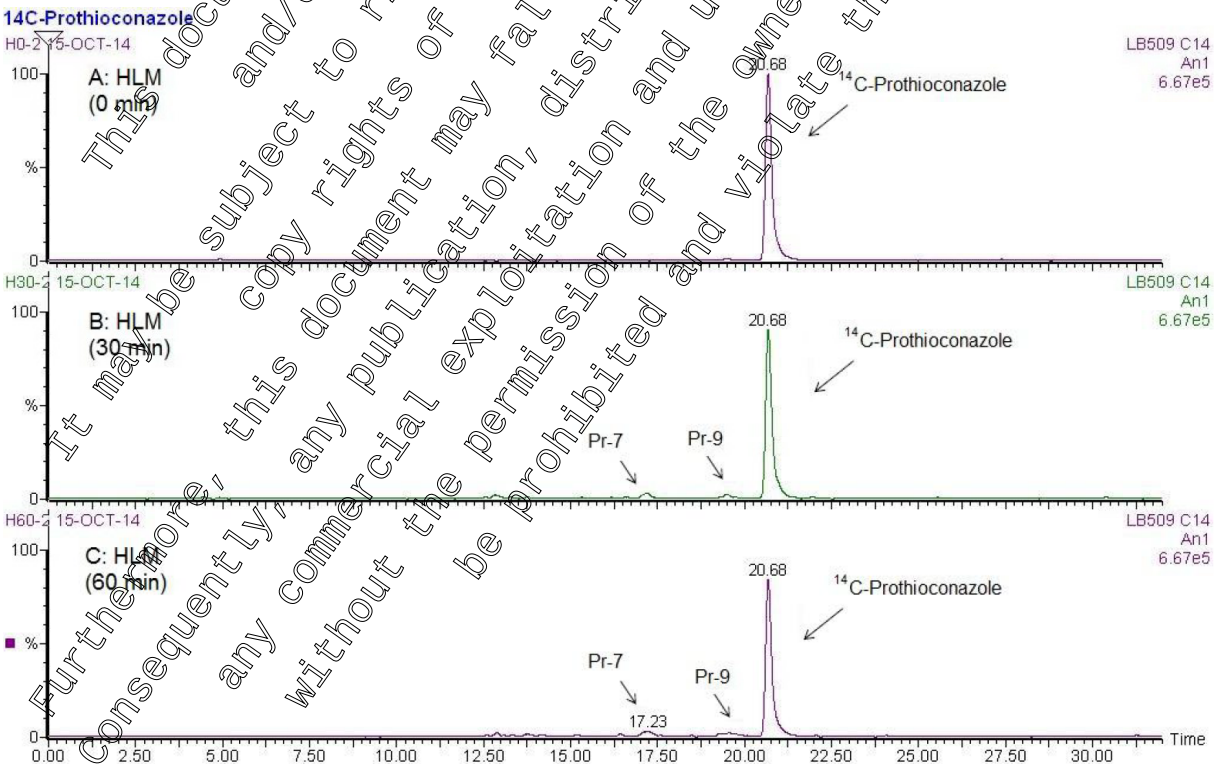


Figure 5.1.2-2: HPLC-radioactivity profiles of ^{14}C-prothioconazole metabolism in human liver microsomes.



Conclusion

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

- The *in vitro* metabolite profile of ^{14}C-prothioconazole when incubated with liver microsomes was found to be slightly different between rats and humans.
- In incubations with rat liver microsomes, between 50 and 60% of the initial ^{14}C-prothioconazole remained unchanged following an incubation period of one hour. Prothioconazole was metabolised towards a number of metabolites; three of them were above 5% of the relative percentage.
- In human liver microsomes, ^{14}C-prothioconazole was metabolised to a lower number of metabolites, and the amount of unchanged ^{14}C-prothioconazole following an incubation period of one hour was considerably higher (ca. 90%) as compared to rat liver microsomes. This indicates a slower metabolism rate in human liver microsomes.

The metabolic pattern in rat and human liver microsomes was qualitatively very similar and no unique human metabolite was detected.

Report:	KCA 5.1.2/03 [redacted],; [redacted]; [redacted]; 2016; M-534556-02-1
Title:	In-vitro metabolism and detoxification of [triazolyl-UL-14C] prothioconazole in human and rat hepatocytes with rat and human liver microsomes
Report No.:	EnSa-16-0065
Document No.:	M-534556-02-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

Executive Summary

The comparative *in vitro* metabolism of [triazolyl-UL-^{14}C]prothioconazole was investigated by incubating the test compound in suspension with hepatocytes from male humans and rats at 37°C. A test compound concentration of 1 µg/mL (2.9 µM) was chosen, the incubation times were 0, 0.5, 1 and 2 hours. Longer incubation times were not considered necessary because the test compound was almost totally metabolised during this period.

Three replicate tests (tests 1 – 3) were conducted for each species. The hepatocyte batches were tested for metabolic activity using different control substrates, and very good enzymatic activities were demonstrated. After protein precipitation with acetonitrile followed by centrifugation, aliquots of the supernatants were analysed by HPLC with radiochemical detection. Parent compound and metabolites were identified in selected samples by LC-MS.

The metabolic profiles were very comparable in the corresponding tests 1 – 3 with human and rat hepatocytes.

The rate of metabolism of ^{14}C-prothioconazole was fast in both *in vitro* systems. The test compound was extensively metabolised during the 2 h incubations: its amount decreased in the tests with human hepatocytes from the initial 100% to about 3.5% during the 2 h incubation period whereas in the tests with rat hepatocytes the unchanged test compound was detectable only in the 0 h sample.

The major metabolite in incubations with both species was the S-glucuronide of the parent compound accounting for max. 34.5% in human and 55.5% in rat hepatocytes. The corresponding O-glucuronide was traceable only in the incubations with rat hepatocytes (max. 3%).

Two further major metabolites were detected in the incubations with human and rat hepatocytes: the glucuronides of hydroxymethoxy and hydroxy metabolites of prothioconazole in human hepatocytes accounting after an incubation time of 2 hours for 30.1% and the glucuronide of the hydroxy metabolite in rat hepatocytes with 9.2%.

Very low amounts of JAU 6476-desthio were found in any of the incubations (max. 4.7% in human and 4.0% in rat hepatocytes).

Besides these above mentioned metabolites, a series of other metabolites were detected in the incubations of both species. The following further biotransformation reactions were found:

- methylation (human hepatocytes) or oxidation (rat hepatocytes) of the sulfur atom of the triazole ring,
- formation of dihydro-diene metabolites of the phenyl ring (human and rat hepatocytes),
- dechlorination linked to conjugation with glutathione (human hepatocytes), and
- glutathione conjugation of the metabolite JAU6476-desthio-dihydroxy-diene (rat hepatocytes).

Overall, the results indicate an extensive metabolism of ^{14}C-prothioconazole in hepatocytes of both humans and rats, leading to a series of phase I and II metabolites.

The glucuronidation of the unchanged parent compound prothioconazole and its hydroxy and hydroxymethoxy metabolites can be considered as a typical detoxification reaction. In addition, these derivatives have a higher polarity than the parent compound, which is also in accordance with their shorter retention times in an animal body, and can thus be easier excreted. The sum of these glucuronide conjugates were in both in vitro systems very similar, i.e. ca. 65%.

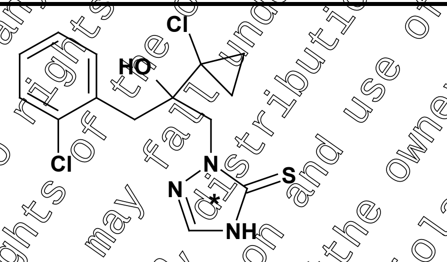
The rapid and extensive glucuronidation of prothioconazole, in conjunction with the negligible formation of JAU6476-desthio, was already identified in the *in vivo* ADME rat study as the "mechanism of detoxification" of prothioconazole.

Because no significant differences in the metabolite patterns were detectable after incubation of the test compound with metabolically active hepatocytes from both species, and also between these *in vitro* results and the results from the previously conducted *in vivo* ADME study in rats, it can be concluded that the rat is a suitable animal model for the assessment of the metabolism and detoxification of prothioconazole in humans and that the same "mechanism of detoxification" prevails in both species.

Materials and Methods

Test substances

The characteristics of the radiolabelled test compound [triazolyl-UL-^{14}C]prothioconazole are given in the table below:

Test Compound Characteristics	
Chemical structure	 * denotes the position of ^{14}C-label
Radiolabel position	Triazolyl-UL-^{14}C
Sample ID	KML 9869
Radiochemical purity	99% (HPLC, radiochemical detector)
Chemical purity	>99% (HPLC, UV-detector, 210 nm)
Specific radioactivity	2.31 MBq/mg
Supplier	[redacted]

All other chemicals and solvents (except water) were obtained from commercial suppliers and were used without additional purification. HPLC-grade water was prepared by a Milli-Q water purification system. Commercial grade solvents were used for HPLC analysis.

Hepatocytes

Rat hepatocytes were isolated in-house from male Wistar rats according to standard procedures.

Human hepatocytes for tests 1 and 2 were obtained from Hepacult GmbH ([redacted], Germany) and tebu-bio GmbH (D-[redacted], Germany). They were isolated from surgical waste tissue obtained from two male patients undergoing partial liver resections. For test 3, cryopreserved

hepatocytes were obtained from [redacted], France and [redacted], Germany.

Methods

Prothioconazole was tested at one concentration (1 µg/mL = 2.9 µM) in cell suspensions of human and rat hepatocytes in three replicates (tests 1 – 3) for each species. The incubation times were 0, 0.5, 1 and 2 hours at 37°C. Each test (1 – 3) was conducted in duplicate.

Viability testing of hepatocytes

The hepatocytes were incubated with phenacetin, amodiaquine, diclofenac, dextromethorphan, and midazolam at a concentration of 1 µM each to assess their metabolic capacity (positive controls). The metabolic conversion of these compounds was analysed and calculated as intrinsic clearance (Cl_{int}).

Stock solution of the test compound

A 5 mM stock solution of the test compound was prepared by dissolving 4.44 MBq of ^{14}C-prothioconazole in 0.55 mL of water. The stock solution was stored at ≤ -80 °C.

Sample preparation and incubation

Primary fresh rat and human hepatocytes were incubated with the radiolabelled test compound in suspension culture.

Incubation buffer: Williams E medium (pH 7.4)
Temperature: 37°C
Substrate: [Triazolyl-UL-^{14}C]prothioconazole (2.9 µM) added from a 5 mM stock solution dissolved in water
Incubation time: 0 - 2 h
Incubation volume: 5 mL total
2 mL aliquot at 0 h incubation
3 mL aliquot at 2 h incubation

Sample processing for analysis

The incubations were terminated by the addition of acetonitrile (approx. 30% (v/v)) and stored at ≤-18°C until analysis. Prior to analysis the samples were thawed, vortexed and afterwards centrifuged. The supernatants were removed and aliquots thereof were used for the analytical investigations.

Expression of the results

The relative percentages of the metabolites were calculated from the radiochromatographic profiles at the different incubation times according to the following equation:

$$\% \text{ Relative } P_i = \frac{(\text{Area } P_i)}{\sum \text{Area } P} \times 100 ,$$

where Area P_i is the mean area of the unchanged ^{14}C-prothioconazole (or metabolites) peak in the radiochemical chromatogram and $\sum$ Area P is the sum of the total radioactive mean peak areas in the chromatogram.

Analytical methodology

Aliquots (10 µL) of the supernatants from all samples were analysed without any further extraction or purification by high performance liquid chromatography (HPLC) with radiometric detection in the reversed phase mode. Selected samples were afterwards investigated for identification of parent compound and metabolites by mass spectrometry.

High Performance Liquid Chromatography (HPLC) and Mass Spectrometry

The electro-spray ionisation MS spectra (ESI) were obtained using a Q-Exactive mass spectrometer. The detailed chromatographic conditions for the MS experiments are described in the report. The HPLC instrument used for chromatography was an Agilent 1290. The effluent of the HPLC column was split between UV-detector followed by a fraction collector and an MS spectrometer.

The HPLC-chromatograms were recorded electronically and quantitatively evaluated using the software package Wallac Top Count. The ^{14}C-trace of a chromatogram was divided into regions of interest (ROI's), corresponding to the separated radioactive peaks. The regions of interest were integrated. Additionally, background regions (BKG's) were defined and used for background correction. The background corrected area counts from all regions of interest were used for calculation of the distribution of components in the sample.

For quantification of radioactive residues, all ^{14}C-signals in HPLC chromatograms from the different tests that were > 2-times of the signal to noise ratio were integrated for quantitative evaluation of the parent compound as well as identified and characterised metabolites. The following strategy was used for identification of parent compound and metabolites:

- comparison of the HPLC-profiles from the samples of the respective tests with each other and designation of peaks to an unique component according to the specific retention time (e.g. Rt ca. 7.5 min = JAU6476), and
- spectroscopic investigations by LC-MS/MS of selected samples from human and rat hepatocytes.

All other peaks or regions additionally detected in the HPLC profiles of the respective chromatograms were assigned as unknown. None of them accounted for >5.8%. They were characterised by their retention times in HPLC chromatograms.

Results and Discussion

The *in vitro* metabolic profile of [triazolyl-UL-^{14}C]prothioconazole was determined in hepatocytes from male humans and rats after 0, 0.5, 1 and 2 hours of incubation. The test concentration was 1 µg/mL (= 2.9 µM). Longer incubation times were not considered necessary because the test compound was almost completely metabolised after this period.

Viability testing of hepatocytes

The hepatocytes were incubated with phenacetin, amodiaquine, diclofenac, dextromethorphan, and midazolam at a concentration of 1 µM each to assess their metabolic capacity. All hepatocyte batches were metabolically competent and exhibited good activities.

Metabolism of ^{14}C-prothioconazole

The metabolism rate of ^{14}C-prothioconazole was fast in the incubations with both, human and rat hepatocytes. Prothioconazole was intensively metabolised during the 2 h incubations: the amount decreased in the tests with human hepatocytes from the initial 100% to about 3.5%, while in the tests with rat hepatocytes the unchanged parent compound was detectable only in the 0 h sample.

The major metabolite in incubations with both species was the S-glucuronide of prothioconazole accounting for 34.5% in human and 55.5% in rat hepatocytes.

The corresponding O-glucuronide was traceable only in the incubations with rat hepatocytes in very low amounts (max. 3%). The structural identification of the S- and O-glucuronides of the test compound was based on LC-MS/MS spectra and on earlier results obtained in the goat metabolism study ([redacted], E.; [redacted], H. and [redacted], K.; 2003; M-116219-02-1) and in the rat ADME study ([redacted], K.; 2001; M-034280-01-1). In the goat metabolism study the exact structure of the JAU 6476-S-Glucuronide was determined by mass- and NMR-spectroscopic methods. By comparison of the metabolic profiles (HPLC) of urine samples from the rat ADME- and goat metabolism studies it was demonstrated that also in the rat the glucuronidation took place at the sulfur atom of the molecule.

Another significant peak in the chromatogram of the human hepatocyte incubation after 2 hours elutes at a retention time of 5.2 minutes (30.1%, Fig. 5.1.2-3). Spectroscopic investigations revealed that this is a mixture of the glucuronides of hydroxymethoxy and hydroxy metabolites of prothioconazole (JAU 6476-hydroxy-glucuronide and JAU 6476-hydroxymethoxy-glucuronide). It was not possible to assign the exact positions of the hydroxy and methoxy groups at the phenyl ring. The corresponding peak in the chromatogram of rat hepatocytes after 2 hours was less pronounced (9.2%).

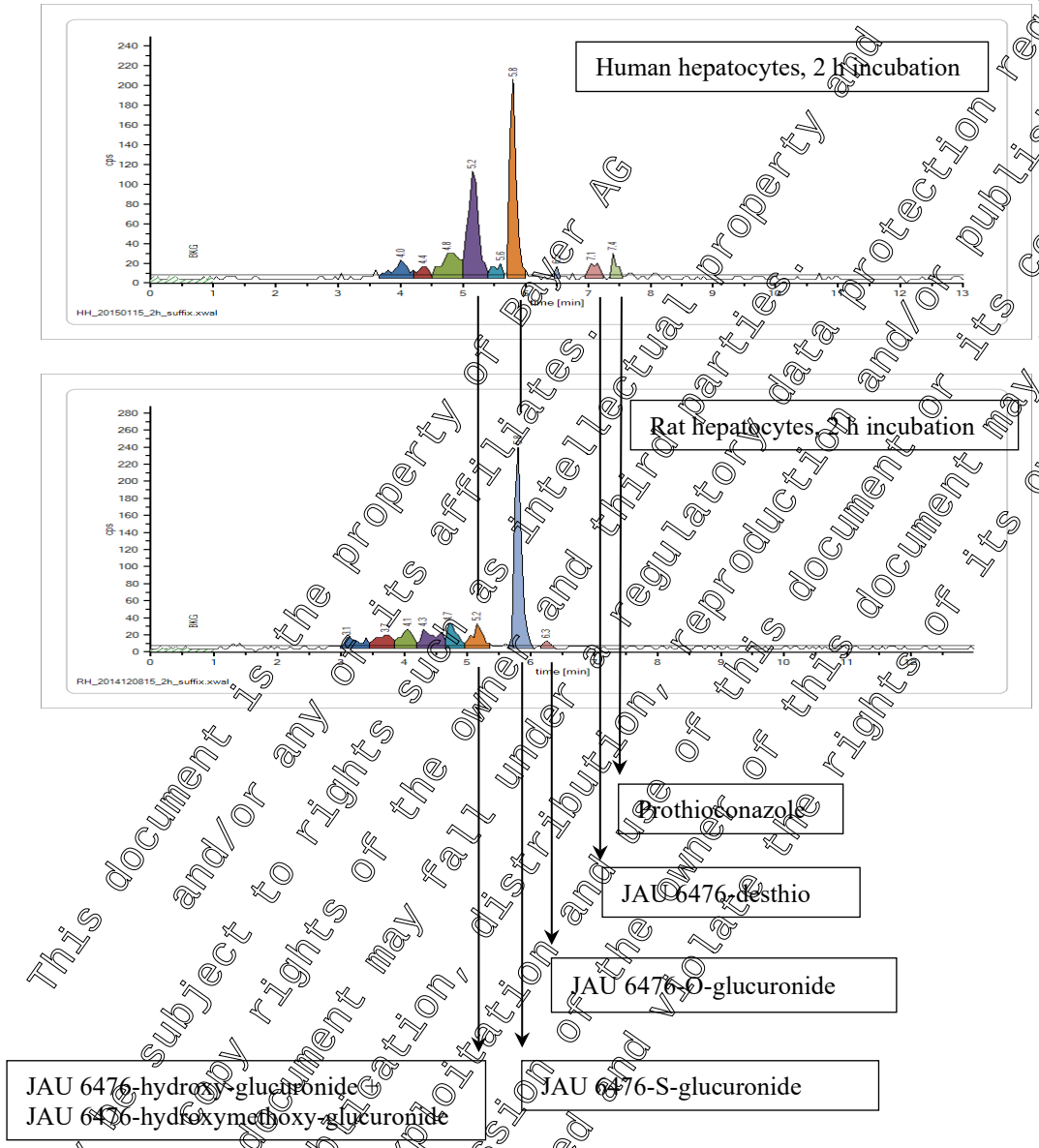
Very low amounts of JAU 6476-desthio were found in any of the incubations (max. 4.7% in human and 4.0% in rat hepatocytes).

The glucuronidation of the unchanged parent compound prothioconazole and its hydroxy and hydroxymethoxy metabolites can be considered as a typical detoxification reaction. In addition, these derivatives have a higher polarity than the parent compound, which is also in accordance with their shorter retention times in an animal body, and can thus be easier excreted. The sum of theses glucuronide conjugates were in both *in vitro* systems very similar, i.e. ca. 65%. The rapid and extensive glucuronidation of prothioconazole, in conjunction with the negligible formation of JAU 6476-desthio, was already identified in the *in vivo* ADME rat study as the "mechanism of detoxification" of prothioconazole. However, care should be taken in quantitatively comparing rat and human *in vitro* systems since they have to be regarded as model systems. Also a quantitative extrapolation to the intact organism should not be made because the effects of absorption, distribution and excretion are not reflected in the *in vitro* systems.

Nevertheless the results of this study clearly demonstrate that the principal metabolic reactions are identical in rat and human *in vitro* systems and in rat *in vivo*. Therefore, it can be concluded that the rat is a suitable animal model for the assessment of the metabolism and detoxification of prothioconazole in humans and that the same mechanisms of detoxification prevail in both species.

A comparison of the metabolic profiles in both *in vitro* systems is provided in Figure 5.1.2-3.

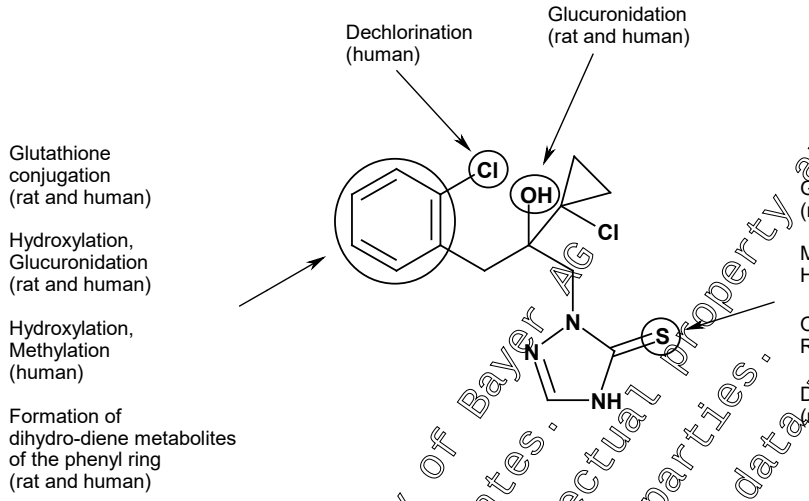
Figure 5.1.2-3: HPLC-radioactivity profiles of ^{14}C-prothioconazole metabolism in rat and human hepatocytes after 2 h incubation.



Beside these above mentioned metabolites a series of other metabolites was detected in the incubations of both species. The following further biotransformation reactions were found:

- methylation (human hepatocytes) or oxidation (rat hepatocytes) of the sulfur atom of the triazole ring,
- formation of dihydro-diene metabolites of the phenyl ring (human and rat hepatocytes),
- dechlorination linked to conjugation with glutathione (human hepatocytes), and
- glutathione conjugation of the metabolite JAU 6476-desthio-dihydroxy-diene (rat hepatocytes).

Overall, the results indicate an extensive metabolism of ^{14}C-prothioconazole in hepatocytes of both humans and rats leading to a series of phase I and II metabolites. The principal metabolic routes are shown in the figure below:



Conclusion

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

- The principal metabolic reactions are identical in both species.
- [Triazole-UL-^{14}C]prothioconazole was intensively metabolised during 2 h incubations at 37°C in human and rat hepatocytes.
- Very low amounts of the test compound remained unchanged in the incubations with rat and human hepatocytes.
- JAU 6476-desthio was found only in very low amounts in any of the incubations.
- Conjugation of the parent compound with glucuronic acid at the sulfur atom of the molecule (JAU 6476-S-glucuronide) was the major detoxification route in both species.
- A further important detoxification route in both species was the conjugation of the hydroxymethoxy and hydroxy metabolites of prothioconazole with glucuronic acid (JAU 6476-hydroxy-glucuronide and JAU 6476-hydroxymethoxy-glucuronide).
- The glucuronidation of the unchanged parent compound prothioconazole and its hydroxy and hydroxymethoxy metabolites can be considered as a typical detoxification reaction. In addition, these derivatives have a higher polarity than the parent compound, which is also in accordance with their shorter retention times in an animal body, and can thus be easier excreted. The sum of these glucuronide conjugates were in both in vitro systems very similar, i.e. ca. 65%.
- The rapid and extensive glucuronidation of prothioconazole, in conjunction with the negligible formation of prothioconazole-desthio, was already identified in the rat *in vivo* as the “mechanism of detoxification” of prothioconazole.
- Because no significant differences in the metabolite patterns were detectable after incubation of the test compound with metabolically active hepatocytes from both species, and also between these *in vitro* results and the results from the previously conducted *in vivo* ADME study in rats, it can be concluded that the rat is a suitable animal model for the assessment of the metabolism and detoxification of prothioconazole in humans and that the same “mechanism of detoxification” prevails in both species.
- The results of this study also demonstrate that hepatocytes are a much more suitable *in vitro* system to investigate the comparative metabolism of prothioconazole than liver microsomes (see 5.1.2/01).

CA 5.2 Acute toxicity

Table 5.2-1: Summary of the acute toxicity of prothioconazole*

Species	Sex	Route/Study	Comments	Classification (EU Directive 93/21/EEC)	Reference
Rat	M F	Oral	LD_{50} >6200 mg/kg bw	-	[redacted] (1995) M-012312-01-1
Mouse	M F	Oral	No data submitted	-	-
Rat	M F	Dermal	LD_{50} >2000 mg/kg bw	-	[redacted] (1999) M-009688-01-1
Rat	M F	Inhalation	LC_{50} > 4990 mg/m^3	-	[redacted] (1999) M-008846-01-1
Rat	M/F	Intraperitoneal	No data submitted[1]	-	-
Rabbit	M/F	Skin irritation	No irritation recorded	-	[redacted] (1996a) M-009890-02-1
Rabbit	M/F	Eye irritation	Minimal irritation recorded	-	[redacted] (1996b) M-009893-02-1
Guinea pig	M/F	Skin sensitisation M&K method	Negative (study features deviations from OECD Guideline 406 (1992))	-	[redacted] (1996) M-009898-03-1
Mouse	*	Skin sensitization Local lymph node assay	Negative	-	[redacted] (2007) M-291490-01-1
BALB/c 3T3 cells	*	In vitro 3T3 NRU phototoxicity test	Negative	-	[redacted] (2014) M-498655-01-1

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

[1] But not considered a data gap.

Prothioconazole does not require classification for acute toxicity, irritancy or sensitisation effects. Furthermore, prothioconazole does not show a phototoxic potential.

CA 5.2.1 Oral

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

CA 5.2.2 Dermal

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

CA 5.2.3 Inhalation

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

[1] But not considered a data gap.

CA 5.2.4 Skin irritation

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

CA 5.2.5 Eye irritation

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

CA 5.2.6 Skin sensitization

In addition to the study on skin sensitization already available in the DAR for the first Annex I inclusion and baseline dossier a new local lymph node assay was performed in 2007 in order to investigate the impact of the increase of a certain impurity with slight sensitizing properties in the technical specification. For further details please see document M-291556-01-1 (confidential information).

Report:	KCA 5.2.6/06 [redacted]; 2007; M-291490-01-1
Title:	Prothioconazole (Project: Prothioconazole (JAU 6476)) - Local lymph node assay in mice (LLNA/IMDS)
Report No.:	AT04016
Document No.:	M-291490-01-1
Guideline(s):	OECD 406 (1992); OECD 429 (2002); Guideline 96/54/EC, Method B.6 (1996)., B.42 (2001)., US-EPA 712-C-03-197, OPPTS 8702600 (2003)
Guideline deviation(s):	none
GLP/GEP:	**yes**

I. Materials and methods

A. Materials

1. Test material: Prothioconazole

Description:	white powder
Batch no:	2007-000236
Purity:	97.2%
Stability of test compound:	guaranteed for study duration; expiry date: 2008-01-31

2. Vehicle: dimethylformamide

3. Test animals

Species:		NMRI mice
Strain:		Hsd Win:NMRI
Age:		9 weeks
Weight at dosing:		26 g - 32 g
Source:		[redacted], Germany
Acclimatisation period:		at least four days
Diet:		PROVIMI KLIBA SA 3883 maintenance diet for rats and mice ([redacted], Switzerland), *ad libitum*
Water:		tap water, *ad libitum*
Housing:	adaptation	up to 8 mice conventional in Makrolon type III cages
	study period	1 animal in type II cages bedding: low-dust wood granulate ([redacted] France)

B. Study design and methods

1. Animal assignment and treatment

Dose	0%-2%-10%-50%.
Application route:	epicutaneously onto the dorsal part of both ears
Application volume:	25 µL/ear
Duration:	three consecutive days (d1, d2, d3)
Group size:	6 females/group
Observations:	local lymph node weight and cell count determination, ear swelling, ear weight, body weight (at beginning and termination of study)

II. Results and discussion

A. Findings

The NMRI mice did not show an increase in the stimulation indices for cell counts or for weights of the draining lymph nodes. The "positive level" of 1.4 for the cell count index was never reached or exceeded in any dose group.

The "positive level" for ear swelling of $2x10^{-2}$ mm increase, i.e. about 10% of the control values, has not been reached or exceeded in any dose group.

No increases of the ear weights could be determined compared to control animals either.

Body weights were not affected by treatment.

No antigen specific activation of the cells of the immune system via dermal route was determined after application of up to and including 50% prothioconazole by the LLNA/IMDS method.

Figure 5.2.6-1: Bar charts (weight and cell count) for the LLNA.

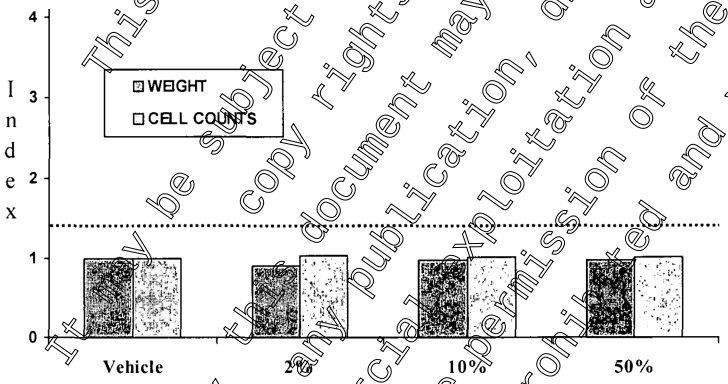


Table 5.2.6-1: Summary of LLNA results

Dose (%)	Direct LLNA Weight index (Mean ± SD in %)	Direct LLNA Cell count index (Mean ± SD in %)	Ear swelling (0.01 mm) Day 1 (Mean ± SD in %)	Ear swelling (0.01 mm) Day 4 (Mean ± SD in %)	Ear swelling (0.01 mm) Index day 4	Ear weight (mg per 8 mm diameter punch) Day 4 (Mean ± SD in %)	Ear weight (mg per 8 mm diameter punch) Index day 4
0*	1.00 ± 28.25	1.00 ± 28.20	18.25 ± 4.75	18.00 ± 4.74	1.00	11.77 ± 10.15	1.00
2	0.92 ± 17.02	1.05 ± 27.82	18.17 ± 2.14	17.83 ± 5.77	0.99	11.33 ± 4.97	0.96
10	0.99 ± 36.37	1.02 ± 49.04	18.08 ± 3.70	17.75 ± 4.25	0.99	11.20 ± 8.09	0.95
50	0.99 ± 33.98	1.02 ± 33.80	17.83 ± 3.24	17.92 ± 2.87	1.00	11.26 ± 5.76	0.96

* = vehicle control (dimethylformamide)

III. Conclusion

After dermal application prothioconazole caused neither a non-specific (irritant) nor a specific immunostimulating (sensitizing) effect.

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), the conduct of an in vitro phototoxicity study is required "*where the active substance absorbs electromagnetic radiation in the range 290-700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance is less than 10 L x mol^{-1} x cm^{-1}, no toxicity testing is required.*" Since this coefficient is >10 L x mol^{-1} x cm^{-1}, the phototoxicity study was conducted.

Report: KCA 5.2.7/01 [redacted]; 2014; M-498655-01-1
Title: Prothioconazole, technical: Cytotoxicity assay in vitro with BALB/c 3T3 cells: Neutral red (NR) test during simultaneous irradiation with artificial sunlight
Report No.: 1646000
Document No.: M-498655-01-1
Guideline(s): Commission Regulation (EC) No. 440/2008 B 41 (2008); Committee for Proprietary Medicinal Products (CPMP) Note for Guidance on Photosafety testing, EMEA, CPMP/SWP/398/01 (2002); OECD 432 (2004)
Guideline deviation(s): none
GLP/GEP: **yes**

I. Materials and methods

A. Materials

1. Test material:

Name:	Prothioconazole, technical
Synonyms:	JAU 6476
Description:	Light beige solid
Lot/Batch no:	HEC 21597-1-1
Purity:	96.7% (w/w)
Stability of test compound:	guaranteed for study duration; expiry date: 2015-07-02

2. Vehicle, positive control:

Solvent control for the positive control: Earle´s Balanced Salt Solution (EBSS)
Solvent control for the test item: EBSS containing 1% (v/v) dimethylsulfoxide (DMSO)
Positive control: chlorpropmazine (Sigma) dissolved in EBSS

3. Test system:

BALB/c 3T3 cells clone 31

Cell cultures:

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 culture medium.
Cells were sub-cultured twice weekly, cell cultures were incubated at 37 ± 1.5 °C in a 7.5 ± 0.5% carbon dioxide atmosphere.

Culture medium:

Dulbecco´s Minimal Essential Medium (DMEM) supplemented with 10% newborn calf serum (NCS).

B. Study design and methods

1. Treatment

Tested concentrations:

Test item	Artificial sunlight	Tested concentrations (µg/mL solvent control)
Prothio-conazole	+/–	1.95, 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 (6 replicates per concentration)
Positive control	+	0.125, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0 (6 replicates per concentration)
	–	6.25, 12.5, 25, 37.5, 50, 75, 100, 200 (6 replicates per concentration)
Solvent controls for prothiocon-azole and for positive control	+/–	pure (12 replicates each)

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

Solar simulator:

Dr. Hönle Sol 500, filter H1, cultures were irradiated in homogeneous irradiation area (verified with UV-meter)

Seeding of cultures:

Per well about 2 x 10^4 cells were seeded in 100 µL culture medium in two 96 well plates (one exposed to artificial sunlight; one kept in the dark)

Treatment & irradiation:

24 h after seeding the cultures were washed with EBSS. 100 µL of solved test item (see table above) was added/well. Both plates were pre-incubated for 1 hour in the dark. Therafter one plate was irradiated at 1.65 mW/cm^2 (4.95 J/cm^2) for 50 min, the other plate was stored for 50 min in the dark (at 25-28 °C).
Therafter the test item was removed and both plates were washed 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 was added to each well. Plates were incubated for another 3 hours, thereafter the medium was

removed completely and the cells were washed with EBSS. For extraction of the dye 0.15 mL of a solution of 49% (v/v) deionised water, 50% (v/v) ethanol and 1% (v/v) acetic acid were added to each well. After approximately 10 minutes at room temperature and a brief agitation, the plates were transferred to a microplate reader (Versamax®, Molecular Devices) equipped with a 540 nm filter to determine the absorbance of the extracted dye. This absorbance showed a linear relationship with the number of surviving cells.

2. Evaluation

Mean absorption (Optical Density (OD_{540}) per concentration was calculated.
ED_{50} values (effective dose where only 50% of the cells survived) were determined by curve fitting software.
Photo-Irritancy-Factor (PIF) and Mean Phototoxic effect (MPE) were calculated according to OECD guideline 432.

Evaluation criteria:

- if PIF <2 or MPE <0.1 no phototoxic potential is predicted
- if PIF >2 and <5 or MPE >0.1 and <0.15 a probable phototoxic potential is predicted
- if PIF >5 or MPE >0.15 a phototoxic potential is predicted

Acceptability criteria:

- after irradiation with a UVA dose of 5 J/cm² the cell viability of solvent control is >80% of non irradiated cells
- the positive control PIF between the two ED_{50} values is >6
- the mean OD_{540} of solvent controls is >0.4

II. Results and discussion

The study results are summarised in Table 5.2.7-1 and Table 5.2.7-2, below.

Table 5.2.7-1: Optical Density and Viability of BALB/c 3T3 cells in main experiment

Concentration [µg/mL]	With artificial sunlight OD_{540} Mean	SD	% of solvent control	Concentration [µg/mL]	Without artificial sunlight OD_{540} Mean	SD	% of solvent control
Treatment with prothioconazole							
Solvent control	0.6577	0.0827	100.00	**Solvent control**	0.7038	0.0259	100.00
1.95	0.7076	0.0506	107.59	1.95	0.7157	0.0140	101.69
3.91	0.6932	0.0094	105.40	3.91	0.7172	0.0164	101.90
7.81	0.6982	0.0182	106.16	7.81	0.7025	0.0197	99.82
15.63	0.6953	0.0337	105.73	15.63	0.6993	0.0298	99.37
31.25	0.6903	0.0433	104.99	31.25	0.6884	0.0193	97.82
62.5	0.4205	0.0342	63.94	62.5	0.4575	0.0510	65.01
125	0.0864	0.0119	13.14	125	0.0871	0.0135	12.38
250	0.0862	0.0180	13.11	250	0.0684	0.0070	9.72
Treatment with positive control chlorpromazine							
Solvent control	0.6823	0.0767	100.00	**Solvent control**	0.6514	0.0717	100.00
0.125	0.6849	0.0106	100.38	6.25	0.6812	0.0161	104.59
0.25	0.1172	0.0173	17.17	12.5	0.0926	0.0058	14.21
0.5	0.0870	0.0176	12.75	25	0.0678	0.0165	10.41

Concen-tration [µg/mL]	With artificial sunlight OD540 Mean	SD	% of solvent control	Concentration [µg/mL]	Without artificial sunlight OD540 Mean	SD	% of solvent control
0.75	0.0924	0.0200	13.54	37.5	0.0677	0.0128	10.39
1	0.0809	0.0072	11.85	50	0.0663	0.0217	10.17
1.5	0.0798	0.0055	11.70	75	0.0627	0.0071	9.63
2	0.0877	0.0065	12.85	100	0.0659	0.0087	10.12
4	0.0824	0.0088	12.07	200	0.0580	0.0037	8.91

Table 5.2.7-2: Summary of results

	Substance	ED_{50} (+artificial sunlight) [µg/mL]	ED_{50} (–artificial sunlight) [µg/mL]	PIF	MPE	% viability of solvent control of irradiated vs. non-irradiated plate
Range finding experiment	Prothioconazole	57.54	56.72	0.986	-0.040	87.5
	Positive control	0.17	20.74	124.24	0.773	89.8
Main experiment	Prothioconazole	64.77	72.38	1.118	-0.012	93.4
	Positive control	0.19	10.26	53.34	0.683	104.8

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

III. Conclusions

The acceptability criteria for study validity are met.

A dose dependent cytotoxicity was observed after treatment with prothioconazole in the presence and absence of irradiation with artificial sunlight in both the range finding and the main experiment. The PIF of prothioconazole was 0.986 in the range finding experiment and 1.118 in the main experiment. The MPE was calculated as -0.040 and -0.012, respectively.

According to the evaluation criteria (PIF <2 or MPE <0.1), prothioconazole has no phototoxic potential.

CA 5.3 Short-term toxicity

Table 5.3-1: Summary of short-term studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
4 week rat (diet) 0, 196, 1480 and 9250 ppm	1480 ppm [146 mg/kg/d (m)] [151 mg/kg/d (f)]	9250 ppm [952 mg/kg/d (m)] [1033 mg/kg/d (f)]	↑ ALT, ↑ ALP, ↓ bodyweight gains, ↑ food and water consumption, ↑ liver weights and histopathological findings in the kidney.	[redacted], 1997 M-012338-01-1
4 week rat (comparison of gavage against diet) 10000 ppm (diet), or 1000 mg/kg/d (gavage)	Not applicable (the study was not intended to establish an NOAEL or a LOAEL)	Not applicable (the study was not intended to establish an NOAEL or a LOAEL)	↓ bodyweight gains, ↑ ALT and ALP, changes in hepatic enzyme activities, ↑ liver weights and histopathological findings in the kidney. Gavage dosing led to more marked effects than dietary administration.	[redacted], 1998 M-012415-01-1
14 week rat (gavage) 0, 20, 100 and 500 mg/kg/d	100 mg/kg/d	500 mg/kg/d	Death (one f), ↑ water consumption, ↓ urinary volume, liver weights, ↓ spleen weights, hepatocytic hypertrophy, histopathological findings in the kidneys.	[redacted], 1999b M-011757-01-1
14 week mouse (gavage) 0, 25, 100 and 400 mg/kg/d	25 mg/kg/d	100 mg/kg/d	↑ liver weights, ↑ hepatic enzyme activities, hepatocytic hypertrophy and other histopathological findings in the liver.	[redacted], 1999a M-012244-01-1
13 week dog (gavage) 0, 25, 100 and 300 mg/kg/d	25 mg/kg/d	100	Histopathological findings in the kidneys. In addition, ↑ ALT and ↑ liver weights were recorded at 300 mg/kg/d.	[redacted], 2001a M-035825-01-1
52 week dog (gavage) 0, 5, 40 and 125 mg/kg/d	5 mg/kg/d	40 mg/kg/d	Histopathological findings in the kidneys (and marginal effects on bodyweight gains and ↑ ALP levels). Increased liver weights and liver histopathology at 125 mg/kg/d.	[redacted], 2001b M-035967-01-1
4 week rat (dermal) 0, 100, 300 and 1000 mg/kg/d	1000 mg/kg/d		No systemic toxicity or effects on the skin at the highest dose tested.	[redacted], 2000 M-044301-01-1

An initial 4 week study in rats indicated that prothioconazole was relatively unstable when formulated with diet, hence gavage dosing was used for the 13 week studies in rats, mice and dogs.

The liver was consistently identified as a target organ in short term studies performed in rats, mice and dogs. Effects on the liver included raised plasma ALT levels, changes in hepatic enzyme activity (generally increases) and increased liver weights. In some cases hepatocellular hypertrophy was also recorded (consistent with hepatic enzyme induction). None of the effects recorded in the liver persisted following the respective recovery periods in rats (4 weeks) or dogs (8 weeks). There were no recovery groups in the mouse studies.

The kidney was also identified as a target organ in rats and dogs, but not in mice. The effects on the kidneys consisted of histopathological changes, namely increased incidence and severity of basophilic

tubules and tubular dilatation in rats, and interstitial fibrosis/inflammation in dogs. These kidney findings did not persist following a 4 week recovery period in rats, but only partial recovery was recorded following an 8 week recovery period in dogs. In rats, these kidney changes correlated with severely increased water intakes, indicating a disturbance of the kidney function and systemic water homeostasis. As a likely consequence, in the subchronic study one female rat died at the highest tested dose of 500 mg/kg bw/day. The proliferation index (frequency of PCNA-containing cells) was also increased in rat kidneys at a very high dose level. In rats the kidneys were more markedly affected in males compared to females. This was also the case in dogs after 13 weeks treatment but not after 52 weeks treatment

Other comparisons between the 13 and 52 week dog studies reveal the following. The 52 week study recorded effects on bodyweights and food consumption which were not apparent in the 13 week study (the bodyweight effects were not obvious over the first 4 weeks of the study). Increased liver and kidney weights were more pronounced following 125 mg/kg bw/day for 52 weeks compared with 100 mg/kg bw/day for 13 weeks, and this was also the case for histopathological findings in the liver. Histopathological findings in the kidneys were recorded at 40 mg/kg bw/day after 52 weeks, but not at 25 mg/kg bw/day after 13 weeks.

In the 4 week rat study performed to compare the effect of different dose routes, it was found that the lowest plasma concentrations were obtained with silica-stabilised diet formulation, that neat diet formulation resulted in plasma concentrations ≈2-fold higher and that gavage dosing produced the highest plasma concentrations (≈6-fold higher). The higher plasma concentrations resulting from gavage dosing were consistent with the more marked effects seen in gavage dosed animals compared to diet-treated animals. Plasma concentrations were consistently higher in females compared to males. In the 13 week rat study (gavage), plasma concentrations of prothioconazole were again consistently higher than males (≈2-fold). In the 13 week dog study, prothioconazole concentrations were analysed in liver and kidneys rather than in plasma, but again levels of prothioconazole in these organs were higher in females compared to males. Levels of the metabolite SXX 0665 were always significantly lower than prothioconazole (10-20 times lower), but generally followed the same pattern (SXX 0665 was below the LOQ in the kidneys in all animals).

It is notable that effects on the liver in dogs were more marked in females compared to males, which is consistent with the higher levels of prothioconazole recorded in the liver of female animals. However, despite consistently higher levels of prothioconazole in the kidneys of female dogs (and higher plasma concentrations in female rats) the adverse kidney effects were more marked in males over 13 weeks. Plasma concentrations of prothioconazole were not analysed in the 52 week dog study and it cannot easily be determined which sex was more adversely affected since the pattern of effects is different between the sexes after 52 weeks.

Rats treated dermally for 4 weeks at dosages up to 1000 mg/kg bw/day showed no adverse effects. The absence of effects was consistent with very low plasma levels of prothioconazole (<LOQ in most animals) recorded after 7 days of treatment.

CA 5.3.1 Oral 28-day study

All necessary short-term toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the DAR for the first Annex I inclusion and the baseline dossier of prothioconazole.

CA 5.3.2 Oral 90-day study

All necessary short-term toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the DAR for the first Annex I inclusion and the baseline dossier of prothioconazole.

CA 5.3.3 Other routes

All necessary short-term toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the DAR for the first Annex I inclusion and the baseline dossier of prothioconazole.

CA 5.4 Genotoxicity testing

Table 5.4- 1: Summary of genotoxicity studies

Test system; study	Concentration / dose levels	Result	Reference
***In vitro* studies**			
Bacterial point mutation assay (Ames test) in *S. typhimurium* strains (TA1535, TA100, TA1537, TA98, TA102)	Plate incorporation assay: 16 - 5000 µg /plate (±S9) Pre-incubation assay: 1.6 - 500 µg/tube (±S9)	**Negative**	[redacted] (1996a) [M-012254-01-1]
Mammalian cell gene mutation assay *in vitro* (HPRT locus, V79 CHL cells)	1st gene mutation assay: 25 - 175 µg/ml (-S9) 75 - 200 µg/ml (+S9) 2nd gene mutation assay: 5 - 150 µg/ml (-S9) 75 - 200 µg/ml (+S9)	**Negative**	[redacted] (1996) [M-012273-01-1]
Rat liver UDS assay *in vitro*	1st UDS assay: 1.0 - 40.0 µg/ml 2nd UDS assay: 0.5 - 20.0 µg/ml	**Equivocal**	[redacted] (1998) [M-012317-01-1]
Mammalian chromosomal aberration assay *in vitro* (V79 CHL cells)	1st chromosome aberration assay: 18 h harvest: 25 - 150 µg/ml (±S9) 30 h harvest: 75 - 150 µg/ml (±S9) 2nd chromosome aberration assay: 8 h harvest time: 75 - 150 µg/ml (±S9) 18 h harvest time: 50 - 100 µg/ml (-S9)	**Positive**	[redacted] (1996c) [M-012277-01-1]
Micronucleus test *in vitro* (human lymphocytes)	1st micronucleus assay (4 h exposure): 30.1, 52.7, 119 µg/ml (-S9) 30.1, 52.7, 79 µg/ml (+S9) 2nd micronucleus assay (20 h exposure): 43.4, 57.8, 69.9 µg/ml (-S9)	**Negative**	[redacted] (2017) [M-588628-01-1]
***In vivo* studies**			
Rat liver UDS assay *in vivo*	2500, 5000 mg/kg bw (oral gavage)	**Negative**	[redacted] (1999a) [M-007155-01-1]
Micronucleus assay (*in vivo* mouse bone marrow)	250 mg/kg bw (i.p.)	**Negative** (PCE/NCE ratio not altered)	[redacted] (1996b) [M-012265-01-1]
Micronucleus assay (*in vivo* mouse bone marrow)	2 x 50, 2 x 100, 2 x 200 mg/kg bw (i.p.)	**Negative** (PCE/NCE ratio altered)	[redacted] (2003) [M-102790-01-1]

Prothioconazole gave negative results when tested up to cytotoxic doses in a battery of *S. typhimurium* strains (TA 1535, TA 100, TA 1537, TA 98, TA 102). When tested in an HPRT locus mammalian cell gene mutation assay in V79 CHL cells, isolated changes were observed in mutant frequency. Those changes were either not reproducible, occurred at extreme cytotoxicity, did not show a dose-response relationship and/or were with the historical control range and therefore are not of toxicological

significance. Hence, an overall negative result was concluded. An *in vitro* rat liver UDS assay provided an equivocal result (increased NNG counts and percentage of cells in repair, but only marginal and/or without a dose-related pattern and below the threshold for a positive response applied by the performing laboratory).

In an *in vivo* rat liver UDS assay an increase in NNG counts was recorded at the top dose after 16 hours, but only in 2 out of 4 animals and the mean NNG counts for the group did not exceed the threshold for a positive response applied by the performing laboratory. Two vehicle control animals from another study (T6040651, compiled in M-078413-01-1) conducted at the same test laboratory, also displayed very similar positive NNG counts. A negative result was therefore concluded for the study.

Overall it is concluded that prothioconazole is not a gene mutagen.

Prothioconazole induced chromosome aberrations in Chinese hamster lung cells in the presence and absence of metabolic activation. It is concluded that prothioconazole is clastogenic *in vitro*. However, since aberrations only occurred at cytotoxic concentrations, since the cytotoxicity may have even been underestimated and since the increases were not concentration-related, it is likely that the clastogenicity seen is an indirect effect triggered by cytotoxicity.

No significant increase in the incidence of micronuclei was induced in the first *in vivo* mouse bone marrow micronucleus assay. The assay was compliant with the contemporary (1983) but not the 1997 OECD guideline 474 (more dose levels and two times the number of PCEs examined for micronuclei would be required for the 1997 guideline). As this study did therefore not provide complete reassurance regarding the potential clastogenicity of prothioconazole (it was also noted that the test material used was of very high purity - 99.5-99.9 %), a second *in vivo* mouse micronucleus assay was conducted according to the 1997 OECD guideline, using technical prothioconazole representative (in terms of overall purity and impurity profile) of material which is likely to be produced commercially. The second *in vivo* mouse micronucleus assay gives the required reassurance that prothioconazole is not clastogenic/aneugenic.

This is further substantiated by the results of a recently conducted micronucleus test *in vitro* (according to the current (2016) OECD guideline 487 and using technical prothioconazole representative (in terms of overall purity and impurity profile) of material which is likely to be produced commercially). In this assay prothioconazole did not show any clastogenic or aneugenic effects.

Photomutagenicity

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

For prothioconazole there is no evidence of a photoreactivity potential (see chapter CA 5.2.7; KCA 5.2.7/01, M-498655-01-1, and the Ultraviolet/visible molar extinction/absorption coefficient is less than 1000 L × mol^{-1} × cm^{-1} (see KCA 7.3.1 /01, M-051279-01-1). Thus photomutagenicity testing is not required.

CA 5.4.1 In vitro studies

Report:	KCA 5.4.1/01[redacted]R; 1996; M-012254-01-1
Title:	JAU 6476 - Salmonella/microsome test plate incorporation and preincubation method
Report No.:	24859
Document No.:	M-012254-01-1
Guideline(s):	OECD 471 (1983), EEC Directive 92/69/EEC B.14, US-EPA PB 84-233295 (1984)
Guideline deviation(s):	none
GLP/GEP:	**yes**
Deviations:	Deviations from the current OECD guideline (1997): Composition of medium used and the number of cells per culture were not reported However, these minor deviations do not compromise the study results.

Executive summary:

In a 1996 GLP study, the potential mutagenicity of prothioconazole (batch no. NLL 6096-4, purity 99.5 %) was investigated in an Ames test (plate incorporation method) using *S.typhimurium* strains TA1535, TA100, TA1537, TA98 and TA102. Bacterial cultures were exposed to the test material (dissolved in DMSO) at concentrations between 1.6 and 5000 µg/plate in the presence and absence of an exogenous metabolic activation system (Aroclor 1254-induced male Sprague Dawley rat liver S9 mix). The results were confirmed in an independently repeated assay incorporating a pre-incubation step. Both assays were performed in triplicate. The test material was shown by analysis to be stable in solvent for at least four hours at room temperature. Except for minor deviations (see above) the study was compliant with OECD Guideline 471 (1997).

The test substance was fully soluble at all concentrations tested. Concentrations of greater than 50 µg/plate induced a marked bacteriotoxic effect such that the highest concentration which could be used for assessment purposes was 500 µg/plate (concentration range in the first experiment was 16-5000 µg/plate). For the second experiment, the concentration range tested was lowered to 1.6-500 µg/tube. There were no increases in mutation frequency induced by the test material either with or without S9 mix for any strain. Positive control substances (sodium azide, nitrofurantoin, 4-nitro-1,2-phenylene diamine, cumene hydroperoxide and 2-aminoanthracene) produced significant increases in the number of revertants in all strains tested.

Prothioconazole was concluded to be non-mutagenic in this assay.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-4
Purity:	99.5 %
Stability of the test compound:	The batch used was analysed prior to study initiation and approved for use during the test period. A stability test in the solvent did not detect a relevant change in the percent active ingredient.
Solvent used:	DMSO
Solvent/final concentration:	0.1 ml/plate

2. Control Materials

Solvent control: DMSO (0.1 ml/plate)

Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 1535	Sodium azide (Na-azide)	DMSO	10 µg/plate
TA 100	Nitrofurantoin (NF)	DMSO	0.2 µg/plate
TA 1537	4-nitro-l,2-phenylene diamine (4-NPDA)	DMSO	10 µg/plate
TA 98			0.5 µg/plate
TA 102	Cumene hydroperoxide (Cumene)	DMSO	50 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 1535	2-aminoanthracene (2-AA)	DMSO	3 µg/plate
TA 100			
TA 1537			
TA 98			
TA 102			

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism of the test substance.

Preparation: The S9 fraction was isolated from the livers of at least six adult male Sprague Dawley rats. For enzyme induction, the animals received a single intraperitoneal injection of Aroclor 1254, dissolved in corn oil, at a dose of 500 mg/kg body weight, five days prior to sacrifice and liver preparation (Preparation of S9 fraction: November 7, 1994; protein content, 26.3 mg/ml).

Prior to first use, the batch was checked for sterility and its metabolizing capacity.

The S9 mix was freshly prepared before use and used only on the same day. For this purpose, a sufficient amount of S9 fraction is slowly thawed and mixed with the cofactor solution.

70 ml of cofactor solution are composed as follows:

$MgCl_2 \times 6\ H_2O$	162.6 mg
KCl	246.0 mg
glucose-6-phosphate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
phosphate buffer	100.0 mM

The S9 mix comprises 70 % cofactor solution, 10 % S9 fraction and additional 20% KCl (0.15 M).

4. Test organisms: *Salmonella typhimurium* strains TA1535, TA100, TA1537, TA98, TA102.

All strains are checked for crystal-violet sensitivity (rfa) and all strains except TA 102 (not required for TA 102) are checked for UV sensitivity (uvrB). In each individual test, histidine dependence of the cultures was automatically checked by the accompanying negative controls. A special test for ampicillin resistance was not necessary since strains TA 100, TA 98 and TA 102 were incubated on ampicillin containing nutrient agar and formed individual colonies. Consequently surviving bacteria were ampicillin resistant. A special test for tetracycline resistance was not necessary since TA 102 was incubated on nutrient agar containing in addition to ampicillin tetracycline and formed individual colonies. Consequently surviving bacteria were also tetracycline resistant.

5. Test concentrations:

Plate incorporation assay: 0, 16, 50, 158, 500, 1581, 5000 µg prothioconazole/plate
Pre-incubation assay: 0, 1.6, 5, 16, 50, 158, 500 µg prothioconazole/tube

B. TEST PERFORMANCE

1. Dates of experimental work: November 17, 1995 – December 04, 1995

2. Salmonella/microsome test

For each test and strain 1 ml portion of stock culture was thawed and 0.2 ml of the thawed culture were added to 10 ml nutrient broth. This culture was incubated overnight at 37°C and used only on the same day. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per milliliter, since the chosen method of incubation normally produces the desired density.

Plate incorporation assay

To test tubes containing 2 ml portions of warm soft agar, 0.1 ml test substance solution or solvent, 0.1 ml bacterial culture, 0.5 ml S9 mix (in tests with metabolic activation) or 0.5 ml buffer (in tests without metabolic activation) were added. After mixing, the samples were poured onto petri dishes with solid agar. After incubation for 48 h at 37°C, his+ revertants were counted. Three test plates per concentration or per control incubation were carried out.

Pre-incubation assay

For the pre-incubation assay 0.1 ml of test substance solution or solvent, 0.1 ml bacterial suspension and 0.5 ml S9 mix or buffer were incubated at 37°C for 20 minutes. The amount of solvent per plate used for 1.6 and 5 µg prothioconazole/plate was reduced to 0.01 ml/plate due to the range of the stability test with prothioconazole. The lacking volume of 0.09 ml solvent was added directly to the tubes. Subsequently, 2 ml of molten soft agar was added and, after mixing, the samples were poured onto petri dishes with solid agar. After incubation in the dark for 48 h at 37°C the bacterial colonies were counted. Three test plates per concentration or per control incubation were carried out.

4. Statistics

Descriptive statistical methods were used to calculate means and standard deviations.

5. Evaluation criteria

Solubility: If observed, precipitation of the test substance would have been recorded.

Toxicity: The toxicity of the test substance was assessed in three ways:

by gross appraisal of background growth on the plates for mutant determination

by mutant count per plate (if marked and dose-dependent reduction in the mutant count per plate compared to the negative controls appears, a toxic effect is assumed)

by titer determination (To establish the number of viable cells total bacterial counts were taken on two plates for each concentration studied with S9 mix. The dilution of bacterial suspensions used for the determination of titers was 1:1,000,000. Titers were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased fivefold to permit the complete growth of bacteria.)

Mutagenicity: A reproducible and dose-related increase in mutant counts of at least one strain is considered to be a positive result, if:
for TA 1535, TA 100 and TA 98 this increase is about twofold compared to negative controls
for TA 1537 this increase is at least threefold compared to negative controls
for TA 102 an increase of about 150 mutants is reached.
Otherwise, the result is evaluated as negative.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations verified that prothioconazole is stable in the solvent at room temperature at concentrations ranging from 0.1 mg/ml to 50 mg/ml for at least four hours.

Table 5.4.1/01- 1: Analysis for stability of prothioconazole in the solvent at room temperature

Nominal value in mg/ml	Content in % after storage time	
	0 hrs	4 hrs
0.1	100.0	100.0
50	92.9	99.2

B. TOXICITY AND SOLUBILITY

Concentrations of greater than 50 µg/plate induced a marked bacteriotoxic effect such that the highest concentration which could be used for assessment purposes was 500 µg/plate.

The test substance was fully soluble at all concentrations tested.

C. MUTATION ASSAY

There were no increases in mutation frequency induced by prothioconazole either with or without S9 mix for any strain in both the plate incorporation assay and the pre-incubation assay. Positive control substances produced significant increases in the number of revertants in all strains tested.

Table 5.4.1/01- 2: Ames test with prothioconazole – Mean number of revertants

Strain	TA 1535		TA 100		TA 1537		TA 98		TA 102	
Metabolic activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Plate incorporation assay										
Negative control	8	15	106	124	8	13	17	31	238	278
Test substance (µg/plate)										
16	8	17	91	121	9	13	23	31	225	287
50	10	13	105	110	10	15	22	37	225	287
158	9 [A]	19 [A]	89 [A]	114 [A]	7 [A]	14 [A]	15 [A]	45 [A]	224 [A]	244 [A]
500	7 [A,B]	12 [A,B]	74 [A,B]	81 [A,B]	6 [A,B]	10 [A,B]	14 [A,B]	47 [A]	185 [A,B]	263 [A,B]
1581	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	5 [A,B]	14 [A,B]	81 [A,B]	126 [A,B]
5000	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]	- [A,B]
Positive control[#]	839	204	276	1543	125	276	146	1542	445	677
Pre-incubation assay										
Negative control	9	14	90	105	9	9	45	58	267	280
Test substance (µg/tube)										
1.6	9	13	82	100	8	7	48	58	268	299
5	8	10	93	85	7	8	43	69	280	322
16	11	12	74	93	10	7	46	62	276	269
50	9	11	92	103	11	7	52	70	207	268
158	9	8	86	89	10	10	48	62	259 [A]	264 [A]
500	6 [A,B]	9 [A,B]	55 [A,B]	67 [A,B]	5 [A,B]	5 [A,B]	24 [A,B]	26 [A,B]	190 [A,B]	228 [A,B]
Positive control[#]	714	258	324	1415	143	254	198	1139	560	598

[A] bacteriotoxic effect observed in titer determination at this concentration
[B] reduced background growth
[#] see Material and Methods (I.A.2.) above for compound and concentrations

III. CONCLUSION

Positive control substances produced significant increases in the number of revertants in all strains tested. There were no increases in mutation frequency induced by prothioconazole either with or without S9 for any strain.

Prothioconazole was concluded to be non-mutagenic in this assay.

Report:	KCA 5.4.1/04[redacted]; 1996; M-012273-01-1
Title:	JAU 6476 - Mutagenicity study for the detection of induced forward mutations in the V79- HPRT assay in vitro
Report No.:	25605
Document No.:	M-012273-01-1
Guideline(s):	OECD 476 (1984); EEC Directive 88/302/EEC; US-EPA OPPTS 870.5300 (1996)
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.4.1/04. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.4.1/02** (also in numbering of tables/figures).

Deviations: The following minor deviations from the current OECD guideline (2016) occurred: The number of cells treated and cultured was lower than required according to the current guideline (1.5×10^6 cells instead of at least 2×10^6 were cultured during the expression period; not 10 spontaneous mutants were maintained in every culture in all phases of the test). However, cell numbers were acceptable according to the contemporary guideline (1984) and differ only slightly from the ones required by the current guideline. Therefore those minor deviations are not considered to have influenced the study outcome.

Executive summary:

In a 1996 GLP study prothioconazole (batch no. NLL 6096-9.1, purity 99.8 %) was investigated in a gene mutation assay in Chinese hamster lung cells (V79) using the HPRT locus. Duplicate flasks of exponentially growing cells (4 x 10^6 cells/flask) were exposed for 5 hours to prothioconazole (dissolved in DMSO) at concentrations of 25-175 µg/ml (without S9 mix) or 75-200 µg/ml (with S9 mix). Cells were incubated for 7 days to allow for expression of mutant phenotype and were then plated in selective medium and incubated for 6-7 days before counting the number of 6-TG resistant colonies. Cytotoxicity and cloning efficiency were also assessed. A similar independent repeat assay was also performed (with slightly lower concentrations for the cells exposed without S9 mix (5-150 µg/ml)). Appropriate negative, vehicle and positive controls were also used and gave acceptable results. The study was compliant with OECD Guideline 476 (1984) and, apart from minor deviations (see above), complied also with the 2016 Guideline.

Dose levels were based on two preliminary cytotoxicity tests. In the first test, precipitation was recorded at ≥1250 µg/ml and marked cytotoxicity was recorded at ≥156 µg/ml with no cells surviving at ≥313 µg/ml. In the second test, dose-related cytotoxicity was recorded both with and without S9 mix, cells exposed without S9 mix showing greater sensitivity. In the gene mutation assays, cytotoxicity (reduced survival and reduced growth) was recorded at concentrations ≥100 µg/ml. There were no increases in mutant frequency at any of the cultures treated in the absence of S9 mix. In the presence of S9 mix, the mutant frequency was increased in one culture at 150 µg/ml in the second assay (the highest concentration used in the second assay). However this was at extreme cytotoxicity and there was no increase recorded in the duplicate culture at this concentration, nor in the equivalent cultures in the first assay therefore this increase is not considered to be toxicologically significant since it was not reproducible. When the results of the first and second assays combined were analysed statistically (Dunnett's test), there was a statistically significant ($p<0.05$) increase in mutation frequency at 125 µg/ml in the presence of S9 mix. However, no significant increase was recorded at 150 µg/ml and the mutant frequency at 125 µg/ml did not exceed the historical vehicle control range for assays with S9 mix. It is concluded that the statistically significant increase recorded at 125 µg/ml is not toxicologically significant.

Prothioconazole is considered non-mutagenic in a V79-HPRT gene mutation assay.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Prothioconazole

Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-9.1
Purity:	99.8 %
Stability of the test compound:	Prothioconazole was checked analytically in advance and the batch used was shown to be stable for the treatment period.
Solvent used:	Dimethylsulfoxide (DMSO)
Solvent/final concentration:	≤1 % (v/v) DMSO

2. Control Materials

Solvent control:	1 % (v/v) DMSO
Positive control -S9:	Ethylmethanesulfonate (EMS), final concentration: 900 µg/ml
Positive control +S9:	Dimethylbenzanthrazene (DMBA), final concentration: 20 µg/ml

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism.

Preparation: The S9 fraction was isolated from the livers of Aroclor 1254 induced Wistar rats. It was purchased from Cytotest Cell Research, Germany (protein content: 33.6 mg/ml).
For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with a cofactor solution (2:3). The S9 mix was kept on ice and used on the same day.
Final concentrations in the S9 mix:

S9 fraction	40.0 % (v/v)
Sodium phosphate buffer	60.0 % (v/v)
$MgCl_2 \times 6\ H_2O$	8 mM
KCl	33 mM
Glucose-6-phosphate (disodium salt)	5 mM
NADP (disodium salt)	1 mM

4. Test organism: Chinese hamster lung cells (V79)

5. Culture media:

Culture medium: Hypoxanthine-free Eagle's minimal essential medium supplemented with nonessential amino acids, 2 mM L-glutamine, MEM-vitamins, $NaHCO_3$-solution, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 % heat inactivated fetal calf serum (FCS)

Treatment medium: Culture medium with reduced serum content (2 % FCS)

Selection medium: Hypoxanthine-free culture medium with 10 µg/mL 6-thioguanine (6-TG)

6. Locus examined: HPRT

7. Test concentrations:

Pre-test for cytotoxicity: 1st test: 0, 19.5, 39.1, 78.1, 156, 313, 625, 1250, 2500 µg/ml (±S9)
2nd test: 0, 75, 100, 125, 150, 175 µg/ml (-S9)
0, 150, 175, 200, 225, 250 µg/ml (+S9)

1st gene mutation assay: 0, 25, 50, 100, 125, 150, 175 µg/ml (-S9)
0, 75, 100, 125, 150, 175, 200 µg/ml (+S9)

2nd gene mutation assay: 0, 5, 25, 50, 100, 125, 150 µg/ml (-S9)

0, 75, 100, 125, 150, 175, 200 µg/ml (+S9)

B. TEST PERFORMANCE

1. Dates of experimental work: February 20, 1996 – April 30, 1996

2. Test substance preparation

Stability of the compound in the vehicle in a range from 0.05 mg/ml to 250 mg/ml was analytically approved for at least twenty-four hours (Table 5.4.1/02- 1). Test substance solutions were prepared in vehicle immediately prior to cell treatment.

Table 5.4.1/02- 1: Analysis for stability of prothioconazole in the vehicle at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 hrs	24 hrs
0.5	93.0	111.5
250	95.5	108.4

3. Pre-test for cytotoxicity

Initially 8 concentrations of prothioconazole in the range 19.5 – 2500 µg/ml, plus vehicle control, with and without S9 activation were tested. Subsequently, 5 concentrations in the range 75 – 175 µg/ml without S9 and 150 – 250 µg/ml with S9 activation were tested.

For both tests exponentially growing V79 cells were plated in culture medium in a 250 ml flask ($4x10^6$ cells per flask, one culture per concentration). After attachment (16-24 hours later), cells were exposed to vehicle alone and to 5-8 concentrations of the test substance ranging from 19.5 µg/ml to 2500 µg/ml for 5 hours in treatment medium, both in the presence and absence of S9 metabolic activation.

Thereafter, cell monolayers were washed with phosphate buffered saline (PBS), trypsinized and replated in culture medium at a density of 200 cells into each of 3 Petri dishes (60 mm). These dishes were incubated for 6 days to allow colony development. Thereafter, colonies were fixed with 95 % methanol, stained with Giemsa and counted either by eye, excluding colonies with 50 cells or less, or with an automatic counter. Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

The concentration range of prothioconazole for the gene mutation assays was chosen according to the results of the cytotoxicity test with prothioconazole.

4. Gene mutation assays

Two independent gene mutation assays were performed as follows:

Cell treatment and expression period

Exponentially growing V79 cells were plated in culture medium in two 250 ml flasks per dose group ($4xl0^6$ cells per flask). This was defined as day 0 of the assay. After attachment (16-24 hours later), the cells were exposed to each test substance concentration for 5 hours in treatment medium. For treatment with metabolic activation 5 % S9 mix was added. The corresponding controls were incubated under the same conditions. An experiment without metabolic activation consisted of one negative control, one vehicle control, one positive control (EMS) and 6 test substance doses. An experiment with metabolic activation consisted of one negative control, one vehicle control, one positive control (DMBA) and 6 test substance doses. Two independent runs (both with and without metabolic activation) were performed.

Thereafter, cell monolayers were washed with PBS, trypsinized and replated in culture medium at a general density of $1.5x10^6$ cells in 250 ml flasks and at 200 cells into each of 3 Petri dishes (60 mm).

The Petri dishes were incubated (as a rule for 7 days) to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment ("Survival to Treatment").

The large flasks were incubated to permit growth and expression of induced mutations; cells were subcultured on days 4 and 7. At the first subculture the 2 cultures for each dose level and the controls were reseeded at generally $1.5x10^6$ cells into two 250 ml flasks each.

Seeding for selection and cloning efficiency

At the end of the expression period, the cultures from each dose level were reseeded at $3x10^5$ cells per dish (100 mm, a total of 8 dishes) in selection medium for selection of mutant cells. In addition, three dishes (60 mm) were seeded in culture medium at 200 cells/dish to determine the absolute cloning efficiency for each dose level. All dishes were incubated at 37°C in a humidified atmosphere with about 5 % CO_2 for 6 to 7 days.

Colony counting and determination of colony size distribution

After incubation for 6 to 7 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes. Colonies with 50 cells or less were excluded.

Calculations and processing of the data

The data listed in the tables of results are calculated as follows:

Relative survival (%)

$$\frac{\text{Average no. of colonies per treated culture}}{\text{Average no. of colonies per vehicle control dish}} * 100$$

Absolute population growth

$$\text{cell number day 4} * \text{cell number day 7 for each culture}$$

Relative population growth (%)

$$\frac{\text{Absol. Pop. Growth of treated culture}}{\text{Absol. Pop. Growth of corresponding vehicle control culture}} * 100$$

Absolute cloning efficiency (CE) (%)

$$\frac{\text{Average no. of viable colonies per dish}}{200} * 100$$

The absolute CE is expressed by the average number of viable colonies per dish (200 cells/dish seeded).

Mutant Frequency

$$\frac{\text{Total Number of Mutant Colonies} * 100}{\text{Number of Evaluated Dishes} * 3 * 10^5 * \text{CE}}$$

The mutant frequency is expressed as 6-TG resistant mutants per 10^6 clonable cells.

5. Statistics

The statistical analysis relies on the mutation frequencies rather than on individual plate counts which are submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights ([redacted]., 1981; [redacted]., 1989). Mutation frequencies based on less than 5 plate counts are very uncertain estimates of the true mutation frequencies and provide no basis for a rational estimation of the variance. Therefore, such values are not included in the statistical analysis. If the relative population growth in an experiment falls below 10 %, the corresponding mutation frequency is discarded.

The two mutation frequency values obtained per group in the standard HPRT assay are considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay requires at least two replicates, the overall analysis per type of metabolic activation is the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses will be run for each assay in order to examine the consistency of the results.

All groups are included in the weighted analysis of variance followed by pairwise comparisons to the vehicle control on a nominal significance level of $\alpha = 0.05$ using the Dunnett test. The regression analysis part is performed on the basis of the actual dose levels thereby omitting the positive, negative and vehicle controls. If there is a significant increase of the mutation frequency with dose ($\alpha = 0.05$) in the main analysis the highest dose group will be dropped and the analysis will be repeated until $p > 0.05$. Dose levels eliminated in that way are flagged correspondingly.

6. Acceptability criteria

Normally, an assay is only considered acceptable for evaluation if the following criteria are met:

- The assay (with and without metabolic activation) is repeated at least once independently.

Cloning efficiency:

- The average cloning efficiency of the negative and vehicle controls should be at least 50 %. Assays below 50 % cloning efficiency will be unacceptable.

Cytotoxicity

- Cytotoxicity is determined after treatment with the test substance by the assay parameter "Survival to Treatment". The highest test substance concentration should produce a low level of relative survival (0 – 30 %) or should be the first concentration where insolubility occurs. The survival at the lowest concentration should approximate the negative control.

Mutant frequency

- The background mutant frequency (average value for vehicle controls) in a trial should not exceed 25×10^6 cells. Assays with higher spontaneous mutant frequencies are not necessarily invalid, however, if all other criteria are fulfilled.
- An experimental mutant frequency is considered acceptable only if the absolute cloning efficiency is 10% or greater.
- Mutant frequencies for at least four concentrations of the test substance are routinely determined in each assay.
- Mutant frequencies are normally derived from sets of 8 dishes per parallel-culture of each dose level. To allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of 5 dishes.
- The positive control must induce an average mutant frequency of at least three times that of the vehicle control.

7. Evaluation criteria

An assay will be considered:

positive
- if a dose-dependent, significant and in parallel cultures reproducible increase in mutant frequency is observed.
  (It is desirable to obtain this dose-relationship for at least 3 doses. To be significant, the mutagenic response to the substance should be at least approximately two to three times that of the highest negative or vehicle control value observed in that trial.)
- if the result can be reproduced in a second assay
- if a reproducible increase greater than two times the minimum criterion is observed for a single dose near the highest testable concentration, the test substance is also considered mutagenic.
- if no significant change in osmolality compared to the vehicle control can be observed (otherwise, unphysiological culture conditions may be the reason for the positive result).

equivocal - if there is no dose-dependency but one or more doses induce a reproducible, significant mutant frequency in all assays.

negative - if none of the doses tested (for a range of applied concentrations which extends to sufficient toxicity) induces a reproducible mutant frequency which is considered significant.

II. RESULTS AND DISCUSSION

A. PRE-TEST FOR CYTOTOXICITY

In the first test, precipitation was recorded at ≥1250 µg/ml and marked cytotoxicity was recorded at ≥156 µg/ml with no cells surviving at ≥313 µg/ml. In the second test, dose-related cytotoxicity was recorded both with and without S9 mix, cells exposed without S9 mix showing greater sensitivity.

Table 5.4.1/02- 2: Results of the pre-tests for cytotoxicity

1st test				2nd test			
Dose (µg/ml)	**± S9**	**Survival (% control)**	**Cloning efficiency (%)**	**Dose (µg/ml)**	**± S9**	**Survival (% control)**	**Cloning efficiency (%)**
0$	-	100.0	73.8	0$	-	100.0	89.8
19.5		112.8	83.3	75		66.9	60.0
39.1		114.9	84.8	100		79.9	71.8
78.1		97.7	72.2	78.1		61.5	55.0
156		12.6	9.3	125		23.8	21.3
313		-	-	175		15.0	13.5
625		-	-	0$	+	100.0	53.3
1250		- P	-	150		88.6	47.2
2500		- P	-	175		89.2	47.5
0$	+	100.0	70.5	200		107.0	57.0
19.5		123.6	87.2	225		21.1	11.3
39.1		118.7	83.7	250		3.3	1.8
78.1		92.4	65.2				
156		99.5	70.2				
313		-	-				
625		-	-				
1250		- P	-				
2500		- P	-				

$ solvent control; - no cell survival; P precipitation of the test substance

B. GENE MUTATION ASSAYS

In the gene mutation assays, cytotoxicity (reduced survival and reduced growth) were recorded at concentrations ≥125 µg/ml or sometimes ≥100 µg/ml in each experiment. There were no increases in mutant frequency at any of the cultures treated in the absence of S9 mix. In the presence of S9 mix, the mutant frequency was increased in one culture at 150 µg/ml in the second assay (the highest concentration used in the second assay; Table 5.4.1/02- 4). However this was at extreme cytotoxicity

and there was no increase recorded in the duplicate culture at this concentration, nor in the equivalent cultures (150 and 175 µg/ml) in the first assay, therefore this increase is not considered to be toxicologically significant since it was not reproducible (Table 5.4.1/02- 3).

Table 5.4.1/02- 3: Results of the first gene mutation assay

Dose (µg/ml)	Metabolic activation	Survival (% control)	Relative growth (% control)	Cloning efficiency (%)	Mutant frequency (x 10⁻⁶)
0	-	117.8	109.7	61.8	1.5
0		100.2	124.6	55.2	1.0
0$		100	100	55.5	1.3
0$		100	100	72.5	9.2
25		112.7	121.4	58.3	1.6
25		127.1	107	68.2	8.6
50		99.0	131.2	55.8	3.0
50		114.9	101.7	68.3	7.0
100		89.8	96.3	78.8	9.7
100		112.3	77.3	83.7	1.5
125		66.2	65.3	61.3	6.8
125		72.4	50.6	86.2	0.0
150		36.3	[a]	97.0	9.5
150		48.0	-	[n]	-
175		-	-	[n]	-
175		10.1	-	[n]	-
EMS 900		102.9	47.2	49.7	839.8
EMS 900		103.9	52.7	66.0	587.8
0	+	99.1	107.2	52.5	0.8
0		92.7	99.7	55.7	0.7
0$		100	100	53.3	4.5
0$		100	100	53.2	5.5
75		-	139.1	46.7	1.8
75		124.8	101.4	50.3	8.3
100		96.1	73.0	48.3	0.9
100		101.7	123.8	41.8	5.0
125		105.7	125.2	46.8	4.4
125		78.9	62.4	72.3	2.3
150		85.2	167.4	50.5	0.8
150		61.6	70.4	76.5	3.6
175		36.6	68.8	59.3	0.7
175		54.7	50.4	79.5	3.7
200		4.1	-	[n]	-
200		0.2	-	[n]	-

Dose (µg/ml)	Metabolic activation	Survival (% control)	Relative growth (% control)	Cloning efficiency (%)	Mutant frequency (x 10^{-6})
DMBA 20		89.3	119.8	39.5	35.9
DMBA 20		99.4	79.4	45.3	35.8

$ solvent control; [a] no calculation due to low cell number; [n] not cloned due to cytotoxicity

Table 5.4.1/02- 4: Results of the second gene mutation assay

Dose (µg/ml)	Metabolic activation	Survival (% control)	Relative growth (% control)	Cloning efficiency (%)	Mutant frequency (x 10^{-6})
0	-	128.7	99.5	62.5	12.0
0		84.4	118.7	71.3	8.7
0$		100	100	82.2	7.1
0$		100	100	72.8	6.9
5		109.6	140.2	69.0	6.0
5		72.5	87.9	73.8	6.8
25		96.6	129.2	59.0	6.4
25		106.7	93.4	66.8	8.1
50		55.6	107.6	51.8	11.3
50		75.5	96.5	68.5	5.5
100		74.8	70.5	53.8	5.4
100		80.4	57.3	47.3	3.5
125		70.8	66.7	56.7	0.7
125		49.0	15.9	65.0	9.0
150		39.5	31.2	57.8	4.3
150		5.9	-	[n]	-
EMS 900		55.0	32.7	47.7	971.2
EMS 900		74.5	23.5	66.7	710.6
0	+	80.5	86.9	82.0	2.0
0		108.7	116.2	66.0	1.3
0$		100	100	77.3	0.5
0$		100	100	83.8	2.0
75		78.1	87.1	62.2	2.0
75		92.7	84.1	54.7	5.3
100		91.2	102.5	117.3	3.6
100		80.8	100.5	68.3	3.0
125		62.1	58.7	96.7	3.9
125		65.9	23.4	85.3	5.4
150		45.2	50.1	68.2	3.1
150		11.9	8.1	69.8	13.7
175		5.4	-	[n]	-
175		0.2	-	[n]	-

Dose (µg/ml)	Metabolic activation	Survival (% control)	Relative growth (% control)	Cloning efficiency (%)	Mutant frequency (x 10-6)
200		0.0	-	[n]	
200		0.0	-	[n]	-
DMBA 20		78.4	65.2	70.7	72.8
DMBA 20		98.0	58.6	60.2	59.6

$ solvent control; [n] not cloned due to cytotoxicity

When the results of the first and second assays combined were analysed statistically (Dunnett's test), there was a statistically significant ($p<0.05$) increase in mutation frequency at 125 µg/ml in the presence of S9.

Table 5.4.1/02- 5: Weighted ANOVA and regression results

± S9	P values weighted		Concentration (µg/ml)						
	ANOVA	Regression	PC	5	25	50	100	125	150	175
-	<0.001	0.019	*				-	-	-	m
			PC	75	100	125	150	175	200	
+	<0.001	0.019	*	-	-		-	-	-	

* significant (α=0.05) increase relative to vehicle control using the Dunnett-test
m missing value due to cytotoxicity of the test substance
PC positive control

However, no significant increase was recorded at 150 µg/ml and the mutant frequencies (per 10^6 clonable cells) at 125 µg/ml (2.3 and 4.4 in the first assay, 3.9 and 5.4 in the second assay) did not exceed the historical vehicle control range for assays with S9 mix (range 0.5 - 26.7, for 17 experiments performed between February 1994 - April 1995). The vehicle control mutant frequencies in this study were 4.5 and 5.5 in the first assay and 0.5 and 2.0 in the second assay. It is concluded that the statistically significant increase recorded at 125 µg/ml is not toxicologically significant.

III. CONCLUSION

The isolated changes observed in mutant frequency were only of statistical but not of toxicological significance. Prothioconazole was not mutagenic in a V79-HPRT gene mutation assay.

Report: KCA 5.4.1/03[redacted]; 1998; M-012317-01-1
Title: JAU 6476 - Test on unscheduled DNA synthesis in rat liver primary cell cultures in vitro
Report No.: 27417
Document No.: M-012317-01-1
Guideline(s): EEC Directive 88/302/EEC; OECD 482 (1986); US-EPA712-C-96-230, OPPTS 870.5550 (1996)
Guideline deviation(s): none
GLP/GEP: **yes**

Deviations: Deviations from the current OECD guideline (1986):

none

Executive summary:

In a 1998 GLP study, the potential for prothioconazole (batch no. NLL 6096-12, purity 99.7 %) to induce unscheduled DNA synthesis (UDS) was investigated in primary rat hepatocyte cultures. Cultures were exposed to prothioconazole (dissolved in DMSO) at concentrations of 0-40 µg/ml (based on a cytotoxicity test) along with tritiated thymidine. The exposure period was 16-24 hours, following which cells were coated with photographic emulsion and stained. Grains were counted from 50 cells/slide and from three slides per group (isolated nuclei and cells with abnormal morphology were not evaluated). An independent repeat assay was performed at concentrations of 0-20 µg/ml and a positive control (2-AAF) was also included. The study was compliant with OECD Guideline 482 (1986).

Due to cytotoxicity 20 µg/ml was the highest concentration which could be evaluated. In the first assay, there was an increase in the nuclear net grain count (NNG) in all treated cultures, but there was no dose-response relationship. The NNG values were greater than zero, but did not exceed +0.5 (+0.5 was the performing laboratory's normal minimum response to be considered a marginal response, with +2.0 considered to be a clear positive). The number of cells in repair was statistically significantly increased (but below the 5 % laboratory criterion for a positive response) at 5.0 and 10.0 µg/ml only but not at higher concentrations and without dose-response relationship. Survival was lower than vehicle controls in all treated cultures but a clear dosage-related response was not established. This result is classified as equivocal since a dose-response relationship was not established for both the only marginal increases in NNG and percentage of cells in repair. A second assay was initiated to clarify these findings. In the second assay, an increase in the NNG was also recorded, with values greater than zero at ≥7.5 µg/ml, but there was no clear dose-response relationship. NNG values ranged from -0.60 to +0.95. Survival was lower at all treated concentrations but again there was no clear dose-response relationship. The number of cells in repair was statistically significantly increased at 10.0 and 15.0 µg/ml, but not at 12.5 µg/ml or 20.0 µg/ml nor at other concentrations. The positive control substance gave a satisfactory response in both assays.

The overall test result is equivocal, based on the fact that the increases in NNG and in cells in repair were marginal and/or there was no dose-response relationship.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Prothioconazole

Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-12
Purity:	99.7 %
Stability of the test compound:	The batch used was analysed prior to study initiation and approved for use during the test period. There was no evidence of a relevant change in the percentage of active ingredient following a stability test in solvent.
Solvent used:	Dimethylsulfoxide (DMSO)
Solvent/final concentration:	1 % (v/v) DMSO

2. Control Materials

Solvent control:	1 % (v/v) DMSO
Positive control:	2-Acetylaminofluorene (2-AAF); final concentration: 1.0 µg/ml (solvent: DMSO)

3. Test organism:	Primary rat hepatocytes from young adult male Sprague-Dawley rats
Preparation of cells:	A single animal was used for each trial. The cells were obtained by perfusing the rat liver in situ with a collagenase solution. Monolayer cultures were established on plastic coverslips in culture dishes and used on the same day to initiate the UDS assay.
Culture medium:	Williams Medium E, supplemented with L-glutamine (2 mM), gentamycin sulfate (50 µg/ml), dexamethasone (2.4 µM) and 10 % heat-inactivated fetal calf serum (FCS).
Treatment medium:	Williams Medium E, supplemented with L-glutamine (2 mM) and a reduced serum content of 1 % FCS.
Culture conditions:	37°C in a humidified atmosphere containing approx. 5 % CO_2
4. Test concentrations:	
Pre-test for cytotoxicity:	0, 2, 3.9, 7.8, 15.6, 31.0, 63.0, 125.0, 250.0 µg/ml
1st UDS assay:	0, 1.0, 5.0, 10.0, 12.5, 15.0, 20.0, 40.0 µg/ml
2nd UDS assay:	0, 0.5, 5.0, 7.5, 10.0, 12.5, 15.0, 20.0 µg/ml

B. TEST PERFORMANCE

1. Dates of experimental work: May 15, 1997 – December 01, 1997

2. Test substance preparation and solubility in culture medium

Prothioconazole was dissolved in DMSO. A clear solution was obtained up to a concentration of 250 mg/ml. However, there was precipitation of the test substance after addition of this solution to the medium starting at a final concentration of 313 µg/ml. Test substance solutions were prepared immediately prior to cell treatment.

The stability of prothioconazole in DMSO at room temperature at concentrations ranging from 0.5 mg/ml to 250 mg/ml for at least twenty-four hours was analytically verified (Table 5.4.1/03- 1).

Table 5.4.1/03- 1: Analysis for stability of prothioconazole in DMSO at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 hrs	24 hrs
0.5	93.0	111.5
250	95.0	108.4

3. Dose selection

A preliminary cytotoxicity test was performed to evaluate the dose range of the test substance in the UDS assay. Treatments were initiated by replacing the culture medium on the hepatocyte cultures with treatment medium containing the test material in the desired concentrations. The experimental design of the cytotoxicity test was identical to the procedure described below for the UDS assay.

A series of 8 concentrations of prothioconazole ranging from 2 µg/ml to 250 µg/ml was applied to the cells (two 60 mm Petri-dishes per dose group, 750000 living cells per dish with a viability of 80.5 % after isolation). After 18.5 hours, a viable cell count (trypan blue exclusion) was obtained. Cytotoxicity was expressed by comparison of viable cells in treated cultures versus vehicle control cultures (relative survival to treatment).

After determining the cytotoxicity of prothioconazole, the concentration range was chosen for the genotoxicity study, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies, and proceeding to successively lower doses.

4. Experimental design

Two independent UDS assays were performed as follows:

Liver perfusion and preparation of rat hepatocytes

A single animal was used for each assay. The cells were obtained by perfusing the rat liver in situ with a collagenase solution. After perfusion, primary hepatocytes were prepared according to the protocol of Butterworth et al. (1987) under sterile conditions. Cell preparations used for the UDS assay were single-cell suspensions with good viability (determination of cell viability and cell concentration by trypan blue exclusion).

The cells were seeded as follows:

1. For determination of cytotoxicity: two 60 mm-Petri dishes (7.5x10^5 viable cells per dish) precoated with collagen were available for each dose group as well as for the positive and negative controls. Two additional dishes were seeded to determine cell viability, attachment rate and morphology about 1.5 hours after establishment of the cultures.
2. For determination of genotoxicity: a 25 mm round plastic coverslip precoated with collagen was placed into each well of 6-well culture dishes. Approx. 3.75x10^5 viable cells were seeded per well (in 2.5 ml culture medium), whereby 3 wells per dose group including the control groups were established.

For cell-attachment all cultures were incubated for 90-120 min in a 37°C incubator in a humidified atmosphere containing approximately 5 % CO_2.

Culture labelling and treatment

After the attachment period, the cultures were washed with phosphate buffered saline (PBS) to remove unattached cells. Cell number and viability of the cultures were determined by the method of trypan blue exclusion employing the two additional 60 mm-Petri dishes.

Test substance solutions were prepared in the vehicle and applied to the cells in two 60 mm dishes per dose group for determination of cytotoxicity. The medium in the 6-well dishes (cultures for determination of genotoxicity) was replaced by treatment medium containing the dissolved test chemical in the indicated concentrations and 10 µCi/ml ^{3}H-thymidine (15.3-15.6 Ci/mmole). The cultures were then placed in the incubator for 16-24 hours.

Thereafter, the cultures were washed twice with PBS in the 6-well dishes. Subsequently, a 1 % sodium citrate solution was added to swell the nuclei. The cells on the coverslips were then fixed, washed with deionized distilled water and air dried.

Determination of cytotoxicity

At 16-24 hours after initiation of treatment, viable cell counts (trypan blue exclusion) were determined in the two 60 mm-Petri dishes available for each treatment condition. Cell survival for the dose groups was estimated in relation to the negative controls.

Autoradiography and Staining

The whole autoradiography-procedure was performed in the dark. Air-dried coverslips were mounted cell-side-up on microscope slides. In a darkroom, these were dipped in a NTB-2 photographic emulsion and left to dry in the air overnight. The next day, the coated slides were stored in light-tight boxes in the presence of a drying agent for 4-10 days at -20°C in order to reduce the cytoplasmic grains background and therefore to increase the sensitivity of the assay. The photographic emulsion was then developed at temperatures below 15°C. The slides were rinsed afterwards with distilled water, fixed and air dried. Slides were then stained with hematoxylin and eosin.

Grain counting

Evaluation was performed with coded slides. Each slide was examined by counting 50 cells per slide, normally 3 slides per dose group (total of 150 nuclei for each treatment condition). Only cells viable at the time of fixation were scored; isolated nuclei and cells with abnormal morphology were excluded. A starting point was randomly selected on each slide, and cells were scored in a regular fashion by bringing new cells into the field of view, moving along the X-axis. If the total number of 50 cells had not been reached before coming to the edge of the slide, the stage was moved on the Y-axis, and counting resumed in the opposite X-direction, parallel to the first line.

UDS was measured by counting nuclear grains and subtracting the average number of grains in 3 cytoplasmatic areas of the same size as the corresponding nucleus. This value was referred to as the net nuclear grain count of the cell. The mean net nuclear grain count per concentration was routinely determined from triplicate coverslips. The number of cells in repair (nuclei with 5 or more net grains) was also determined.

5. Data presentation and assay evaluation

Net grains per nucleus:	Average no. of the mean net nuclear grain counts of each evaluable coverslip, 50 cells per coverslip
Mean grains per nucleus:	Average no. of the mean nuclear grain counts of each evaluable coverslip, 50 cells per coverslip.
Mean cytoplasmic grain count:	Average no. of the mean cytoplasmic grain counts (3 areas per cell) of each evaluable coverslip, 50 cells per coverslip
% Nuclei with 5 or more grains:	(No. of cells with 5 or more net nuclear grain counts per dose/ no. of evaluated cells per dose) x 100
Survival (%):	No. of viable cells relative to vehicle control.

1. For each of the 50 cells on each slide, the number of nuclear grains (NG) was scored, as well as numbers of three cytoplasmic grain counts from nuclear-sized areas adjacent to each nucleus.

2. A number of 5 net nuclear grains or more was chosen as a conservative estimate as to whether a particular cell is responding (cell "in repair").

3. A minimum of 4 - 5 dose levels were analysed for NG. Repeat trials need only augment the number of analysed dose levels in the first trial to achieve a total of five different concentrations.

4. Only cells viable at the time of fixation and with nuclei evenly coated with emulsion were scored. Cells with abnormal morphology, such as those with pyknotic or lysed nuclei, were not counted. Isolated nuclei not surrounded by cytoplasm were not counted.

5. S-phase cells having dense NG were excluded, however, the number of S-phase cells was noted.

6. Statistics

The mean cytoplasmic count from each evaluated cell was subtracted from the nuclear count to derive the NG. For each slide, the mean and standard deviation for NG was calculated, as was the percentage of cells in repair.

An evaluation was made of the percentage of cells in repair per dose group compared to the negative control using a one-sided 2x2-chi^2-test corrected for continuity. To assess the statistical significance of a result, the square root of the test statistic was compared to the upper 95 % quantile ($P \leq 0.05$) of the normal standard distribution.

7. Acceptance and assessment criteria

An assay is normally considered acceptable for evaluation only if the following criteria are satisfied. However, these criteria may be overruled by good scientific judgment.

Viability

- The viability of the hepatocytes collected by this process normally exceeds 70 %, although values between 50 % and 70 % viability can also be acceptable. Trials below 50 % are considered unacceptable, to avoid the possible use of a damaged cell population.
- The viability of the monolayer cell cultures used for the UDS assay must be 75 % or greater. Normally, the viability of attached cells is about 85 %.
- The number of viable cells in the negative (vehicle) control cultures should remain reasonably stable over the experimental time period because rapidly declining (dying) cultures may not respond in a representative manner to the test substance treatments. Therefore, the number of viable cells in the negative control cultures must be 60 % or greater after 16-24 hours.

Nuclear grain counts

- Grain count data obtained for a given treatment are acceptable as part of the evaluation if obtained from at least two replicate cultures and at least fifty cells per culture.
- The highest analysed dose must approach an excessive cytotoxicity of about 50 % or result in test material insolubility, or reach the highest applicable dose of 5 mg/ml.
- The average NG in the negative control cultures should range between -6 to 0. No more than 5 % of the cells should be in repair.
- The positive control 2-AAF is used to demonstrate the responsiveness of the cell population employed and the adequacy of the method for the detection of UDS. For the positive control 2-AAF (1.0 µg/ml), one might expect mean values of 6-20 NG with 60-100 % of the cells with greater than or equal to 5 NG.
- An experiment is considered invalid if cytoplasmic background counts of control cultures exceed 30 grains per nuclear-sized area.

For the conditions described a response is considered positive if a chemical yields +2 NG or more (population average) and more than 5 % of the cells responding.

A population average of between +0.5 NG and +2 NG can be considered a marginal response. A positive dose-response relationship in both NG and the percentage of cells in repair is required as additional information to confirm a positive response for counts below +2 NG. In this case, an additional experiment might be necessary.

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

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

There was a dose-related decrease in cell viability at all concentrations with marked cytotoxicity at 31 µg/ml; hence a dose range of 0-40 µg/ml was selected for the main study.

Table 5.4.1/03- 2: Cytotoxicity assay for dose selection

Dose (µg/ml)	Dish No.1 (Cells x10^6)	Dish No.2 (Cells x10^6)	Average number of cells (x10^6)	Viable cells (%)	Relative survival (%)[a]
0$	0.61	0.54	0.58	80.3	100.0
2	0.83	0.52	0.67	74.0	92.2
3.9	0.66	0.52	0.59	67.2	83.7
7.8	0.44	0.47	0.46	68.8	85.7
15.6	0.62	0.35	0.48	52.1	64.8
31.0	0.18	0.19	0.19	6.9	8.6
63.0	0.11	0.21	0.16	0.0	0.0
125.0	0.25	0.16	0.20	0.0	0.0
250.0	0.27	0.18	0.23	0.0	0.0

[a] relative to solvent control; $ solvent control

B. UNSCHEDULED DNA SYNTHESIS ASSAY

Cytotoxicity

Viability after isolation and attachment was 89.3 % and 85.1 % respectively in the first assay, and 74.9 % and 80.5 % respectively in the second assay, which is acceptable.

In the first assay at a concentration of 40 µg/ml no slides were evaluable due to cytotoxicity of prothioconazole. Therefore, 6 concentrations of prothioconazole covering a good range of cytotoxicity (77.6 % to 89.2 % survival) were available for analysis of nuclear labelling (Table 5.4.1/03- 3). Cytotoxicity and moderate cytotoxicity were observed at concentrations ranging from 5 µg/ml to 20 µg/ml, whereas 1 µg/ml was non-toxic. The positive control 2-AAF was non-cytotoxic in this experiment.

In the second assay 7 concentrations of prothioconazole ranging from 0.5 µg/ml to 20 µg/ml were tested. The viability in the control cultures was 86.6 %, which corresponds to 107.6 % of the viability after attachment. This high viability and the normal morphological appearance of the cells indicated that the hepatocyte cultures were in good condition for the UDS assay. All 7 concentrations of prothioconazole covering a good range of cytotoxicity (65.3 % to 85.0 % survival) were available for analysis of nuclear labelling. Cytotoxicity and moderate cytotoxicity were observed at concentrations ranging from 5 µg/mL to 20 µg/ml, whereas 0.5 µg/ml was non-toxic. The positive control 2-AAF was moderately cytotoxic in this experiment.

UDS assay

In the first assay, there was an increase in the nuclear net grain count (NNG) in all treated cultures, but there was no dose-response relationship (Table 5.4.1/03- 3). The NNG values were greater than zero, but did not exceed +0.5. The number of cells in repair was statistically significantly increased at 5.0 and 10.0 µg/ml, but not at other concentrations and again there was no relationship to dose. Survival was lower than vehicle controls in all treated cultures (ranging from 89.2% at 1.0 µg/ml to 78.3 % at 20 µg/ml) but a clear dosage-related response was not established. This result is classified as equivocal since a positive dose-response relationship was not established for both the increase in NNG and the percentage of cells in repair. A second assay was initiated to clarify these findings.

In the second assay, an increase in the NNG was also recorded, with values greater than zero at ≥7.5 µg/ml, but there was no clear dose-response relationship. NNG values ranged from -0.60 to +0.95. Survival was lower at all treated concentrations but again there was no clear dose-response relationship.

The number of cells in repair was statistically significantly increased at 10.0 and 15.0 µg/ml, but not at 12.5 µg/ml or 20.0 µg/ml nor at other concentrations.

The positive control substance gave a satisfactory response in both assays.

Table 5.4.1/03- 3: Rat liver UDS assay

Concentration (µg/ml)	Net grains per nucleus ± SD	Mean grains per nucleus ± SD	Mean cytoplasmic grain count ± SD	Mean cells in repair (%)	Survival (%)[a]
1st assay					
0 (solvent)	-1.41 ± 1.74	2.74 ± 2.01	4.15 ± 1.88	0.00	100
1.00	0.28 ± 2.34	5.60 ± 3.11	5.32 ± 2.62	2.67	89.2
5.00	0.44 ± 2.82	8.20 ± 3.71	7.76 ± 2.57	4.00*	83.3
10.0	0.45 ± 2.24	5.94 ± 2.80	5.49 ± 2.07	3.33*	85.9
12.5	0.48 ± 2.09	4.41 ± 2.37	3.93 ± 1.46	2.67	81.8
15.0	0.24 ± 1.95	3.57 ± 2.83	3.33 ± 1.90	2.67	77.6
20.0	0.07 ± 1.59	2.39 ± 1.77	2.32 ± 0.96	0.00	78.3
40.0	-	-	-	-	1.50
2-AAF 1µg/ml	7.51 ± 3.66	12.55 ± 4.20	5.04 ± 1.88	78.0*	92.0
2nd assay					
0 (solvent)	-1.72 ± 2.17	5.65 ± 2.94	7.37 ± 2.52	0.00	100
0.50	-0.60 ± 2.54	5.63 ± 3.26	6.23 ± 2.73	0.67	85.0
5.00	-0.54 ± 2.13	4.95 ± 2.47	5.49 ± 2.12	0.67	80.6
7.50	0.22 ± 2.38	6.11 ± 3.02	5.89 ± 2.49	2.00	73.3
10.0	0.51 ± 2.28	5.57 ± 3.11	5.06 ± 2.24	3.33*	71.5
12.5	0.27 ± 2.01	6.53 ± 2.99	6.26 ± 2.42	1.33	75.5
15.0	0.95 ± 2.33	8.21 ± 3.26	7.26 ± 2.66	6.00*	65.3
20.0	0.06 ± 2.16	5.42 ± 2.90	5.36 ± 2.17	2.00	75.4
2-AAF 1µg/ml	7.32 ± 3.46	14.39 ± 4.29	7.07 ± 2.49	74.67*	83.7
Historical control data ranges [b]					
DMSO	-2.2 – -0.6	0.4 – 4.3		0.0 – 2.0	
2-AAF 1µg/ml	6.1 – 9.95	7.5 – 14.2		67.3 – 93.3	

[a] relative to solvent control; [b] controls of 13 experiments performed Feb. 1995 – May 1997
* $p < 0.05$ (chi^2 test); - no slides evaluable

III. CONCLUSION

The test result is equivocal, based on the fact that the increases in NNG and in cells in repair were marginal and/or there was no dose-response relationship.

Report:	KCA 5.4.1/02[redacted],; 1996; M-012277-01-1
Title:	JAU 6476 - In vitro mammalian chromosome aberration test with Chinese hamster V79 cells
Report No.:	25718
Document No.:	M-012277-01-1
Guideline(s):	OECD 473 (1983); EEC Directive 92/69/EEC B.10.; US-EPA "In vitro mammalian cytogenetics" (1986)
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.4.1/02. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.4.1/04** (also in numbering of tables/figures).

Deviations: The following deviations from the current OECD guideline (2016) were noted:
- Only 200 metaphases were scored (instead of 300).
- In this assay the mitotic index was used to detect cytotoxicity. According to the current guideline this is not the appropriate method to detect cytotoxicity in cell lines. Instead, Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) are recommended as appropriate methods for the assessment of cytotoxicity in cell lines in cytogenetic tests. In addition in this assay the survival index was determined. This parameter is no longer recommended by the current guideline because it can underestimate cytotoxicity.

However, these deviations do not compromise the study results.

Executive summary:

In a 1996 GLP study, the potential clastogenicity of prothioconazole (batch no. NLL 6096-9.1, purity 99.8 %) was investigated in Chinese hamster lung cells (V79). Duplicate cultures were exposed to the test substance (dissolved in DMSO) in the presence and absence of an exogenous metabolic activation system. Colcemid was used to arrest mitosis two hours prior to the end of the incubation period. Chromosomes of 100 metaphases per culture (200 metaphases per concentration) were scored for aberrations. Mitotic index was determined from 1000 cells per culture. The stability of the test substance in the solvent was confirmed by analysis. The study was compliant with OECD guideline 473 (1983). The deviations from the current (2016) guideline do not compromise the study results.

Based on the results of an initial cytotoxicity test, concentrations up to 150 µg/ml were used in two independent chromosome aberration assays. The first assay was performed using concentrations of 0-150 µg/ml, with a 4 hour exposure period and 18 hour and 30 hour harvest times. The mitotic index was reduced at 150 µg/ml at the 30 hour harvest. Cell survival was statistically significantly reduced at ≥50 µg/ml. There were marked increases in the number of cells with aberrations at 150 µg/ml without S9 (at both harvest times). There were also smaller but statistically significant increases at 75 and 100 µg/ml without S9 (considered to be equivocal due to the small magnitude of the increases and the absence of a dose-response relationship). With S9 in the first assay, there were clear increases at 150 µg/ml only (at both harvest times). On the basis of the results of the first assay, a second assay was performed as an attempt to clarify whether the increases without S9 at 75 and 100 µg/ml were biologically relevant and to investigate whether the effects were due to excessive cytotoxicity. In the second assay, cells were harvested after 8 hours (with and without S9) and 18 hours (without S9 only), respectively, following 4 hours exposure to 75-150 µg/ml or 50-100 µg/ml, respectively. The mitotic index was significantly reduced at the 8 hour harvest time, but not at the 18 hour harvest time, thus suggesting that in the absence of S9 there was a marked cytotoxic effect early in the culture phase only. Only cultures at 18 hours harvest (without S9) were assessed for aberrations. Increases in cells with aberrations were recorded at all concentrations in the second assay. However, the increases were not dosage-related. The positive control substances (mitomycin C without S9 and cyclophosphamide with S9) gave satisfactory results in both assays. The number of cells with polyploidy was similar between treated cultures and controls.

Prothioconazole induced chromosome aberrations (with and without metabolic activation) under the conditions of this study. Since aberrations only occurred at cytotoxic concentrations, since the cytotoxicity may have been underestimated (see guideline deviations) and since the increases were not concentration-related, it is likely that the clastogenicity seen is an indirect effect triggered by cytotoxicity.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Prothioconazole
Synonym: JAU 6476
Description: White powder
Batch No.: NLL 6096-9.1
Purity: 99.8-99.9 %
Stability of the test compound: The batch used was analytically examined and was approved for use during the test periods. A stability test in the solvent did not reveal significant degradation of the active ingredient.
Solvent used: DMSO
Solvent/final concentration: 1 % (v/v)

2. Control Materials
Negative control: Culture medium
Solvent control: 1 % (v/v) DMSO in
Positive control -S9: Mitomycin C (MMC; solvent: Hanks' balanced salt solution), concentration in the culture medium: 0.1 µg/ml
Positive control +S9: Cyclophosphamide (CP; solvent: Hanks' balanced salt solution), concentration in the culture medium: 2.0 µg/ml

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism.
Preparation: The S9 fraction was isolated from the livers of Aroclor 1254 induced Wistar rats. It was purchased from CCR, Roßdorf, Germany (protein content: 32.0 mg/ml).
For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with a cofactor solution (4:6). The S9 mix contained 40 % S9 fraction and was kept on ice and used on the same day.
Cofactor solution per 100 ml S9 mix:

Sodium phosphate buffer	60.0 ml
$MgCl_2 \times 6\ H_2O$	162.6 mg
KCl	246.0 mg
Glucose-6-phosphate (disodium salt)	152.0 mg
NADP (disodium salt)	78.8 mg

4. Test organism: Chinese hamster lung cells (V79)
Culture medium: Eagle's minimal essential medium supplemented with nonessential amino acids, 2 mM L-glutamine, MEM-vitamins, $NaHCO_3$-solution (final conc. 0.225 %), 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 % heat inactivated fetal calf serum (FCS)
Treatment medium: Culture medium with reduced serum content (2 % FCS)
Culture conditions: 37°C in a CO_2-incubator (5 % CO_2)

5. Test concentrations:

Pre-test for cytotoxicity:	0, 10, 25, 50, 100, 200, 400, 800 µg/ml
1st chromosome aberration assay:	18 h harvest time: 0, 25, 50, 75, 100, 150 µg/ml (±S9) 30 h harvest time: 0, 75, 100, 150 µg/ml (±S9)
2nd chromosome aberration assay:	8 h harvest time: 0, 75, 100, 150 µg/ml (±S9) 18 h harvest time: 0, 50, 75, 100 µg/ml (-S9)

B. TEST PERFORMANCE

1. Dates of experimental work: 1st assay: March 05, 1996 – April 23, 1996
2nd assay: June 04, 1996 – July 11, 1996

2. Test substance preparation and solubility in culture medium

For prothioconazole, DMSO was selected as solvent. In this solvent prothioconazole was soluble up to 250 mg/ml and formed a clear colourless solution. The stability of prothioconazole in the solvent DMSO at room temperature at concentrations ranging from 0.5 mg/ml to 250 mg/ml for at least twenty-four hours was analytically approved (Table 5.4.1/04- 1).

Table 5.4.1/04- 1: Analysis for stability of prothioconazole in DMSO at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 hrs	24 hrs
0.5	93.0	111.5
250	95.0	108.4

Precipitation was observed in culture medium at 781.3 µg/ml, but concentrations up to 800 µg/ml did not change either the pH or the osmolality of the medium.

3. Culturing of V79 cells

Chinese hamster V79 cells can be kept in culture as established cell lines with a generation time of approximately fourteen hours. The cells were normally grown in 20 ml medium and 75 cm^2 flasks and incubation of the cells was always performed at 37 °C in a CO_2-incubator (5 % CO_2). The karyotype of the V79 cells (modal chromosomes number: 22) was confirmed. There was no evidence of mycoplasma contamination.

4. Pre-test for cytotoxicity and determination of cytotoxicity in the main study

An initial pre-test for cytotoxicity was performed with 4 hours exposure and 24 hour harvest time at concentrations of 0-800 µg/ml. Both cell survival and mitotic index were determined in duplicate cultures (in the presence and absence of S9 mix). The mitotic index was also determined within the main study as indicator of cytotoxic effects. Cell survival was determined as an additional indicator of cytotoxicity in the main study if the mitotic index was considered to be no sufficient indicator of cytotoxicity.

At the end of the treatment period, cells of all cultures were trypsinized, and an appropriate dilution was counted using a haemocytometer to determine cell survival. The mitotic index was determined for all cultures. The number of mitotic cells among a total of 1000 cells per culture was determined. All cells which were not in interphase were defined as mitotic.

5. Chromosome aberration assay

All cultures were set up in duplicate. Two independent chromosome aberration assays were performed as follows:

Cell treatment

The chinese hamster V79 cells were passaged on the day prior to treatment. Approximately 1 x 10^5 cells were seeded in 20 ml of medium per 75 cm^2 flasks and incubated. All cultures were set up in duplicate. Immediately before treatment with test substance, the culture medium was removed from the cultures. For the treatment without metabolic activation, 0.2 ml test substance solution and 20 ml treatment medium was added. For treatment with metabolic activation 0.2 ml test substance, 1 ml S9-mix and 19 ml treatment medium was added.

The cells were incubated for 4 hours at 37°C. After this treatment period, the medium was removed, the cells were washed with pre-warmed phosphate buffered saline (PBS) (about 37°C), 20 ml of fresh culture medium was added to the flasks and the flasks were placed in a CO_2-incubator for the remaining incubation time. 0.2 ml Colcemid-solution (40 µg/ml water) were added to each flask two hours prior to the end of the incubation period to arrest the cells in a metaphase-like stage of mitosis (c-metaphase).

Positive controls and solvent controls (0.2 ml solvent per culture), and negative controls (no addition of solvent) were set up in parallel and handled as described for prothioconazole-treated cultures.

Chromosome preparations

The medium was removed from each flask and cells were removed from the bottom of the flask by trypsinization and suspended in medium. Cells were pelleted by centrifugation, the supernatant was removed and hypotonic solution (0.56 % KCl; 37°C) was added. The cells were resuspended, centrifuged again, and after removal of the supernatant cold fixative was added. The mixture was incubated at room temperature for 20-30 minutes. Cells were pelleted by centrifugation as before and the supernatant was discarded. Cells were again resuspended in fixative as before and centrifuged. Pelleted cells were resuspended carefully in a small volume of fresh fixative. This suspension was dropped onto clean slides which had been frozen at -20°C previously.

The slides were allowed to dry for at least 2 hours. Thereafter, they were submerged in pure methanol for 3 minutes and stained for 20-30 minutes in 5 % Giemsa solution. Slides were rinsed twice in water and once in acetone and were then kept in xylene for about 30 minutes. The slides were allowed to dry completely and covered. At least two slides were generated per culture.

All solutions used during this preparation were freshly prepared each time. The Giemsa solution was filtered before usage

6. Evaluation criteria

Coded slides were evaluated using a light microscope.

Mitotic index:	The mitotic index was determined by counting 1000 cells per culture. The numbers of mitotic and non-mitotic cells were noted. Duplicate cultures were processed and examined.
Chromosome aberrations:	Chromosomes of approximately 200 metaphases per concentration, 100 metaphases from each of two parallel cultures, were examined. Only metaphases containing the modal chromosome number (22) were analysed unless exchanges were detected. In this case, metaphases were evaluated even if the chromosome number was not equal to 22. The classes of structural chromosome damage were defined and recorded. Both chromatid and chromosome-type aberrations were assessed. Chromatid-type aberrations are clastogenic effects restricted to one of the two corresponding chromatids. Chromosome-type aberrations are defined as changes expressed in both corresponding sister chromatids at the same locus. The distinction between chromatid and chromosome type aberrations was not made for exchanges.

The different classes of aberrations are characterized as follows:

Gap: A gap is an achromatic lesion within a chromatid arm without obvious dislocation of the chromatid end(s). Gaps are found on one chromatid ("gap") or on both chromatids at apparently identical sites ("isogap"). The biological relevance of gaps of both types is not clear.

Break: A break is defined as a discontinuity of one chromatid ("break") or both chromatids, at apparently the same locus ("isobreak"), with dislocation of the chromatid ends. The dislocated chromatid end(s) has (have) to be present within the respective metaphase.

Fragment: Fragments are parts of chromosomes without centromer. A fragment is the result of a break. The corresponding defective chromosome is not detectable among the chromosomes of the same metaphase. Fragments can be derived from one chromatid ("fragment") or from both corresponding chromatid regions of a chromosome ("isofragment").

Deletion: A deletion occurs as the result of a break. In case of a deletion, one chromatide ("deletion") or both corresponding terminal chromatide parts of a chromosome ("isodeletion") are missing within the metaphase under assessment.

Exchange: This is an exchange of chromatid parts between different chromosomes (interchange) or within the same chromosome (intrachange).

Multiple aberration: A cell was assessed as to contain "multiple aberrations" when five or more structural changes (excluding gaps) occur within one metaphase.

In addition to these aberrations, metaphases showing chromosome disintegration as an indication of a cytotoxic effect were also recorded if they were observed. They were counted separately and were not included among the cells that were assessed for aberrations. "Chromosome disintegration" was recorded if fewer than half of the chromosomes reveal characteristic structural features within a given metaphase. Additionally observed polyploid metaphases were recorded.

7. Statistics

The statistical analysis was performed in the 1st and 2nd chromosome aberration assay by pair-wise comparison of prothioconazole-treated and positive control groups to the respective solvent control group.

Statistical test	Parameter
One sided chi² test:	- mitotic index (provided that it was reduced compared to the respective negative control mean)
Fisher's exact test:	- number of metaphases with aberrations (including and excluding gaps) - number of metaphases with exchanges (provided that these data superseded the respective negative control)

A difference was considered to be significant if the probability of error was below 5 %.

8. Assessment and acceptability criteria

A test was considered positive if there was a relevant and statistically significant increase in the aberration rate. An increased incidence of gaps of both types without concomitant increase of other aberration types was not considered as indication of a clastogenic effect. A test was considered negative if there was no such increase at any time interval. A test was considered equivocal if there was an increase which was statistically significant but not considered relevant, or if an increase occurred, which was considered relevant, but which was not statistically significant.

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

II. RESULTS AND DISCUSSION

A. PRE-TEST FOR CYTOTOXICITY

As indicators of cytotoxic effects, numbers of surviving cells (survival index) and mitotic indices were used. The mitotic index was determined by counting a total of 1000 cells per concentration. The results of the solvent controls were set 100 % and compared to the prothioconazole treated cultures. The number of surviving cells was reduced at ≥25 µg/ml without S9 and at ≥200 µg/ml with S9. The mitotic index was reduced at ≥100 µg/ml (Table 5.4.1/04- 2).

Table 5.4.1/04- 2: Cell survival and mitotic index in the pre-test for cytotoxicity

Exposure period (hr)	Harvest time (hr)	Dose (µg/ml)	Survival index (%)[a]		Mitotic index (%)[a]	
			-S9	+S9	-S9	+S9
4	24	0$	100.0	100.0	100.0	100.0
		10	95.5	103.4	118.3	108.1
		25	70.6	102.7	129.6	90.9
		50	56.5	90.4	132.5	101.3
		100	57.1	95.9	138.2	76.8
		200	9.2	17.9	0	39.1
		400	0	0	nn	0
		800	0	0	nn	nn

[a] relative to solvent control; $ solvent control; nn: no nuclei

B. CHROMOSOME ABERRATION ASSAYS

Cytotoxicity

On the basis of the results of the pre-test for cytotoxicity, the first assay was performed using concentrations of 0-150 µg/ml, with a 4-hour exposure period and 18 hour and 30 hour harvest times. The mitotic index was reduced at 150 µg/ml at the 30 hour harvest (Table 5.4.1/04- 3). Cell survival was statistically significantly reduced at ≥50 µg/ml and ≥75 µg/ml without S9 at the 18 and 30 hour harvest times respectively. On this basis, concentrations of 75, 100 and 150 µg/ml (18 hour harvest) and 150 µg/ml (30 hour harvest) were selected for metaphase analysis.

On the basis of the results of the first assay, the second assay was performed. Cells were harvested after 8 hours (with and without S9) and 18 hours (without S9 only), respectively, following 4 hours exposure to 75-150 µg/ml or 50-100 µg/ml, respectively. The narrow concentration range selected for the second assay was to clarify the results obtained in the first assay. In the second assay the mitotic index was significantly reduced at the 8 hour harvest time, but not at the 18 hour harvest time (Table 5.4.1/04- 3), thus suggesting that in the absence of S9 there was a marked cytotoxic effect early in the culture phase only. The metaphase analysis in the second assay was confined to the 18 hour harvest cultures only.

Table 5.4.1/04- 3: Cell survival and mitotic index in the chromosome aberration assays

Exposure period (hr)	Harvest time (hr)	Dose (µg/ml)	Survival index (%)[a]		Mitotic index (%)[a]	
			-S9	+S9	-S9	+S9
1st assay						
4	18	0$	100	100	100	100
		0	114.4	110.8	116.7	107.8
		25	85.3	101.4	119.7	100
		50	66.7*	102.3	131.6	116.8
		75	63.5*	108.9	117.5	148.0
		100	63.9*	106.6	129.8	141.4
		150	34.7*	62.0*	93.0	154.1
		MMC	62.8*	-	103.5	-
		CP	-	81.7	-	213.5
4	30	0$	100	100	100	100
		75	66.6*	85.2	98.2	153.3
		100	57.1*	81.0	118.2	138.0
		150	22.8*	34.2*	67.6**	156.0
2nd assay						
4	8	0$	100	100	100	100
		75	84.2	83.2	172***	136.5
		100	73.1	82.6	16.4***	98.4
		150	78.6	69.5*	24.6***	42.9***
4	18	0$	100	-	100	-
		0	121.7	-	104.0	-
		50	88.4	-	108.0	-
		75	71.3	-	103.1	-
		100	82.2	-	114.3	-
		MMC	87.6	-	84.4**	-

$ solvent control; [a] relative to solvent control; - not evaluated; nn: no nuclei; * biologically relevant decrease in survival (based on historical control data); ** $p < 0.05$; *** $p < 0.01$ (chi^2 test)

Chromosome aberrations

The numbers of cells with aberrations from each assay are shown in Table 5.4.1/04- 4.

In the first assay, there were marked increases in the number of cells with aberrations at 150 µg/ml without S9 (at both the 18 hour and 30 hour harvest times). There were also smaller but statistically significant increases at 75 and 100 µg/ml without S9 (considered to be equivocal due to the small magnitude of the increases and the absence of a dose-response relationship). With S9 in the first assay, there were clear increases at 150 µg/ml only (at both harvest times). The second assay was an attempt to clarify whether the increases without S9 at 75 and 100 µg/ml were biologically relevant and to investigate whether the effects were due to excessive cytotoxicity.

In the second assay, increases in cells with aberrations were recorded at all concentrations (only cultures without S9 at 18 hours harvest were assessed for aberrations). However, the increases were not dosage-related. Since cytotoxicity had been demonstrated early in culture, since aberrations only occurred at cytotoxic concentrations, since the cytotoxicity may have been underestimated (see guideline

deviations) and since the increases seen were not concentration-related, it is likely that the clastogenicity seen is an indirect effect triggered by cytotoxicity.

The positive control substances (mitomycin C without S9 and cyclophosphamide with S9) gave satisfactory results in both assays. No increase in polyploid metaphases was observed neither at any concentration of prothioconazole nor after treatment with the positive controls.

Table 5.4.1/04- 4: Numbers of cells with aberrations

Exposure / harvest (hours)	±S9	Dose (µg/ml)	Gaps	Number of cells with structural chromosome aberrations								Polyploid meta-phases (%)
				Chromatid-type		Chromosome type		Others[a]	Aberrant metaphases (%)			
			g + ig	b	f + d	ib	if + id		-g	+g	±	
1st assay												
4 / 18	-	0$	0	0	0	0	0	0	0.0	0.0	0.0	7.5
		0	1	0	0	1	4	0	1.0	1.5	0.0	4.5
		75	1	2	2	1	4	7	7.5*	8.0*	3.5*	18.5
		100	0	1	0	1	2	15	6.5*	6.5*	5.5*	12.5
		150	14	13	4	47	28	38	41.0*	42.5*	17.0*	11.5
		MMC	5	16	6	26	10	17	29.5*	30.5*	7.5*	9.5
4/18	+	0$	0	0	1	2	2	2	2.5	2.5	0.5	10.5
		0	1	0	0	0	3	2	2.0	2.5	1.0	5.0
		75	1	1	0	0	3	4	2.5	3.0	1.5	6.0
		100	3	4	1	1	0	4	3.0	4.5	1.5	10.0
		150	2	9	2	31	6	18	22.0**	24.0**	7.0**	10.0
		CP	3	7	1	13	7	5	13.5**	15.0**	2.5*	9.5
4 / 30	-	0$	0	1	0	0	1	0	1.0	1.0	0.0	5.5
		150	4	10	6	4	15	43	28.0*	29.0*	18.5*	8.5
	+	0$	0	0	0	1	4	0	2.5	2.5	0.0	6.5
		150	2	4	5	9	15	12	15.0*	15.5*	4.5*	8.5
2nd assay												
4 / 18	-	0$	0	1	0	1	3	0	2.5	2.5	0.0	9.0
		0	0	0	0	1	2	0	1.5	1.5	0.0	5.0
		50	3	7	4	4	5	16	11.0*	11.5*	6.5*	9.0
		75	2	9	0	4	3	20	13.5*	14.0*	8.5*	5.0
		100	2	3	2	3	2	15	10.0*	10.5*	6.5*	5.5
		MMC	6	16	7	23	9	20	28.5*	29.5*	9.5*	7.0

$: solvent control; * $p < 0.05$; ** $p < 0.01$ (Fisher's exact test);
gaps/isogaps (g/ig)/breaks/isobreaks (b/ib); fragments/ isofragments (f/if); deletions/isodeletions (d/id); exchanges (x);
[a] includes exchanges, multiple aberrations, multiple aberrations + exchanges, and cell disintegrations

III. CONCLUSION

Prothioconazole induced chromosome aberrations (with and without metabolic activation) under the conditions of this study. Since aberrations only occurred at cytotoxic concentrations, since the cytotoxicity may have been underestimated (see guideline deviations) and since the increases were not

concentration-related, it is likely that the clastogenicity seen is an indirect effect triggered by cytotoxicity.

Report:	KCA 5.4.1/05[redacted].; 2017; M-588628-01-1
Title:	Prothioconazole, technical: Micronucleus test in human lymphocytes In vitro
Report No.:	1825700
Document No.:	M-588628-01-1
Guideline(s):	OECD 487 (2016)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Deviations: Deviations from the current OECD guideline (2016):
None

Executive summary:

Prothioconazole (Batch SES 12535-1-1; Purity: 97.6 %) was tested for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of hepatic S9 mix from induced rats (metabolic activation). Two independent assays were performed in which the cells were incubated for 4 (±S9 mix) or 20 hours (-S9 mix) with the test substance at concentrations in the range of 5.6 to 800 µg/ml. The vehicle DMSO served as negative control, mitomycin C (MMC, 4 h) and demecolcin (20 h) as positive controls in the absence of metabolic activation and cyclophosphamide (CPA) as positive control in the presence of metabolic activation. Exposure was started after a 48 hour stimulation period with phytohemeagglutinine. After exposure (and recovery) cytochalasin B was added and the cultures were fixed and stained finally after another 20 hours. Cytotoxicity (% cytostasis) was determined in 500 binucleated cells/culture and the number of micronucleated cells was determined in 1000 binucleated cells/culture for evaluation of mutagenicity. Based on precipitation and cytotoxicity results concentrations from 30.1 to 119 µg/ml were evaluated for mutagenicity.

In both assays in the absence and presence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentrations. Osmolarity and pH values were not influenced by test substance treatment. In the 1st assay precipitation of prothioconazole in the culture medium was observed at 400 µg/ml and above in the absence and presence of S9 mix at the end of treatment. In the 2nd assay no precipitation occurred.

The test substance prothioconazole led to neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells either without S9-mix or after the addition of a metabolizing system in the two assays. In both assays, either CPA or demecolcin and MMC showed distinct increases in cells with micronuclei, and thus demonstrating sensitivity of the test system towards known mutagens that do or do not require metabolic activation, respectively. Furthermore, the number of micronucleated cells induced by the vehicle control DMSO was within the range of the historical control data.

In conclusion, prothioconazole is considered to be non-mutagenic in this *in vitro* micronucleus test when tested up to cytotoxic concentrations.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Prothioconazole

Synonyms:	JAU 6476; AE 1344248
Description:	Solid light beige
Batch No.:	SES 12535-1-1
Purity:	97.6 %
Stability of the test compound:	Approved for at least the time of study duration
Solvent used:	Dimethylsulfoxide (DMSO)
Solvent/final concentration:	≤0.5 % (v/v)

2. Control Materials

Solvent control:	0.5 % DMSO
Positive control -S9:	Mitomycin C (MMC, 1.0 µg/ml; 4 h exposure) dissolved in deionized water Demecolcin (125 ng/ml; 20 h exposure) dissolved in deionized water
Positive control +S9:	Cyclophosphamide (CPA, 15 µg/ml) dissolved in saline

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism.

Preparation: Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. The S9 was prepared and stored according to the currently valid version of the laboratory's SOP for rat liver S9 preparation.

Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test. The protein concentration of the S9 preparation used for this study was 30.1 mg/ml.

An appropriate quantity of S9 supernatant was thawed and mixed with S9 cofactor solution to result in a final protein concentration of 0.75 mg/ml in the cultures

Composition of the S9 mix:

S9 fraction	0.75 mg/ml
Sodium-ortho-phosphate-buffer	100 mM, pH 7.4
$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate	5 mM
NADP	4 mM

4. Test organism: Human peripheral blood lymphocytes from healthy non-smoking donors not receiving medication: male donor (25 years old) for the 1st assay, male donor (23 years old) for the 2nd assay (A) and male donor (33 years old) for the 2nd assay (B).

5. Culture conditions:

Culture medium: Dulbecco's Modified Eagles Medium/Ham's F12 (DMEM/F12, mixture 1:1) already supplemented with 200 mM GlutaMAX™. Additionally, the medium was supplemented with penicillin/streptomycin (100 U/ml/100 µg/ml), the mitogen PHA (3 µg/ml), 10 % fetal bovine serum (FBS), 10 mM HEPES and the anticoagulant heparin (125 U.S.P.-U/ml)

Incubation: At 37 °C with 5.5 % CO_2 in humidified air.

6. Test concentrations:

1st micronucleus assay (4 h exposure):	5.6, 9.8, 17.2, **30.1**, **52.7**, 79, **119**, 178, 267, 400, 800 µg/ml (-S9) 5.6, 9.8, 17.2, **30.1**, **52.7**, **79**, 119, 178, 267, 400, 800 µg/ml (+S9)
2nd micronucleus assay (20 h exposure):	A) 4.7, 7, 10.5, 15.8, 23.7, 35.6, 53.3, 80, 120, 180 µg/ml (-S9) B) 19.7, 39.5, **43.4**, 47.8, 52.5, **57.8**, 63.6, **69.9**, 76.9, 84.6, 110 µg/ml (-S9)

Concentrations written **in bold** were evaluated for cytogenetic damage.

B. TEST PERFORMANCE

1. Dates of experimental work: February 22, 2017 - April 25, 2017

2. Test substance preparation

Stock formulations of the test item and serial dilutions were made in DMSO. The solvent was chosen due to its solubility properties and its relative non-toxicity to the cell cultures. All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation is assumed to be stable for this period.

The osmolarity and pH were determined in the solvent control and the maximum concentration without metabolic activation:

Table 5.4.1/05- 1: Analysis for osmolarity and pH of solvent controls and maximum concentrations

		Concentration [µg/ml]	Osmolarity [mOsm]	pH
1st micronucleus assay	Solvent control	-	401	7.7
	Prothioconazole, technical	800	396	7.7
2nd micronucleus assay (A)	Solvent control	-	n.d.	7.4
	Prothioconazole, technical	180	n.d.	7.4
2nd micronucleus assay (B)	Solvent control	-	n.d.	7.6
	Prothioconazole, technical	110	n.d.	7.6

n.d. Not determined

3. Dose selection

Dose selection was performed according to the current OECD Guideline 487 for the in vitro micronucleus test. The highest test item concentration should be 10 mM, 2 mg/ml or, 2 µl/ml, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

In case of test item induced cytotoxicity, measured by a reduced cytokinesis-block proliferation index (CBPI) and expressed as cytostasis, or precipitation (observed at the end of test item exposure by the unaided eye) the dose selection should reflect these properties of the test item. Where cytotoxicity occurs the applied concentrations should cover a range from no to approximately 55 ± 5 % cytostasis. For poorly soluble test items, which are not cytotoxic at concentrations lower than the lowest insoluble concentration, the highest concentration analysed should produce turbidity or visible precipitation (phase separation for liquid test items).

With regard to the solubility properties of prothioconazole, 800 µg/ml were applied as top concentration for treatment of the cultures in the pre-test. Prothioconazole concentrations ranging from 5.6 to 800 µg/ml (with and without S9 mix) were chosen for the evaluation of cytotoxicity. In the pre-test for

cytotoxicity, precipitation of prothioconazole was observed at the end of treatment at 400 µg/ml and above in the absence and presence of S9 mix. Since the cultures fulfilled the requirements for cytogenetic evaluation, this preliminary test was designated the 1st micronucleus assay.

Clear toxic effects were observed after 4 hours treatment with 119 µg/ml and above in the absence of S9 mix and with 79.0 µg/ml in the presence of S9 mix. Considering the toxicity and precipitation data of the 1st micronucleus assay, 180 µg/ml (without S9 mix) were chosen as top concentration in the 2nd micronucleus assay (A). Since no concentrations were evaluable in a cytotoxic range, the experiment was repeated with a top dose of 110 µg/ml and designated 2nd micronucleus assay (B).

4. Micronucleus test

The cultures were treated according to the following scheme:

	Without S9 mix		**With S9 mix**
	1st assay	**2nd assay**	**1st assay**
Stimulation period	48 h	48 h	48 h
Exposure period	4 h	20 h	4 h
Recovery	16 h	–	16 h
Cytochalasin B exposure	20 h	20 h	20 h
Total culture period	88 h	88 h	88 h

Pulse exposure (4h):

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

Continuous exposure (20h):

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

Preparation of cells

The cultures were harvested by centrifugation 40 h after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 ml saline G and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 ml KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 ml of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The

slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

5. Cytotoxicity and genotoxicity evaluation

Evaluation of the slides was performed using microscopes with 40 x objectives. The micronuclei were counted in cells showing a clearly visible cytoplasm area. The micronuclei have to be stained in the same way as the main nucleus. The area of the micronucleus should not extend the third part of the area of the main nucleus. At least 1000 binucleate cells per culture were scored for cytogenetic damage on coded slides. The frequency of micronucleated cells was reported as % micronucleated cells. To describe a cytotoxic effect the CBPI was determined in 500 cells per culture and cytotoxicity is expressed as % cytostasis. A CBPI of 1 (all cells are mononucleate) is equivalent to 100 % cytostasis.

$$CBPI = \frac{(MONC \times 1) + (BINC \times 2) + (MUNC \times 3)}{n}$$

CBPI	Cytokinesis-block proliferation index
n	Total number of cells
MONC	Mononucleate cells
BINC	Binucleate cells
MUNC	Multinucleate cells

$$\text{Cytostasis \%} = 100 - 100\ [(CBPI_T - 1) / (CBPI_C - 1)]$$

T Test item
C Solvent control

6. Acceptability criteria

- The rate of micronuclei in the solvent control falls within the historical laboratory control range.
- The rate of micronuclei in the positive controls is statistically significant increased and is within the laboratory historical positive control data range.
- Cell proliferation criteria in the solvent control are considered to be acceptable.
- All experimental conditions described in section 'Experimental performance' were tested unless one exposure condition resulted in a clearly positive result.
- The quality of the slides must allow the evaluation of an adequate number of cells and concentrations.
- The criteria for the selection of top concentration are consistent with those described in section 'Dose selection'.

8. Assessment criteria

A test item can be classified as **non-clastogenic** and **non-aneugenic** if:

- the number of micronucleated cells in all evaluated dose groups is in the range of the historical laboratory control data
- no concentration-related increase of the number of micronucleated cells is observed in comparison to the respective solvent control
- no statistically significant increase of the number of micronucleated cells is observed in comparison to the respective solvent control.

A test item can be classified as **clastogenic** and **aneugenic** if:

- the number of micronucleated cells is outside the range of the historical laboratory solvent control data and
- a concentration-related increase of the number of micronucleated cells is observed in at least one experimental condition and

- at least one of the test item concentrations exhibits a statistically significant increase in the number of micronucleated cells in comparison to the respective solvent control.

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

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

In case the response is neither clearly negative nor clearly positive as described above and/or in order to assist in establishing the biological relevance of a result, the data should be evaluated by expert judgement and/or further investigations. However, results may remain questionable regardless of the number of times the assay is repeated. If the data set will not allow a conclusion of positive or negative, the test item will therefore be concluded as equivocal.

An increase in the number of micronucleated mononucleate cells may indicate that the test item has aneugenic potential.

9. Statistical analysis

Statistical significance was confirmed by the Chi square test ($\alpha < 0.05$), using a validated test script of "*R*", a language and environment for statistical computing and graphics. Within this test script a statistical analysis was conducted for those values that indicated an increase in the number of cells with micronuclei compared to the concurrent solvent control.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS AND TREATMENT CONDITIONS

The highest treatment concentration in this study, 800 µg/mL was chosen with regard to the solubility properties of the test item and with respect to the OECD Guideline 487 for the in vitro mammalian cell micronucleus test.

In the 1st assay, precipitation of the test item in the culture medium was observed at 400 µg/mL and above in the absence and presence of S9 mix at the end of treatment. In the 2nd assay (bot A and B) no precipitation occurred.

No relevant influence on osmolarity or pH was observed.

B. CYTOTOXICITY

In the 1st and 2nd assay in the absence and presence of S9 mix, clear cytotoxicity was observed at the highest evaluated concentrations.

C. MICRONUCLEUS ASSAY

In both independent assays, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed after treatment with prothioconazole. The percentage of micronucleated cells of the solvent controls as well as cells treated with prothioconazole was within the range of historical laboratory control data and also clearly below the respective historical laboratory control mean in both assays.

In both assays, either Demecolcin (125 ng/ml), MMC (1.0 µg/ml) or CPA (15.0 µg/ml) were used as positive controls and showed distinct increases in cells with micronuclei.

Table 5.4.1/05- 2: Results of the *in vitro* micronucleus test in human lymphocytes

Assay	Test item concentration in µg/ml	Proliferation index CBPI	Cytostasis in %[a]	Micronucleated cells in %[b]	Historical laboratory control data[c]
Exposure period 4 hrs without S9 mix					
1	Solvent control[1]	1.97		0.15	0.60 (0.08 – 1.12)
	30.1	1.85	11.7	0.15	
	52.7	1.67	30.7	0.20	
	119	1.43	55.0	0.30	
	Positive control[2]	1.49	49.0	**6.60***	12.48 (1.44 – 23.52)
Exposure period 20 hrs without S9 mix					
2B	Solvent control[1]	1.78		0.15	0.57 (0.12 – 1.03)
	43.4	1.63	18.9	0.10	
	57.8	1.45	41.9	0.00	
	69.9	1.31	60.6	0.15	
	Positive control[3]	1.37	52.2	**2.25***	3.72 (1.43 – 6.01)
Exposure period 4 hrs with S9 mix					
1	Solvent control[1]	1.86		0.40	0.62 (0.16 – 1.08)
	30.1	1.76	11.7	0.20	
	52.7	1.51	41.3	0.35	
	79.0	1.40	53.2	0.45	
	Positive control[4]	1.41	52.8	**2.65***	5.16 (0.84 – 9.49)

a For the positive control groups and the test item treatment groups the values are related to the solvent controls
b The number of micronucleated cells was determined in a sample of 2000 binucleated cells
c Percentage of micronucleated cells in human lymphocyte cultures (2015-2016), mean (95 % Ctrl limit)
* The number of micronucleated cells is statistically significantly higher than corresponding control values
1 DMSO 0.5 % (v/v)
2 MMC 1.0 µg/ml
3 Demecolcin 125 ng/ml
4 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, Prothioconazole is considered to be non-mutagenic in this *in vitro* micronucleus test, when tested up to cytotoxic concentrations.

CA 5.4.2 In vivo studies in somatic cells

Report:	KCA 5.4.2/03 [redacted]; 1999; M-007155-01-1
Title:	JAU 6476 - Test on unscheduled DNA synthesis with rat liver cells in vivo
Report No.:	28905
Document No.:	M-007155-01-1
Guideline(s):	OECD 486 (1997),
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.4.2/03. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.4.2/01** (also in numbering of tables/figures).

Deviations:	Deviations from the current OECD guideline (1997): None

Executive summary:

In a 1998 GLP study, male Wistar rats (4/group) were orally gavaged with prothioconazole (batch no. NLL 6096-12, purity 99.5-99.7 %) in 0.5 % aqueous Cremophor at dose levels of 0 (vehicle control), 2500 or 5000 mg/kg bw. Animals were sacrificed at 4 or 16 hours post-administration. Positive control animals were gavaged with 40 mg/kg bw N,N'-dimethylhydrazine (4 hour sacrifice) or 100 mg/kg bw 2-acetylaminofluorene (16 hour sacrifice). Livers were removed at sacrifice, hepatocytes harvested and cultured in the presence of ^{3}H-thymidine for 4 hours. UDS was assayed by autoradiography of 150 cells/animal (50 cells on three slides/animal – coded slides). Cells in repair (nuclei with ≥5 net grains) were also recorded. The study was compliant with OECD Guideline 486 (1997).

Clinical signs of toxicity were recorded in animals treated at 5000 mg/kg bw (roughened fur in the 4 hours sacrifice group; roughened fur, apathy and partially closed eyes in the 16 hours sacrifice group). No cytotoxicity was evident in any group and cell viability was acceptable. The mean net nuclear grain (NNG) counts in both dose groups after 16 hours were higher than controls, but the mean values did not exceed zero which was the performing laboratories minimum threshold for a possible positive response. The number of cells in repair was very low. When the results for individual animals from the 16 hours sacrifice group are examined, the animals treated at 2500 mg/kg bw all had negative NNG counts with only one animal having a higher NNG count than the highest vehicle control animal. At 5000 mg/kg bw, two animals had positive NNG counts, the remaining 2 animals had NNG counts comparable with the concurrent vehicle controls. The increase is therefore not consistent within the group. Furthermore, two vehicle control animals from another study (T6040651) conducted at the same test laboratory, also displayed positive NNG counts very similar to those of the two high-dose animals, hence this finding is not critical. In the 4 hour sacrifice assay, there was no increase in NNG counts in either treated group, nor any treated animals with positive NNG counts.

It is therefore concluded that a negative result was obtained in this study.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-12
Purity:	99.5 % and 99.7 %

Stability of the test compound:	The batch used was analyzed prior to study initiation and approved for use during the study period.
Vehicle used:	0.5 % aqueous Cremophor emulsion

2. Control Materials:

Vehicle control:	0.5 % aqueous Cremophor emulsion
Positive controls:	2-Acetylaminofluorene (2-AAF) for a sacrifice time of 16 h, dissolved in corn oil N,N'-Dimethylhydrazine (DMH) for a sacrifice time of 4 h, dissolved in DMSO (1 ml) and subsequently mixed with Polyethyleneglycol (9 ml)

3. Test animals:

Species:	Rat
Strain:	Wistar Crl:(WI)BR
Sex:	Males
Age:	10-14 weeks
Weight at dosing:	100-130 g
Source:	[redacted] Germany
Acclimation period:	At least 5 days
Diet:	Altromin 1324 Standard Diet ([redacted] Germany), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Rats were kept singly in Makrolon type II cages during acclimatization and treatment. Bedding of soft wood granules was used.
Environmental conditions:	
Temperature:	22-23 °C
Humidity:	41-72 %
Air changes:	About 10 times per hour
Photo period:	12 h of electrical lighting daily

4. Test compound doses: 0, 2500, 5000 mg prothioconazole/kg bw

5. Test conditions for primary hepatocytes:

Culture medium:	Williams Medium E, supplemented with L-glutamine (2 mM), gentamycin sulfate (50 µg/ml), dexamethasone (2.4 µM) and 10 % heat-inactivated fetal calf serum (FCS).
Treatment medium:	Williams Medium E, supplemented with L-glutamine (2 mM) and 1 % heat-inactivated fetal calf serum (FCS).
Culture conditions:	37 °C in a humidified atmosphere containing approx. 5 % CO_2

B. STUDY DESIGN

1. Dates of work:

January 26, 1998 – March 24, 1998	(16 h sacrifice)
June 17, 1998 – July 30, 1998	(4 h sacrifice)

2. Test substance preparation and analysis

Prothioconazole was suspended in 0.5 % aqueous Cremophor emulsion. The stability of prothioconazole in the vehicle was confirmed by analysis which verified that prothioconazole is stable in the vehicle at room temperature at concentrations ranging from 125 mg/ml to 250 mg/ml for at least four hours.

Table 5.4.2/01- 1: Analysis for stability of prothioconazole in the vehicle at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 h	4 h
125	94	93
250	111	113

3. Unscheduled DNA synthesis (UDS) assay

Animal assignment and treatment

Each group comprised four rats. They were divided into groups by a randomization plan. Each respective substance was administered once. Food was withdrawn before treatment (16 h sacrifice: approximately 6 h before treatment; 4 h sacrifice: the night before treatment). Four animals were treated per day. At each treatment day at least one control animal was exposed (vehicle control and/or positive control).

Table 5.4.2/01- 2: Treatment overview

	Prothioconazole			Positive control	
				2-AAF	DMH
(mg/kg bw)	0	2500	5000	100	40
16 h sacrifice					
Volume (ml/kg bw)	20	20	20	10	-
Route of application	p.o.	p.o.	p.o.	p.o.	-
No. of animals treated	4	4	4	4	-
4 h sacrifice					
Volume (ml/kg bw)	20	20	20	-	10
Route of application	p.o.	p.o.	p.o.	-	p.o.
No. of animals treated	4	4	4	-	4

For each animal the respective amount of prothioconazole was separately supended in a syringe and was administered orally by stomach tube. The positive controls and vehicle control were administered in the same way. Approximately 1 h after treatment animals received food again.

Liver perfusion and preparation of rat hepatocytes

After the respective exposure time rats were anesthetized by i.p. injection with Nembutal Sodium Solution.

The liver cells were obtained by perfusing the rat liver in situ with a collagenase solution. After perfusion, primary hepatocytes were prepared according to the protocol of Butterworth et al. (1987) under sterile conditions. An aliquot was used for the determination of cell viability and cell concentration by the method of trypan blue exclusion. The obtained viability value of the cell suspension after perfusion is a measure for the substance-induced cytotoxicity during *in vivo* exposure.

The cells were seeded as follows:

- For determination of cell viability, attachment rate and morphology about 1.5 h after establishment of the cultures: two 60 mm-Petri dishes (7.5×10^5 viable cells per dish) precoated with collagen were available for each dose group as well as for the positive and negative controls.

4. For determination of genotoxicity: a 25 mm round plastic coverslip precoated with collagen was placed into each well of 6-well culture dishes. Approx. $3.75x10^5$ viable cells were seeded per well (in 2.5 ml culture medium), whereby 6 wells per animal including the control groups were established.

For cell-attachment all cultures were incubated for 90 min. in a 37°C incubator in a humidified atmosphere containing approximately 5 % CO_2.

Culture labelling

After the attachment period, the cultures were washed with phosphate buffered saline (PBS) to remove unattached cells. Cell number and viability of the cultures were determined by the method of trypan blue exclusion employing the two additional 60 mm-Petri dishes.

The medium in the 6-well dishes was replaced by treatment medium. To each culture 10 µCi/ml ^{3}H-thymidine (15.0 Ci/mmole) was added. The cultures were then placed in the incubator. After 4 h incubation hepatocytes were washed twice with treatment medium containing unlabelled thymidine and subsequently cultivated in this medium overnight (cold chase).

Thereafter, the cultures were washed twice with PBS in the 6-well dishes. Subsequently, a 1 % sodium citrate solution was added to swell the nuclei. The cells on the coverslips were then fixed, washed with deionized distilled water and air dried.

Autoradiography and Staining

The whole autoradiography-procedure was performed in the dark. Air-dried coverslips were mounted cell-side-up on microscope slides. In a darkroom, these were dipped in a NTB-2 photographic emulsion and left to dry in the air overnight. The next day, the coated slides were stored in light-tight boxes in the presence of a drying agent for 11-14 days at -20°C in order to reduce the cytoplasmic grains background and therefore to increase the sensitivity of the assay. The photographic emulsion was then developed at temperatures below 15°C. The slides were rinsed afterwards with distilled water, fixed and air dried. Slides were then stained with hematoxylin and eosin.

Grain counting

Evaluation was performed with coded slides. Each slide was examined by counting 50 cells per slide. 3 slides per animal were evaluated (total of 150 nuclei for each rat). Only cells viable at the time of fixation were scored; isolated nuclei and cells with abnormal morphology were excluded. A starting point was randomly selected on each slide, and cells were scored in a regular fashion by bringing new cells into the field of view, moving along the X-axis. If the total number of 50 cells had not been reached before coming to the edge of the slide, the stage was moved on the Y-axis, and counting resumed in the opposite X-direction, parallel to the first line.

UDS was measured by counting nuclear grains and subtracting the average number of grains in 3 cytoplasmatic areas of the same size as the corresponding nucleus. This value was referred to as the net nuclear grain count of the cell. The mean net nuclear grain count per concentration was routinely determined from triplicate coverslips. The number of cells in repair (nuclei with 5 or more net grains) was also determined.

4. Data presentation and assay evaluation

Net grains per nucleus:	Average no. of the mean net nuclear grain counts of each evaluable coverslip, 50 cells per coverslip
Mean grains per nucleus:	Average no. of the mean nuclear grain counts of each evaluable coverslip, 50 cells per coverslip.
Mean cytoplasmic grain count:	Average no. of the mean cytoplasmic grain counts (3 areas per cell) of each evaluable coverslip, 50 cells per coverslip

% Nuclei with 5 or more grains:	(No. of cells with 5 or more net nuclear grain counts per dose/ no. of evaluated cells per dose) x 100
Absolute survival (%):	% of viable cells after isolation

1. For each of the 50 cells on each slide, the number of nuclear grains was scored, as well as numbers of three cytoplasmic grain counts from nuclear-sized areas adjacent to each nucleus.

2. A number of 5 net nuclear grains (NNG) or more was chosen as a conservative estimate as to whether a particular cell is responding (cell "in repair").

3. Only cells viable at the time of fixation and with nuclei evenly coated with emulsion were scored. Cells with abnormal morphology, such as those with pyknotic or lysed nuclei, were not counted. Isolated nuclei not surrounded by cytoplasm were not counted.

4. Cells with heavily labelled nuclei (= S-phase cells) were excluded from scoring.

5. Statistics

Descriptive statistical methods were used to calculate means and standard deviations. The means and standard deviations in the tables were calculated from the means calculated individually for each of the three coverslips per animal.

6. Acceptance and assessment criteria

An assay is normally considered acceptable for evaluation only if the following criteria are satisfied.

Viability

- The viability of the hepatocytes of the vehicle control animals collected by this process normally exceeds 70 %, although values between 50 % and 70 % viability can also be acceptable. Vehicle control animals with hepatocyte preparations below 50 % are considered unacceptable in order to avoid the possible use of a damaged cell population.
- The viability of the monolayer cell cultures of animals treated with 0.5 % aqueous Cremophor should be 65 % or greater after attachment. Normally, the viability of attached cells is about 75 %.

Nuclear grain counts

- Grain count data obtained for a given treatment are acceptable as part of the evaluation if obtained from at least two slides per animal and at least 100 cells per animal.
- An experiment is considered invalid if cytoplasmic background counts in hepatocytes of vehicle control animals exceed 30 grains per nuclear-sized area.
- The average NNG value in hepatocytes of vehicle control animals should range between -8 and 0. No more than 5 % of the cells should be in repair.
- The positive controls 2-AAF and DMH are used to demonstrate the responsiveness of the cell population employed and the adequacy of the method for the detection of UDS after in vivo treatment of rats. For the positive controls 2-AAF (100 mg/kg bw) and DMH (40 mg/kg bw), one might expect mean values of 2-15 NNG per dose group with 5 %-80 % of the cells with greater than or equal to 5 NNG.

For the conditions described a response is considered positive if a chemical yields +2 NNG or more (dose group average) and 11 % or more of the cells responding.
A dose group average equal to or below 0 NNG is considered to be a negative response.
Results between 0 NNG and 2 NNG have to be assessed on a case by case basis.

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

II. RESULTS AND DISCUSSION

A. CLINICAL OBSERVATIONS

After single oral administration of prothioconazole in doses of 2500 mg/kg bw and 5000 mg/kg bw, in the 5000 mg/kg 16 h sacrifice group roughened fur, apathy and partially closed eyes were recorded. In the 5000 mg/kg 4 h sacrifice group roughened fur was recorded for one animal. No treatment-related clinical signs were observed at 2500 mg/kg bw.

Animals of the control groups showed no clinical symptoms with the exception of one animal of the positive control group (4 h sacrifice) which showed diarrhoea.

B. UNSCHEDULED DNA SYNTHESIS ASSAY

Cytotoxicity

After cell isolation, no cytotoxic effects could be observed in hepatocytes of rats exposed to prothioconazole and sacrificed after 16 h or 4 h. The same was true for hepatocytes of the positive control animals.

Table 5.4.2/01- 3: Cytotoxicity (mean ± standard deviation)

Treatment group / dose level	4 h sacrifice		16 h sacrifice	
	Absolute viability[a]	Relative viability (%)[b]	Absolute viability[a]	Relative viability (%)[b]
Vehicle control / 0 mg/kg	80.4 ± 1.8	100	83.2 ± 3.6	100
Prothioconazole / 2500 mg/kg	74.5 ± 3.7	91.5	76.8 ± 3.7	93.6
Prothioconazole / 5000 mg/kg	69.9 ± 0.7	92.7	77.8 ± 4.0	92.3
Positive control[#]	73.6 ± 2.1	86.9	77.9 ± 1.9	93.5

[a]: mean viability of cell preparation after perfusion
[b]: relative to vehicle control animal
[#] DMH (40 mg/kg) for 4 h sacrifice and 2-AAF (100 mg/kg) for 16 h sacrifice

UDS assay

The mean grain counts and individual animal grain counts (16 h sacrifice groups only) are presented in Table 5.4.2/01- 4 and Table 5.4.2/01- 6.

The mean NNG counts in both dose groups at 16 h sacrifice were higher than controls, but the mean values did not exceed zero which was the performing laboratories minimum threshold for a possible positive response. The number of cells in repair was very low. Historical vehicle control values for 16 h sacrifice times are also presented in Table 5.4.2/01- 5 for comparison.

In the 4 h sacrifice assay, there was no increase in NNG counts in either treated group, nor any treated animal with positive NNG counts.

Table 5.4.2/01- 4: *In vivo* rat liver UDS assay – group mean values ± standard deviation

Treatment group / dose level	Net grains per nucleus ± SD	Mean grains per nucleus ± SD	Mean cytoplasmic grain count ± SD	Mean cells in repair (%)
4 h sacrifice				
Vehicle control / 0 mg/kg	- 1.11 ± 1.6	2.59 ± 1.8	3.69 ± 0.9	0.17
Prothioconazole / 2500 mg/kg	- 0.98 ± 1.7	2.82 ± 2.0	3.80 ± 0.8	0.00
Prothioconazole / 5000 mg/kg	- 1.01 ±2.0	4.15 ± 2.3	5.16 ± 0.9	0.17
Pos. control / DMH 40 mg/kg	3.18 ± 2.0	6.60 ± 2.3	3.42 ± 1.1	18.7
16 h sacrifice				
Vehicle control / 0 mg/kg	- 0.82 ± 1.15	0.73 ± 0.86	1.55 ± 0.91	0.00
Prothioconazole / 2500 mg/kg	- 0.37 ± 1.25	1.11 ± 1.15	1.48 ± 0.76	0.00
Prothioconazole / 5000 mg/kg	- 0.16 ± 1.28	1.29 ± 1.31	1.45 ± 0.87	0.18
Pos. control / 2-AAF 100 mg/kg	3.54 ± 2.32	5.07 ± 2.70	1.53 ± 1.06	24.8

Table 5.4.2/01- 5: Historical control data (16 h sacrifice time)

Year of study and number of animals (n)	Mean net grains per nucleus	Mean grains per nucleus	Mean cytoplasmic grain count	Mean % cells in repair
1994 (n=4)	-0.73	0.77	1.51	0
1995 (n=3)	-0.74	0.37	1.11	0
1996 (n=4)	-1.22	0.72	1.94	0
1996 (n=4)	-1.30	3.92	5.21	0
1997 (n=6)	-1.06	1.94	3.00	0

All studies used 0.5 % aqueous Cremophor and a 16 h sacrifice time

When the results for individual animals (16 h sacrifice) are examined, the animals treated at 2500 mg/kg bw all had negative NNG counts with only one animal having a higher NNG count than the highest vehicle control animal. At 5000 mg/kg bw, two animals had positive NNG counts, the remaining 2 animals had NNG counts comparable with the concurrent vehicle controls.

Two vehicle control animals from another study (T6040651, conducted at the same test laboratory), also displayed positive NNG counts. The NNG counts for the animals from study T6040651 were 0.14 (± 0.68) and 0.44 (± 0.63), and are very similar to the positive counts observed in this study. Hence this finding is not considered critical.

Table 5.4.2/01- 6: *In vivo* rat liver UDS assay – individual values ± standard deviation of 16 h sacrifice groups

Individual animal	Net grains per nucleus[a] ±SD	Mean grains per nucleus[a] ±SD	Mean cytoplasmic grain count[a] ±SD	Mean % cells in repair[b]	Absolute survival[c] (%)
Vehicle control					
Animal 1	-1.00 ± 1.18	0.56 ± 0.79	1.56 ± 1.03	0	79.9
Animal 2	-0.68 ± 1.09	0.60 ± 0.87	1.28 ± 0.91	0	87.8
Animal 3	-0.32 ± 1.13	0.84 ± 0.88	1.16 ± 0.71	0	80.7
Animal 4	-1.29 ± 1.20	0.91 ± 0.90	2.21 ± 0.95	0	84.3
2500 mg/kg bw prothioconazole					
Animal 1	-0.15 ± 1.30	0.97 ± 1.08	1.12 ± 0.66	0	71.9
Animal 2	-0.37 ± 1.36	1.37 ± 1.26	1.73 ± 0.83	0	76.7
Animal 3	-0.56 ± 1.18	1.19 ± 1.09	1.75 ± 0.76	0	77.7
Animal 4	-0.41 ± 1.17	0.89 ± 1.16	1.30 ± 0.80	0	80.8
5000 mg/kg bw prothioconazole					
Animal 1	0.41 ± 1.48	1.61 ± 1.61	1.21 ± 0.89	0.7	71.9
Animal 2	0.12 ± 1.19	1.45 ± 1.12	1.34 ± 0.79	0	81.0
Animal 3	-0.81 ± 1.29	1.21 ± 1.07	2.02 ± 1.04	0	78.4
Animal 4	-0.37 ± 1.14	0.87 ± 0.97	1.24 ± 0.74	0	79.7
Positive control (2-AAF)					
Animal 1	3.66 ± 2.36	5.25 ± 2.71	1.60 ± 1.08	29.3	77.2
Animal 2	4.19 ± 2.67	5.81 ± 3.13	1.62 ± 1.14	33.3	78.2
Animal 3	3.41 ± 2.08	4.69 ± 2.23	1.29 ± 0.76	20.7	75.7
Animal 4	2.91 ± 2.17	4.51 ± 2.68	1.60 ± 1.21	16.0	80.3
Historical control values (Study T6040651, 1992)[d]					
Animal 10	0.14 ± 0.68	-	-	-	-
Animal 15	0.44 ± 0.63	-	-	-	-

[a] Average values of triplicate coverslips

[b] Average values of percentage of cells with 5 or more net nuclear grains on triplicate coverslips

[c] Percentage of viable cells after isolation

[d] values were submitted in M-078413-01-1 ("RESPONSE TO COMMENTS FROM PSD / IDS DATED JANUARY 7, 2003 ON DEVELOPMENTAL TOXICITY (AND IMPLICATIONS FOR RISK ASSESSMENT) AND ON GENOTOXICITY, INCLUDING COMMENTS ON THE RESPECTIVE CHAPTERS OF THE DRAFT PSD-MONOGRAPH (VERSION DATED NOVEMBER 25 (JAU 6476) OR NOVEMBER 26 (SXX 0665), 2002)"). Together with this information, scanned raw data pages were submitted that contain individual NNG cell counts (plus mean values for the three separate slides per animal) for the two animals with increased NNG counts from the study conducted with prothioconazole as well as individual and mean NNG counts per slide for the two historical control animals with positive NNG counts from study T6040651 – these data are not compiled here.

III. CONCLUSION

The results for the 4 hour sacrifice animals were clearly negative. The NNG counts of the 16 hour sacrifice animals are generally higher than controls but this is not considered to represent a positive result for the following reasons:

1) The NNG counts are only positive in 2/4 animals, the counts in the other two animals are within the range of the concurrent controls, therefore the increase is not consistent within the group.

2) The NNG counts in this group do not exceed the criteria provided by the performing laboratory for a positive response (mean NNG counts less than zero are considered to be negative). The proportion of cells in repair was also very low.

3) Two vehicle control animals from another study (T6040651) conducted at the same test laboratory, also displayed very similar positive NNG counts, hence this finding is not critical.

Based on the results of this study prothioconazole is considered negative in the *in vivo* UDS Assay with rat liver cells.

Report:	KCA 5.4.2/01 [redacted] 1996, M-012265-01-1
Title:	JAU 6476 - Micronucleus test on the mouse
Report No.:	25572
Document No.:	M-012265-01-1
Guideline(s):	OECD 474 (1983), EEC Directive 92/69/EEC B.12, US-EPA OPPTS 870.5395 (1996)
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.4.2/01. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.4.2/02** (also in numbering of tables/figures).

Deviations: The following deviations from the current OECD guideline (2016) occurred:
Treatment and sampling: As required by the 1983-guideline, which was valid at the time of the study conduct, the test substance was administered only once. The way of administration was not specified in the 1983-guideline and intraperitoneal injection of JAU 6476-desthio was chosen to maximise systemic exposure (instead of oral gavage as recommended nowadays). Intraperitoneal injection was still one of the suggested routes of exposure in the 1997 guideline. Only a single dose level was used as required by the 1983 guideline, whereas the current guideline specifies a minimum of 3 dose levels However, the maximum tolerated dose (MTD) was reached in the current study, which is also required by the current guideline. The method of euthanasia was not reported.
Observations: 1000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei as required by the 1983 guideline. According to the current guideline a minimum of 4000 PCE/animal should be scored for the incidence of micronuclei.

Executive summary:

In a 1996 GLP study, groups of 5/sex albino mice (Hsd/Win:NMRI strain) were treated with prothioconazole (batch no. NLL 6096-9.1, purity 99.9 %) by intraperitoneal injection at a dosage of 250 mg/kg bw. The vehicle used was 0.5 % aqueous Cremophor. Femoral bone marrow was prepared at sacrifice after 16, 24 or 48 hours. Vehicle and positive (cyclophosphamide) controls were also used (sacrificed after 24 hours only). Coded slides were evaluated for micronuclei from 1000 polychromatic erythrocytes (PCEs) per animal. In addition, the number of normochromatic erythrocytes (NCEs) per

1000 PCEs was noted. The study was conducted according to the original OECD guideline 474 (1983), deviations from the current (2016) guideline are noted above.

The dose level (250 mg/kg bw) was selected on the basis of a pilot study in which 5 animals (unspecified mixture of males and females) received 100, 250, 350 or 500 mg/kg bw intraperitoneally with clinical signs starting at the lowest dose and high mortality at 500 mg/kg bw (4/5 animals died).

In the main study there were no deaths. Clinical signs of toxicity were recorded for up to 24 hours. After 24 hours, no signs were apparent and there was no effect on food consumption over the course of the study. No signs were apparent in control animals. There was no significant increase in the incidence of micronucleated polychromatic erythrocytes (PCEs) over vehicle controls at any time point. The PCE/NCE ratio was not affected by treatment (the ratios at each time point were within the range of laboratory historical control values). The positive control gave satisfactory results.

Exposure of the bone marrow to prothioconazole or its metabolites was demonstrated in rats in the whole body autoradiography study. Measurable (>LOQ) quantities of material were present in bone marrow after oral administration of 4 mg/kg bw. Thus, the bone marrow is likely to have been exposed to a 'biologically relevant concentration' after an i.p. dose of 250 mg/kg bw. The clinical signs recorded support the conclusion that there was significant systemic exposure.

The study recorded a negative result. Though the study was not fully compliant with the current OECD Guideline, it was compliant with the 1983 Guideline. Given the positive results in the *in vitro* chromosome aberration assay in Chinese hamster lung cells, further clastogenicity data was considered necessary by a regulatory authority to provide reassurance regarding the potential clastogenic effects of prothioconazole. This is presented in the form of a second micronucleus assay conducted according to the 1997 OECD guideline (see 5.4.2/03).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Prothioconazole

Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-9.1
Purity:	99.9 %
Stability of the test compound:	The batch used was analysed prior to study initiation and approved for use during the test period. A stability test in the vehicle did not detect a relevant change in the percent active ingredient.
Vehicle used:	0.5 % aqueous Cremophor emulsion

2. Control Materials:

Vehicle control:	0.5 % aqueous Cremophor emulsion
Positive control:	Cyclophosphamide (CP) in deionized water; 20 mg/kg bw

3. Test animals:

Species:	Mouse	
Strain:	Hsd/Win:NMRI	
Sex:	Males and females, (females were nulliparous)	
Age:	6-12 weeks	
Weight:	Males	38-42 g
	Females	27-33 g
Source:	[redacted], Germany	
Acclimation period:	At least one week	

Number of animals per dose
Range finding test: 5/group (unspecified mixture of males and females)
Micronucleus assay: 5/sex/ group
Diet: Altromin 1324 Standard Diet ([redacted] Germany), *ad libitum*
Water: Tap water, *ad libitum*
Housing: The animals were kept singly in type I cages. Bedding of soft wood granules was used.

Environmental conditions:
Temperature: 22.5-23 C
Humidity: 52-56 %
Air changes: About 10 times per hour
Photo period: 12 h of electrical lighting daily

4. Test compound doses
Range finding test: 100, 250, 350, 500 mg prothioconazole/kg bw
Micronucleus assay: 0, 250 mg prothioconazole/kg bw
Prothioconazole was administered once by intraperitoneal injection.

B. TEST PERFORMANCE

1. Dates of experimental work: July 09, 1996 – July 30, 1996

2. Preliminary range finding test

5 animals (unspecified mixture of males and females) received 100, 250, 350 or 500 mg/kg bw intraperitoneally. Clinical signs were recorded for up to 48 hours.

3. Micronucleus assay

Treatment and sampling

Each group comprised ten mice, five males and five females. Each respective substance was administered once.

Table 5.4.2/02- 1: Treatment overview

	Vehicle control	Prothioconazole			Positive control CP
Dose (mg/kg bw)	**0**	**250**	**250**	**250**	**20**
Volume (ml/kg bw)	10	10	10	10	10
Route of application	i.p.	i.p.	i.p.	i.p.	i.p.
No. of animals treated	10	10	10	10	10
Time of sacrifice	24 hrs	16 hrs	24 hrs	48 hrs	24 hrs

At least one intact femur was prepared from each sacrificed animal. The femur was separated from all soft tissue. The proximal end of the femur was opened at its extreme end and the bone marrow was flushed out in a tube with fetal calf serum and subsequently centrifuged at approximately 1000 rpm for five minutes. The supernatant was discharged leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension.

Slide preparation

One drop of the viscous suspension was placed on a wellcleaned slide and spread with a suitable object to allow proper evaluation of the smear. The slides were then dried overnight. After drying the smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry. Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly to be covered and a small amount of covering agent was applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

Slide evaluation

In general, 1000 polychromatic erythrocytes (PCEs) were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern.

The number of normochromatic erythrocytes per 1000 polychromatic ones was noted to establish the ratio of polychromatic to normochromatic erythrocytes, which is expedient for two reasons:

1. Individual animals with pathological bone marrow depressions may be identified and excluded from the evaluation.
2. An alteration of this ratio may show that the test compound actually reaches the target.

In addition the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways:

1. It permits the detection of individuals already subject to damage before the start of the test.
2. Combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances.

4. Statistics

The number of polychromatic erythrocytes with micronuclei and the number of normochromatic erythrocytes of the dose groups and the positive control were analyzed by Wilcoxon's non-parametric rank sum test. A variation was considered statistically significant if its error probability was below 5 % and the treatment group figure was higher than that of the negative control.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided chi^2-test. A variation was considered statistically significant if the error probability was below 5 % and the treatment group figure was higher than that of the negative control.

In addition, standard deviations (1s ranges) were calculated for all the means.

5. Evaluation criteria

The assay is considered positive, negative or equivocal if the following criteria apply:

positive - if there is a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.

negative - if there is no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes at any time.
- if there is a significant increase in that rate which, according to the laboratory's experience, is within the range of negative controls.

equivocal - if there is an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls (provided the increase is not

significant and the result of the negative control is not closely related to the data of the respective treatment group)

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The stability of prothioconazole in vehicle was confirmed by analysis. The analytical determinations verified that prothioconazole is stable in the vehicle at room temperature at concentrations ranging from 1 mg/ml to 50 mg/ml for at least twenty-four hours.

Table 5.4.2/02- 2: Analysis for stability of prothioconazole in the solvent at room temperature

Nominal value in mg/ml	Content in % after storage time	
	0 hrs	24 hrs
1.0	90.9	92.8
50	94.9	97.3

B. PRELIMINARY RANGE FINDING TEST

5 animals (unspecified mixture of males and females) received 100, 250, 350 or 500 mg/kg bw intraperitoneally. The following clinical signs were recorded for up to 48 hours:

100 mg/kg bw: apathy, staggering gait, spasm and difficulty in breathing.

≥ 250 mg/kg bw : sternal recumbency and semi-anaesthestised state.

≥ 500 mg/kg bw: lateral recumbency, extension spasm, leaping spasm, twitching In addition, 4/5 animals died in the 500 mg/kg bw group.

Based on these results, 250 mg/kg prothioconazole was chosen as maximum tolerated dose for the micronucleus assay.

C. MICRONUCLEUS ASSAY

Clinical findings

In the main study there were no deaths. The following clinical signs of toxicity were recorded for up to 24 hours: apathy, semi-anaesthetized state, staggering gait, sternal recumbency, spasm and difficulty in breathing. After 24 hours, no signs were apparent and there was no effect on food consumption over the course of the study. No signs were apparent in control animals.

Micronucleus assay results

There was no significant increase in the incidence of micronucleated PCEs over vehicle controls at any time point (Table 5.4.2/02- 3). The PCE/NCE ratio was not affected by treatment (the ratios at each time point were within the range of laboratory historical control values). The positive control gave satisfactory results.

Table 5.4.2/02- 3: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE

Treatment group / sampling interval	No. of animals	Total no. PCE scored	No. NCE / 1000 PCE ± SD	No. micronucleated cells/1000 ±SD	
				NCE	PCE
Vehicle control / 24 h	10	10000	775 ± 201	2.0 ± 1.7	1.7 ± 1.3
Prothioconazole / 250 mg/kg / 16 h	10	10000	911 ± 274	3.0 ± 2.5	2.6 ± 1.6
Prothioconazole / 250 mg/kg / 24 h	10	10000	968* ± 221	1.7 ± 1.1	2.0 ± 1.6
Prothioconazole / 250 mg/kg /48 h	10	10000	877 ± 270	1.8 ± 1.2	2.3 ± 1.3
CP / 20 mg/kg / 24 h	10	10000	707 ± 244	0.5 ± 0.7	17.1** ± 9.2
Historical control range[a]	-	-	695 - 1159	0.1 - 1.9	0.9 - 2.6

* $p < 0.05$; ** $p < 0.01$ (Wilcoxon's non-parametric rank sum test)

[a] mean values from 12 studies performed during 1993 - 1995 using 0.5 % Cremophor vehicle

Exposure of the bone marrow to prothioconazole or its metabolites was demonstrated in rats in the whole body autoradiography study. Measurable (>LOQ) quantities of material were present in bone marrow after oral administration of 4 mg/kg bw (M-034660-01-1). Thus, the bone marrow is likely to have been exposed to a 'biologically relevant concentration' after an i.p. dose of 250 mg/kg bw. The clinical signs recorded support the conclusion that there was significant systemic exposure.

III. CONCLUSION

The study recorded a negative result. Though the study was not fully compliant with the current OECD guideline, it was compliant with the contemporary guideline. Given the positive results in the in vitro chromosome aberration assay in Chinese hamster lung cells, further clastogenicity data was considered necessary by a regulatory authority to provide reassurance regarding the potential clastogenic effects of prothioconazole. This is presented in the form of another micronucleus assay conducted to the, at that time most up-to-date OECD guideline (1997).

Report:	KCA 5.4.2/02[redacted],; 2003; M-102790-01-1
Title:	JAU 6476 - Micronucleus-test on the male mouse
Report No.:	AT00605
Document No.:	M-102790-01-1
Guideline(s):	OECD 474 (1997), Commission Directive 2000/32/EC B-12 (2000); US-EPA712-C-98-226, OPPTS 870.5395 (1988)
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.4.2/02. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.4.2/03** (also in numbering of tables/figures).

Deviations: The following deviations from the current OECD guideline (2016) occurred:
Treatment and sampling: Intraperitoneal injection of prothioconazole was chosen to maximise systemic exposure (instead of oral gavage as recommended nowadays). According to the 1997 guideline administration via intraperitoneal injection was still accepted. The method of euthanasia was not reported.
Observations: 2000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei as required by the 1997 guideline. According to the current guideline a minimum of 4000 PCE/animal should be scored for the incidence of micronuclei. Nonetheless, since there was clearly no effect on the number of micronucleated PCE in the current study, the result obtained is still considered valid.

Executive summary:

Prothioconazole (batch no. 6023/0016, purity 98.7 %), was tested for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts of male mice (Hsd/Win: NMRI). Males only were used, because there were no substantial differences between the sexes in a range finder test for this study. Animals received two intraperitoneal administrations of 50, 100 and 200 mg/kg bw, respectively, separated by 24 hours. The femoral marrow of all groups was prepared 24 hours after the last administration. Negative and positive control (cyclophosphamide, 20 mg/kg bw i.p.) groups were also included. The study was conducted according to OECD guideline 474 (1997) and was compliant to GLP. The deviations from the current OECD guideline listed above are not found to compromise the scientific outcome of the study.

A relevant systemic exposure to prothioconazole was indicated by clinical signs starting at 50 mg/kg bw (apathy, roughened fur, sternal recumbency, spasm, twitching, periodically stretching of body, difficulty in breathing) and by the altered ratio between polychromatic and normochromatic erythrocytes at 200 mg/kg bw. No increase in micronucleated cells after treatment with prothioconazole was observed. No test has been performed to identify the nature of the micronuclei in order to determine whether the mechanism of micronucleus induction is due to clastogenic and/or aneugenic activity. However, the fact that prothioconazole did not cause any increase in micronuclei formation implies that prothioconazole did exhibit neither clastogenic nor aneugenic activity in this assay.

The positive control cyclophosphamide had a clear clastogenic effect, and thus demonstrated the sensitivity of the test.

Prothioconazole was not clastogenic or aneugenic in this *in vivo* test system in male mice.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476

Description:	Fine yellowish powder
Batch No.:	6023/0016
Purity:	95.7 %
Stability of the test compound:	The batch used was analytically examined prior to study initiation and was approved for use for the test period. A stability test in the vehicle did not reveal significant degradation of the active ingredient.
Vehicle used:	0.5 % aqueous Cremophor emulsion

2. Control Materials:

Vehicle control:	0.5 % aqueous Cremophor emulsion
Positive control:	Cyclophosphamide (CP); solvent: deionized water

3. Test animals:

Species:	Mouse
Strain:	Hsd/Win:NMRI
Sex:	Males
Age:	6-12 weeks
Weight:	37-43 g
Source:	[redacted] Germany
Acclimation period:	At least 5 days
Number of animals per dose:	5/group
Diet:	[redacted] CH-[redacted], *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	The animals were kept singly in type I cages. Bedding of soft wood granules was used.

Environmental conditions:

Temperature:	23°C
Humidity:	44-58 %
Air changes:	About 10 times per hour
Photo period:	12 h of electrical lighting daily

4. Test compound doses

Micronucleus assay:	0, 50, 100, 200 mg prothioconazole/kg bw Prothioconazole was administered twice by intraperitoneal injection.

B. TEST PERFORMANCE

1. Dates of experimental work: July 09, 2003 - July 21, 2003

2. Dose selection

The selection of the prothioconazole doses was based on a pilot test (data taken from study T2063355). This pilot test was performed in the laboratory which conducted the main study using animals of the same source, strain and age. Groups consisting each of three males and three females received two intraperitoneal injections separated by 24 hours. 250 mg/kg were used.

In males the following symptoms were recorded for up to at least 24 hours after the second application, starting at apathy, roughened fur, loss of weight, sternal recumbency, spasm, periodically stretching of body and difficulty in breathing. In addition, 1 of 3 males died. In females the following symptoms were recorded for up to at least 24 hours after the second application: apathy, roughened fur, sternal recumbency, spasm and difficulty in breathing. No female died.

Based on these findings, 200 mg/kg prothioconazole were chosen as MTD for males. Due to the results of the dose range finder it is concluded, that there are no substantial differences between sexes in toxicity. Therefore, no females were used.

3. Micronucleus assay

Treatment and sampling

Each group comprised five male mice. They were divided into groups by a randomization plan. Mice treated with prothioconazole received two intraperitoneal administrations separated by 24 hours. Cyclophosphamide was administered only once.

Table 5.4.2/03- 1: Treatment overview

	Vehicle control	Prothioconazole			Positive control
					CP
Dose (mg/kg bw)	0	50	100	200	20
Volume (ml/kg bw)	10				
Route of application	intraperitoneal application (i.p.)				
No. of applications (separated by 24 hours)	2	2	2	2	1
No. of animals treated	5	5	5	5	5
Time of sacrifice	24 hours after last treatment				

The femoral marrow of all groups was prepared 24 hours after the last administration. At least one intact femur was prepared from each sacrificed animal. The femur was separated from all soft tissue. The proximal end of the femur was opened at its extreme end and the bone marrow was flushed out in a tube with fetal calf serum and subsequently centrifuged at approximately 1000 rpm for five minutes. The supernatant was discharged leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension.

Slide preparation

One drop of the viscous suspension was placed on a well cleaned slide and spread with a suitable object to allow proper evaluation of the smear. The slides were then dried overnight. After drying the smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry. Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly to be covered and a small amount of covering agent was applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

Slide evaluation

In general, 2000 polychromatic erythrocytes (PCEs) were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern.

The number of normochromatic erythrocytes (NCE) per 2000 polychromatic ones was noted to establish the ratio of polychromatic to normochromatic erythrocytes, which is expedient for two reasons:

3. Individual animals with pathological bone-marrow depressions may be identified and excluded from the evaluation.
4. An alteration of this ratio may show that the test compound actually reaches the target.

In addition to the number of normochromatic erythrocytes per 2000 polychromatic ones, the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways:

3. It permits the detection of individuals already subject to damage before the start of the test.
4. Combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances.

4. Statistics

The number of polychromatic erythrocytes with micronuclei and the number of normochromatic erythrocytes of the dose groups and the positive control were analysed by Wilcoxon's non-parametric rank sum test. A variation was considered statistically significant if its error probability was below 5% and the treatment group figure was higher than that of the negative control.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided chi²-test. A variation was considered statistically significant if the error probability was below 5 % and the treatment group figure was higher than that of the negative control.

In addition, standard deviations (1s ranges) were calculated for all the means.

5. Evaluation criteria

The assay is considered positive, negative or equivocal if the following criteria apply:

positive	-	if there is a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.
negative	-	if there is no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes.
	-	if there is a significant increase in that rate which, according to the laboratory's experience, is within the range of negative controls.
equivocal	-	if there is an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls (provided the increase is not significant and the result of the negative control is not closely related to the data of the respective treatment group)

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The stability of prothioconazole in vehicle was confirmed by analysis. The analytical determinations verified that prothioconazole is stable in the vehicle at room temperature at concentrations ranging from 1 mg/ml to 50 mg/ml for at least twenty-four hours, a time interval, which covers the time range from preparation of the formulation to last treatment.

Table 5.4.2/03-2: Analysis for stability of prothioconazole in the solvent at room temperature

Nominal value in mg/ml	Content in % after storage time	
	0 hrs	24 hrs
1	90.9	92.8
50	94.9	97.3

B. MICRONUCLEUS ASSAY

Clinical findings

After two intraperitoneal administrations of 50, 100 and 200 mg/kg prothioconazole, treated males showed the following compound-related symptoms until sacrifice: apathy, roughened fur, sternal recumbency, spasm, twitching, periodically stretching of body and difficulty in breathing. These symptoms demonstrate relevant systemic exposure of males to prothioconazole. There was no substance-induced mortality. No symptoms were recorded for the control groups. No animals died in these groups.

Micronucleus assay results

The ratio of polychromatic to normochromatic erythrocytes in males was altered by the treatment with prothioconazole, being 2000: 1650 in the negative control, 2000:1811 in the 50 mg/kg group, 2000:1711 in the 100 mg/kg group and 2000:2582 in the 200 mg/kg group (Table 5.4.2/03- 3). Biologically relevant variations were thus noted for males treated with 200 mg/kg prothioconazole. This finding demonstrates relevant systemic exposure of the males to prothioconazole.

No biologically important or statistically significant variations existed for males between the negative control and the groups treated intraperitoneally with prothioconazole, with respect to the incidence of micronucleated polychromatic erythrocytes. No relevant variations between the negative control and prothioconazole groups in the number of micronucleated normochromatic erythrocytes were observed.

Table 5.4.2/03- 3: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE

Treatment group	No. of animals	Total no. PCE scored	No. NCE / 2000 PCE ± SD	No. micronucleated cells/2000 ± SD	
				NCE	PCE
Vehicle control	5	10000	1650 ± 551	4.0 ± 1.7	4.4 ± 2.4
Prothioconazole 2x50 mg/kg	5	10000	1811 ± 475	3.4 ± 2.3	4.2 ± 3.3
Prothioconazole 2x100 mg/kg	5	10000	1711 ± 323	4.2 ± 3.1	4.2 ± 1.8
Prothioconazole 2x200 mg/kg	5	10000	2582[#] ± 926	3.0 ± 1.6	2.2 ± 0.8
CP 1x20 mg/kg	5	10000	1017 ± 847	4.5 ± 2.9	27.4* ± 2.5

* $p < 0.05$ (Wilcoxon's non-parametric rank sum test) [#] biologically relevant increase
PCE: polychromatic erythrocytes NCE: normochromatic erythrocytes

The positive control, cyclophosphamide, caused a clear increase in the number of polychromatic erythrocytes with micronuclei.

Cyclophosphamide did not affect the number of micronucleated normochromatic erythrocytes and no effect was found concerning the ratio of polychromatic to normochromatic erythrocytes, since this ratio did not vary to a biologically relevant degree. This clearly demonstrates that an alteration of the ratio of polychromatic to normochromatic erythrocytes is not necessary for the induction of micronuclei.

III. CONCLUSION

Prothioconazole did not induce increased incidences of micronucleated polychromatic erythrocytes while relevant systemic exposure was demonstrated by an altered ratio of polychromatic to normochromatic erythrocytes at 200 mg/kg bw and clinical signs starting at 50 mg/kg bw.

Therefore it can be concluded that prothioconazole had no clastogenic or aneugenic effects in this *in vivo* test system in male mice.

CA 5.4.3 In vivo studies in germ cells

Since overall it was concluded that prothioconazole did not show a genotoxic potential, and since no evidence of an effect on germ cells was seen in other studies, an *in vivo* genotoxicity study in germ cells was not regarded as necessary.

CA 5.5 [redacted]-term toxicity and carcinogenicity

All necessary long-term toxicity studies were presented and evaluated during the EU process for Annex I listing. Please refer to the DAR for the first Annex I inclusion and the baseline dossier of prothioconazole.

Table 5.5-1 Summary of long term studies

Study	NOAEL	LOAEL	Findings at LOAEL	Reference
1 year rat (gavage) 0, 5, 50 and 750 mg/kg bw/day	50 mg/kg bw/day	750 mg/kg bw/day	↓ Bodyweight gains and gross necropsy and microscopic findings in the liver and kidneys and urinary bladder (with accompanying clinical chemistry and urinalysis effects), strongly increased water consumption, isolated deaths (possibly related to kidney failure)	[redacted], 2000 M-030441-01-1
2 year rat (gavage) 0, 5, 50 and 750/500 mg/kg bw/day	5 mg/kg bw/day	50 mg/kg bw/day	Gross necropsy and microscopic findings in the liver and kidneys. Similar but more marked findings were also recorded at the high dose level, along with mortality (obviously related to kidney failure) and numerous wide-ranging effects.	[redacted], 2001 M-084962-01-1
Supplementary information: Clinical Pathology, Reference-Values, Edition 2002	---	---	This document contains clinico chemical and hematological historical control values applicable for the 2 year rat study.	[redacted], 2002 M-068712-01-1
18 month mouse (gavage) 0, 10, 70 and 500 mg/kg bw/day	10 mg/kg bw/day	70 mg/kg bw/day	↓ Body weight gains and gross necropsy and microscopic findings in the liver and kidneys. Similar but more marked findings recorded at the high dose level.	[redacted], 2001 M-085068-01-1

In a two year rat study, the MTD was exceeded at the high dosage (wide-ranging adverse effects and increased mortality). The most notable effects were in the liver and kidneys, which were also recorded to a lesser extent at the mid-dose level. The liver effects included increased liver weights, centrilobular hepatocellular hypertrophy with cytoplasmic change and eosinophilic/clear cell foci with cytoplasmic change.

The kidney findings included increased weights and increased severity of chronic progressive nephropathy. They were accompanied by increased water consumption, urinalysis effects, crystalline material in the urine sediment and transitional cell hyperplasia in the urinary bladder.

Despite these treatment-related findings, there were no notable neoplastic findings in the liver, kidneys or urinary bladder, or in any other organs and tissues. The overall incidence of tumour-bearing animals, the time of occurrence and the pattern of neoplastic findings did not indicate a carcinogenic effect.

The liver and kidney findings in the two year rat study were consistent with a one year rat study performed using the same initial dose levels. In the one year study, effects were confined to the 750 mg/kg bw/day dose level only and the mortality recorded in the two year study was observed only in a few cases (two males in weeks 40 and 51, respectively, and one female in week 37) ~~did not occur~~. The severity of chronic progressive nephropathy was increased by treatment for one year, and it would seem that continued exposure to prothioconazole beyond one year leads to deteriorating health and

eventual mortality due to the prolonged and increasingly more severe breakdown of kidney function (which the eventual lowering of the high dose level in the later part of the chronic rat study attempted to address). Prolonged treatment also results in adverse kidney effects becoming apparent at the intermediate dose level of 50 mg/kg bw/day. This accounts for the lower NOAEL in rats from chronic treatment compared to the one year study.

In an 18 month mouse study, the effects recorded at the high dose were less marked than in the rat study, but the adverse bodyweight effects and a high incidence of histopathological findings in the kidneys suggests that the high dose level was close to the MTD.

Consistent with rats, the liver and kidneys were the target organs in mice. The liver effects were consistent with the chronic rat study (hypertrophy/cytoplasmic change). The kidney effects were decreased weights, tubular degeneration/regeneration and subcapsular tubular degeneration with interstitial fibrosis. There was no increase in neoplastic findings in the liver or kidneys, and the pattern of neoplastic findings in all organs and tissues did not indicate a treatment-related effect.

Comparing the results between the sexes, males were more markedly affected than females in both rats and mice, especially with respect to the effects on the kidneys. It was often the case that effects would be recorded in males at both the intermediate and high dose levels, but only at the high dose level in females. It is noted that the eventual high dose levels were different for males and females in the chronic rat study, but the intermediate dose levels were the same between the rat and mouse studies.

It can be concluded that prothioconazole is not carcinogenic in rats or mice.

CA 5.6 Reproductive toxicity

Table 5.6- 1: Summary of reproductive studies

Study	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Findings at LOAEL	Ref.
Pilot reproductive study in rats (gavage) 0, 10, 100, 250 and 500 mg/kg bw/d	Parental toxicity: 250 Offspring: 250 Reproductive effects: 500	Parental toxicity: 500 Offspring: 500 Reproductive effects: >500	Urine stain, indicating severe disturbance of kidney function, ↓ body weights (males) ↓ pup weight gain -	[redacted], 1999, [M-018760-01-1]
2-generation study in rats (gavage) 0, 10, 100 and 750 mg/kg bw/d	Parental toxicity: 10 Offspring: 100 Reproductive effects: 100	Parental toxicity: 100 Offspring: 750 Reproductive effects: 750	Slight bodyweight and organ weight effects. At 750 mg/kg bw/d, the maternal toxicity is considered to be very high, even sublethal, based primarily on kidney dysfunction and resulting dehydration. ↓ pup weight gain, ↓ pup spleen weights (both generations), ↑ number of days to preputial separation in F1 males, (attributed to retarded growth) Affected oestrus cycling, ↓ implantation sites and litter size, ↑ time to insemination and ↑ duration of gestation (all secondary to maternal toxicity).	[redacted], 2001a [M-036206-01-1]
Supplementary information; New historical control data on F1 number of pre-antral follicles and F1 testicular sperm count	F1 pre-antral follicles and F1 testicular sperm count: 750	F1 pre-antral follicles and F1 testicular sperm count: 750	F1 number of pre-antral follicles and F1 testicular sperm count deviated in all dose groups from the concurrent controls. Since these changes were not dose-related, were not affected in the P-generation, were not confirmed by other corresponding parameters (epididymal sperm count, spermatology, testes histology, reproductive outcome) and since the dose group values are within the new historical control data, they are not considered as a treatment-related effect.	[redacted], 2015 [M-525951-01-1]

Study	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Findings at LOAEL	Ref.
Supplementary information: A new compilation of the individual F1 pup body weights at the respective individual day of preputial separation was generated as a direct download from the electronic raw data of this GLP-monitored study.	Preputial separation: 100	Preputial separation: 750	The delayed preputial separation is secondary to the clearly retarded growth (e.g., on post partum day 21 body weight was decreased by 15.5% in high dose male F1 pups). At the respective individual day of preputial separation, the high dose pups had reached a practically identical (-4%) body weight as the controls. This data constellation is clearly different from that to be expected for an endocrine-mediated effect – in that case a delay in preputial separation would be connected with a higher body weight at the day of preputial separation due to continuous growth over time.	[redacted], 2015 [M-524357-01-1]
Developmental toxicity study in rats (gavage) 0, 80, 500 and 1000 mg/kg bw/d	Maternal toxicity: 80 Feto- and developmental toxicity: 80 (conservative)*	Maternal toxicity: 500 Feto- and developmental toxicity: 500	↓ bodyweight gains, ↑ water consumption and urination Slightly ↑ incidence of rudimentary 14th ribs compared to concurrent control (but within historical control range) At 1000 mg/kg bw/d: ↓ fetal weights, ↑ incidence of engorged placentas, renal pelvis dilatation and incomplete ossification, ↑ incidence of microphthalmia and rudimentary 14th ribs (all secondary to maternal tox)	[redacted], 1997 [M-012279-01-1]

Study	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Findings at LOAEL	Ref.
Supplementary information: positive correlation between maternal toxicity and the occurrence of microphthalmia in the developmental toxicity study in rats ([redacted] 1997, M-012279-01-1)	Microphthalmia: 500*	Microphthalmia: 1000 (secondary to maternal toxicity)	At 1000 mg/kg, grouping of the dams separately for those that produced pups with microphthalmia and for those that did not have any pups with microphthalmia showed: • maternal toxicity was clearly more pronounced in the group of dams that had fetuses with microphthalmia • fetal weight (as a correlating, unspecific developmental toxic effect secondary to maternal toxicity) was more decreased in fetuses from litters that included fetuses with microphthalmia It can therefore be concluded that the severity of maternal toxicity correlates positively with the degree of fetal toxicity (body weight decrease) and with the occurrence of microphthalmia.	[redacted] 2007 [M-285563-01-1]
Supplementary information: Inhalative developmental toxicity study with an "irritating" compound (cyfluthrin) in the same rat strain and laboratory as the [redacted], 1997 study	Increased microphthalmia: 2.55 mg/m³ (cyfluthrin)	Increased microphthalmia: 11.9 mg/m³ (cyfluthrin; secondary to maternal bradypnea)	Reflectory induced maternal bradypnea / hypoxia / hypothermia / respiratory alkalosis caused secondarily fetal retardation and an enhancement of the (in this rat strain) common spontaneous malformation microphthalmia. Percent fetal (litter) incidences of microphthalmia were 5.4 (34.8) and, thus, even higher than those of 4.6 (33.3) as observed at 1000 mg prothioconazole/kg in the [redacted] (1997) study. Oxygen enrichment of the breathed air diminished the enhancement of microphthalmia to 2.9 (21.7). This demonstrates that microphthalmia can be enhanced by maternal toxicity in this rat strain.	[redacted] et al., 1996 [M-041671-02-1]

Study	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Findings at LOAEL	Ref.
Supplementary information: benchmark dose analysis of rudimentary 14th ribs observed in the developmental toxicity study in rats ([redacted] 1997, M-012279-01-1), using US-EPA software	Rudimentary 14th ribs: 80 (conservative)*	Rudimentary 14th ribs: 500 (conservative)	With a $BMDL_{10}$ value of > 300 mg/kg bw/d, the benchmark dose analysis supports that the NOAEL for rudimentary 14th ribs in the first rat developmental toxicity study ([redacted] 1997, M-012279-01-1) should be conservatively set at 80 mg/kg bw/d.	[redacted] et al., 2015 [M-531958-01-1]
Supplementary information: benchmark dose analysis of rudimentary 14th ribs observed in the developmental toxicity study in rats ([redacted] 1997, M-012279-01-1), following EFSA (2016) rules	Rudimentary 14th ribs: 80 (conservative)*	Rudimentary 14th ribs: 500 (conservative)	With $BMDL_{10}$ values of > 300 mg/kg bw/d, the benchmark dose analysis supports that the NOAEL for rudimentary 14th ribs in the first rat developmental toxicity study ([redacted] 1997, M-012279-01-1) should be conservatively set at 80 mg/kg bw/d.	[redacted] & 2017 [M-579365-01-1]
Developmental toxicity study in rats (gavage) using a strain with a virtually zero incidence of microphthalmia 0, 20, 80 and 750 mg/kg bw/d	Maternal toxicity: 80 Feto- and developmental toxicity: 80*	Maternal toxicity: 750 Feto- and developmental toxicity: 750	↓ net body weight gain, ↑ water consumption (up to +170 % of control), ↓ feed consumption and clinical chemical indications for functional impairments of kidneys and liver (a 25 % mortality, related to dehydration, was observed in the pilot developmental toxicity study at 1000 mg/kg) ↑ incidence of rudimentary (comma-shaped) 14th ribs (secondary to maternal tox)	[redacted], 2004 [M-067839-01-1]

Study	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Findings at LOAEL	Ref.
Supplementary information: Non-GLP "positive control" study (All Trans-Retinoic Acid, 15 mg/kg bw/d, daily on pregnancy days 6-15) conducted in 2001 in the same rat strain and laboratory as the [redacted], 2004 study	Feto- and developmental toxicity: < 15 (All Trans-Retinoic Acid)	Feto- and developmental toxicity: 15 (All Trans-Retinoic Acid)	The study showed certain types of malformations known to be caused by All Trans-Retinoic Acid. Specifically, ocular malformations like anophthalmia, microphthalmia, and small lens were observed in this study; this demonstrates the sensitivity of this rat strain to a direct, specific oculo-teratogenic effect of All Trans-Retinoic Acid.	[redacted] 2015 [M-517045-01-1]
Developmental toxicity study in rats (dermal) 1000 mg/kg bw/d - technical material - EC250 formulation - diluted EC250 formulation	1000 (technical) 250 (formulation) 62.5 (diluted formulation)	-	No systemic toxic effects and no effects on developing offspring at the dosages tested	[redacted] 2001b [M-035764-01-1]
Developmental toxicity range finding study in rabbits (gavage) 80, 100, 300 and 480 mg/kg bw/d	Maternal toxicity: 100 Developmental toxicity: 300 (limited investigations)	Maternal toxicity: 300 Developmental toxicity: 480	Study is of very limited value to establish a toxicological profile incl. a dose-response relationship and the setting of NOAELs since no concurrent control group was included and the number of investigated pregnant females in the dose groups was only 2-3. Observations with plausible link to treatment: mortality, bodyweight effects, ↓ feed consumption (at 300 and 480 mg/kg bw/d) ↑ post-implantation loss , ↓ fetal weights (due to number of runts in one litter)	[redacted], 1997 [M-012332-01-1]

Study	NOAEL (mg/kg bw/d)	LOAEL (mg/kg bw/d)	Findings at LOAEL	Ref.
Developmental toxicity study in rabbits (gavage) 0, 10, 30, 80 and 350 mg/kg bw/d	Maternal toxicity: 80 Developmental toxicity: 80	Maternal toxicity: 350 Developmental toxicity: 350	Mortality, ↓ feed consumption, bodyweight loss / ↓ gain, minimally ↓ absolute liver weights Abortions, total litter losses, ↓ gravid uterus and fetal weights (all secondary to maternal toxicity)	[redacted] 1998 [M-012227-01-1]

* At PRAPeR 04 Meeting (25–29 September 2006), the experts considered the results of two studies ([redacted] 1997 and [redacted], 2004) in combination to determine the developmental NOAEL of 20 mg/kg bw/d in rats (EFSA Scientific Report (2007), Conclusion on the peer review of prothioconazole). But a conservative developmental NOAEL in rats should be set at 80 mg/kg bw/d; this should trigger a revision of the currently established ARfD and AOEL.

In a multigeneration study performed in Wistar rats using gavage dosing parental general systemic toxicity was observed at the intermediate and high dose levels. Effects at the intermediate dose included lower body weight gains, decreased thymus weights and increased liver weights. At the high dose of 750 mg/kg bw/d similar but more marked effects were recorded, along with reduced efficiency of feed utilisation, increased kidney weights and histopathological findings in the liver and kidneys consistent with effects seen in previous repeated dose rat studies (hepatocytomegaly, multifocal chronic nephrosis). The maternal toxicity at 750 mg/kg bw/d is considered to be very strong, even sublethal, based primarily on the kidney dysfunction and resulting dehydration (compare maternal toxicity in rat developmental toxicity studies). Secondary to this strong maternal toxicity, there were reproductive effects recorded at the high dose (affected oestrous cycling, increased time to insemination, reduced implantation sites and increased duration of gestation), but these effects did not result in effects on mating, fertility or gestation indices.

Effects on developing pups were restricted to the high dose level and consisted of reduced pup weight gain, reduced spleen weights and delayed preputial separation (which was considered secondary to retarded pup growth). Since these effects occurred at a dose level also giving rise to clear effects in parental animals, a selective effect on offspring was not indicated. There was a clear margin between the NOAEL for parental toxicity and the NOAELs for reproductive effects and effects on offspring, therefore prothioconazole is not considered to be selectively toxic to the reproductive system or developing offspring.

In a developmental toxicity study in the Wistar rat (M-012279-01-1), an increased incidence of microphthalmia and of rudimentary 14th ribs together with a retarded fetal development (lower fetal weights, incomplete ossification, renal pelvic dilatation) and engorged placentas was observed at the high dose level (1000 mg/kg bw/d). Very strong (sublethal) maternal toxicity was also evident at this dose level, indicated by transient bodyweight loss, reduced bodyweight gains, drastically increased water consumption, and increased urination. Reduced bodyweight gain, strongly increased water consumption and increased urination were already present at 500 mg/kg bw/d. Consistent with the results obtained in other repeated dose studies in rats, severe disturbance of the kidney function and systemic water / electrolyte homoeostasis (dehydration) is the primary maternal toxicological target. Since this even caused mortalities at doses between 500 and 1000 mg/kg bw/d in other repeated dose rat studies (at 1000 mg/kg in pregnant dams of another Wistar substrain (25 % mortality related to dehydration), at 500 mg/kg bw/d in a 90-day study and at 750 mg/kg bw/d in the oncogenicity study, both conducted with rats of the same Wistar substrain as in the present study), 1000 mg/kg bw/d is considered as a highly (sublethal) maternally toxic dose in the rat developmental toxicity study. The

developmental effects at 1000 mg/kg bw/d are therefore considered to be secondary unspecific consequences of the observed very strong maternal toxicity at this dose.

This includes the enhancement of microphthalmia and of rudimentary supernumerary ribs, which are common spontaneous malformations / variations in untreated rats of this strain. For the same rat strain and test laboratory as used in the present study, an INHALATIVE developmental toxicity study with exposure to a another, sensory irritating compound caused, secondarily to reflectory induced maternal hypoventilation / hypoxia, a retarded fetal development together with an increased incidence of the common spontaneous malformation microphthalmia (at even higher fetal / litter incidences than in the prothioconazole study). Oxygen enrichment of the inhaled air partially compensated the bradypnea-related hypoxia and, thus, resulted in a reduction of the number of fetuses with microphthalmia at the same highest dose tested. In an ORAL developmental toxicity study, this test compound did not cause any microphthalmia at an approx. 10-fold higher systemic dose, excluding a specific teratogenic potential of this compound and demonstrating the possible occurrence of an unspecific enhancement of microphthalmia secondary to disturbed maternal health for this Wistar substrain (described by [redacted] et al., 1996, M-041671-02-1).

The correlation between the degree of maternal toxicity and the occurrence of microphthalmia in the prothioconazole rat developmental toxicity study at 1000 mg/kg bw/d was substantiated by grouping the maternal toxicity results separately for those dams that produced pups with microphthalmia and for those that did not have any pups with microphthalmia (M-285563-01-1). The outcome of this evaluation shows that

- clear maternal toxicity was present at 1000 mg/kg; but between the two subgroups, maternal toxicity was clearly more pronounced in the group of dams that had fetuses with microphthalmia (group +MO) than in the group of dams that had no fetuses with microphthalmia (group -MO)
- fetal weight (as a correlating, unspecific developmental toxic effect secondary to maternal toxicity) was decreased at 1000 mg/kg; between the two subgroups, fetal weight was more decreased in fetuses from litters that included fetuses with microphthalmia (group +MO) than in group –MO

It can therefore be concluded that the severity of maternal toxicity correlates positively with the degree of fetal toxicity (body weight decrease) and with the occurrence of microphthalmia.

A slightly increased incidence of microphthalmia was also recorded at the low dose level, but only marginally at the intermediate dose level, and this absence of a dosage-related trend and the fact that the incidence at the low and the intermediate dose was within the historical control range (the rat strain used for this study was a "high-background incidence" strain for spontaneous microphthalmia) suggests that this finding at the low- and intermediate dose levels was spontaneous rather than treatment-related.

Slightly increased incidences of rudimentary 14th ribs at the low and intermediate doses were within the historical control range respectively (historical control ranges were only slightly exceeded at 1000 mg/kg bw/d).

Despite this, in 2003 the UK Advisory Committee on Pesticides (ACP) was concerned that

- the microphthalmia / rudimentary 14th ribs observed at the highest dose could have been a specific developmental toxic effect of prothioconazole and not a non-specific enhancement of a common spontaneous malformation / variation secondary to maternal toxicity at this dose level
- the maternal toxicity observed at the highest dose may not have been sufficient to cause any such enhancements of common spontaneous malformation / variation
- respective slightly increased incidences for microphthalmia and rudimentary supernumerary ribs in the low- and mid-dose were not necessarily incidental biological variations (within the historical control range) and unrelated to prothioconazole

In order to investigate the specificity of microphthalmia and rudimentary 14th rib formation, in January 2004 a new (second) developmental toxicity study with prothioconazole was required by the UK Advisory Committee on Pesticides (ACP). Further requirements were the use of a "low-background

incidence" rat strain for microphthalmia and "objective measurements" for microphthalmia. Therefore the OECD-guideline study design was adapted with the assistance of an external expert for developmental toxicity ([redacted] Ph.D. (developmental anatomy (teratology)), at that time director of the Academy of Toxicological Sciences, President and CEO of Argus International, Inc., with more than 35 years of experience in industrial toxicology, personally involved in over 1200 developmental, reproductive and general toxicology evaluations) and was approved by the ACP.

The second prothioconazole developmental toxicity toxicity study (M-067839-01-1) was conducted in a different Wistar rat substrain for which the available historical control database reveals a virtually-zero background incidence of microphthalmia; thus, a non-specific enhancement of microphthalmia secondary to maternal toxicity could not be elicited in this strain. Since the strain was nevertheless sensitive to a direct, specific oculo-teratogenic effect (as shown with the positive control substance All Trans-Retinoic Acid (M-517045-01-1)) it was well suited to decisively investigate the specificity of microphthalmia formation caused by prothioconazole. In order to establish an objective measure for the ocular size, rather than the more subjective standard guideline observation according to the Wilson freehand slicing technique, fresh fetal eyes were extracted, weighed and morphometrically investigated.

In this second rat developmental toxicity study prothioconazole caused no specific malformations or abnormalities, including microphthalmia and anophthalmia, up to the maternally maximum tolerated dose of 750 mg/kg bw/d. There was a marginal increase in the incidence of comma shaped rudimentary supernumerary 14th ribs at the maternally maximum tolerated dose which is considered to be an enhancement of a common spontaneous variation due to maternal toxicity. The results from the second rat developmental toxicity study confirm that the increase in microphthalmia seen in the first rat developmental toxicity study was an unspecific enhancement of a common spontaneous malformation due to maternal toxicity rather than a direct teratogenic effect mediated by prothioconazole. The only marginal increase of rudimentary 14th ribs in the second study at the high dose of 750 mg/kg bw/d (which caused a comparably marked maternal toxicity as the high dose of 1000 mg/kg bw/d in the first study) confirms that the increase in supernumerary 14th ribs at the high dose of the first study can also be interpreted as being due to maternal toxicity and that the mid dose (500 mg/kg bw/d) in the first study can be interpreted as a NOAEL for this effect. A NOAEL for rudimentary 14th ribs of 500 mg/kg bw/d would also be supported by public literature which concludes that rudimentary 14th ribs should not be considered biologically significant in the absence of more profound signs of developmental toxicity – which are clearly absent in the first study at 500 mg/kg bw/d.

It was therefore conclusively demonstrated that there was no evidence of a teratogenic effect of prothioconazole in rats.

After the submission of the second rat developmental toxicity study, the ACP reconsidered their original concerns and concluded that

- the top dose used in the first study was clearly above the maximum tolerated dose
- it was possible to discount the observed microphthalmia at the low and intermediate dose levels in the first study because greater confidence could be placed in the objective measurements used in the second study
- the rudimentary 14th ribs were likely a result of maternal toxicity and the NOAEL for rudimentary 14th ribs in the first study is 500 mg/kg bw/d.

Based on the results of the second study, the British Authorities (ACP, PSD) approved prothioconazole with a NOAEL for developmental toxicity in the first study of 500 mg/kg bw/d and without a classification proposal for developmental toxicity.

At the PRAPeR 04 Meeting (September 2006), the experts defined a "combined" NOAEL of 20 mg/kg bw/d for the formation of rudimentary 14th ribs in rats, considering the results of both the first and the second developmental toxicity studies in rats. This NOAEL of 20 mg/kg bw/d is not considered appropriate for the following reasons:

- it is obvious that in the second study the clear NOAEL for rudimentary 14th ribs is 80 mg/kg bw/d,
- this NOAEL of the second study is highly significant since the fetal sample size in this second study was clearly higher (approx. 200 fetuses per dose group since all fetuses were used for skeletal examination of ribs) as in the first study (approx. 150 fetuses per dose group since the remaining fetuses were used for visceral investigation according to the standard guideline study protocol)
- in the second study the LOAEL for a marginal increase of only comma-shaped (but not punctiform) rudimentary 14th ribs is the highest tested dose of 750 mg/kg bw/d – this dose caused a comparably marked maternal toxicity as the high dose of 1000 mg/kg bw/d in the first study (the slightly more sensitive Wistar substrain used in the second study exhibited a 25% mortality at 1000 mg/kg bw/d in the pilot developmental toxicity study)
- this confirms that the increase in supernumerary 14th ribs at the high dose of the first study can also be interpreted as being due to maternal toxicity and that the mid dose (500 mg/kg bw/d) in the first study can be interpreted as a NOAEL for this effect (the incidences of rudimentary 14th ribs at the low and mid dose are within the historical control range)

Therefore, considering the results of both the first and the second developmental toxicity studies in rats, a "combined" NOAEL for the formation of rudimentary 14th ribs could be set at 500 mg/kg bw/d.

Meanwhile (after the PRAPeR 04 Meeting in 2006) a NOAEL of at least 80 mg/kg for rudimentary 14th ribs was stated by the following Regulatory Bodies:

- PMRA Canada (Regulatory Note REG2007-03, Prothioconazole, 31 January 2007, p. 67)
- US-EPA (Prothioconazole: Human Health Risk Assessment, Jan. 23, 2007, p. 33; Pesticide Fact Sheet, Prothioconazole, March 14, 2007, p. 7)
- UK HSE as ECB-Rapporteur (REACH ANNEX XV, Proposal for Harmonised Classification and Labelling, March 2007, p. 13)
- FAO/WHO (Joint Meeting on Pesticide Residues, Report 2008, 193: 265, p. 271-272)
- EFSA PPR Panel (EFSA Journal 2009; 7(9):1167, Scientific Opinion on Risk Assessment for a Selected Group of Pesticides from the Triazole Group to Test Possible Methodologies to Assess Cumulative Effects from Exposure through Food from these Pesticides on Human Health, p. 117)

Furthermore, two benchmark dose analyses of the rudimentary 14th ribs observed in the first rat developmental toxicity study in rats were conducted (one using US-EPA software (2015, [M-531958-01-1], and one according to EFSA (2016, [M-579365-01-1]); both determined $BMDL_{10}$ values of > 300 mg/kg bw/d and, thus, support that the NOAEL for rudimentary 14th ribs in the first rat developmental toxicity study should be conservatively set at 80 mg/kg bw/d.

This should also trigger a revision of the currently established ARfD and AOEL.

A rat developmental toxicity study was also performed using dermal administration, with a limit dose level of 1000 mg/kg bw/d prothioconazole technical and also 1000 mg/kg bw/d of an EC formulation and an aqueous dilution of an EC formulation. The systemic effects recorded in the gavage study at 1000 mg/kg bw/d were not recorded in the dermal study. In addition, there were no adverse effects on offspring. Similarly, there were no adverse effects recorded using the EC formulation (containing 250 mg/kg bw/d prothioconazole plus co-formulants) or the diluted formulation other than skin irritation. Hence, the use of the formulation was not associated with an obvious increase in toxicity via the dermal route, but the dose levels in terms of prothioconazole are not comparable.

In a developmental toxicity study in rabbits, there was very strong maternal toxicity (including mortality) at the high dose level. The only adverse effects on offspring (abortions/total litter loss, reduced fetal weights) were also recorded at the high dose level and were likely to have been secondary to the maternal toxicity. There was no evidence of a teratogenic effect in rabbits.

All observed reproductive and developmental effects in rats and rabbits are considered as unspecific enhancements secondary to strong to very strong maternal toxicity. Therefore, these findings are no indications for a specific or direct reproductive toxic, developmental toxic or teratogenic potential of

prothioconazole. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, such a constellation of maternal and reproductive / offspring effects does not warrant any reproductive / developmental toxicity classification. Specifically, the Guidance states: *"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species."*

CA 5.6.1 Generational studies

Report:	KCA 5.6.1/01 [redacted]; 1999; M-018760-01-1
Title:	A pilot reproductive toxicity study with JAU 6476 technical in the Wistar rat
Report No.:	109079
Document No.:	M-018760-01-1
Guideline(s):	US EPA OPPTS Guideline No. 870.3800 Health Canada PMRA DAC No. 4.5.1 OECD, Section 4, Guideline 416 Japan MAFF, 59 NohSan No. 4200 EU, 91/414/EEC
Guideline deviation(s):	not specified
GLP/GEP:	**yes**

Deviations: As a pilot study the study was not intended to comply with OECD guidelines.

Executive summary:

In a 1998 GLP pilot study (to determine dose levels for a multigeneration study), groups of 10/sex Wistar rats received prothioconazole (batch no. 6233/0031, purity 98.1-98.8 %) daily by gavage from pre-mating through to weaning of offspring. The dose levels were 0, 10, 100, 250 and 500 mg/kg bw/d and the vehicle was 0.5 % aqueous methylcellulose/Tween 80. Treatment was administered daily for 4 weeks before mating, for one week during mating, and throughout gestation and lactation, except during parturition, until 21 days post partum. Investigations included bodyweights, feed consumption and clinical signs in adults, litter parameters and pup weights and clinical signs during lactation. Animals were sacrificed when pups reached 21 days post partum. Adults received a gross necropsy and the following organs were weighed – liver, kidneys, adrenals, thyroid, testes, uterus and ovaries. As a pilot study the study was not intended to comply with OECD Guidelines.

Since the present range finding study included only a limited group size, the study is only of limited value to establish a reprotoxicological profile including the setting of NOAELs. Nevertheless, it is plausible to assume that clear parental toxicity was indicated at 500 mg/kg bw/d by the observed urine stain, which is interpreted as a clear sign for severe disturbance of kidney function and systemic water / electrolyte homeostasis. Minimally decreased paternal body weights and slightly decreased pup body weights were also observed at this dose level. Parental feed consumption was unaffected by treatment. Necropsy of parental animals did not reveal any treatment-related gross lesions and there were no effects on the absolute and relative organ weights at any dose level. Reproductive parameters were not affected up to the highest dose tested. There were no treatment-related clinical signs amongst pups during the postnatal period, and no effect on litter parameters. Gross necropsy of pups did not reveal any notable findings. Thus, possible NOAELs have been established at 250 mg/kg bw/d (parental and offspring toxicity) and at 500 mg/kg bw/d (reproductive toxicity).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476 Technical
Description:	Beige powder
Batch No.:	6233/0031
Purity:	98.1-98.8 %
CAS No.:	178928-70-6
Stability of the test compound:	Guaranteed for at least for study duration
2. Vehicle:	0.5 % (w/v) methylcellulose and 0.4 % (v/v) Tween 80 (MCT)
3. Test animals:	
Species:	Rat
Strain:	Wistar Hannover, Crl:(Glx/BRL/Han) IGS BR
Sex:	Males and females (nulliparous and non-pregnant)
Age:	Approx. 12-weeks
Weight at dosing:	Males: 275.1-348.1 g Females: 173.3-216.7 g
Source:	[redacted] USA
Acclimation period:	At least six days
Diet:	Purina Mills Rodent Lab Chow 5001-4, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were individually housed (except during the mating phase) in suspended stainless steel cages. During the gestation and lactation phases, individual dams and their litters were housed in plastic cages with corn cob bedding
Environmental conditions:	
Temperature:	18-26 °C
Humidity:	30-70 %
Air changes:	Not reported
Photo period:	12 h light/dark cycle

B. STUDY DESIGN

1. In-life dates: October 19, 1998 – January 05, 1999

2. Animal assignment and treatment

Following one week of acclimation, male and female rats were randomly assigned to either a control or one of three chemically-treated groups (10 rats/sex/group) based on their body weight.

Prothioconazole was administered via oral gavage at nominal dosages of 0, 10, 100, 250, and 500 mg/kg bw/d in the MCT vehicle at a dosage volume of 10 ml/kg bw (based on the body weight measured prior to dosing). The rats were treated continuously (seven days/week) from pre-mating through to weaning with the exception that females were not dosed from day 21 of gestation until completion of delivery.

3. Test substance preparation and analysis

Stock dosing suspensions were prepared prior to the first day of dosing by adding prothioconazole to an aqueous 0.5 % (w/v) methylcellulose and 0.4 % (v/v) Tween 80 (MCT) suspension. Following preparation, the concentration of the test compound at each dose level was determined ([redacted] and [redacted]

[redacted], 1998 [M-091268-01-1]) and the stock suspensions refrigerated. On the days of dosing, the stock suspensions were thoroughly mixed and an aliquot of each stock solution was taken, from which the appropriate animals were dosed. The stock formulations were then returned to the refrigerator. Any unused portion of the daily aliquot was discarded. During the study, new stock dosing suspensions were prepared, and the concentration of the test compound verified, at least every four weeks (Table 5.6.1/01-1). Homogeneity and stability of the test substance in the MCT vehicle at the storage conditions were verified prior to dosing (Table 5.6.1/01- 2, Table 5.6.1/01- 3).

Table 5.6.1/01- 1: Verification of the test compound concentration in stock dosing suspensions

Week	Control	1 mg/mL	10 mg/mL	25 mg/mL	50 mg/mL
1	ND	0.94	9.5	21.3	47.4
2	ND	1.0	9.8	24.7	50.3
6	ND	1.0	10.0	24.9	50.1
10	ND	1.0	10.1	26.1	51.8
Mean		1.0	9.9	24.3	49.9
SD		0.03	0.3	2.1	1.8
CV (%)		3.0	2.7	8.5	3.7
% of Nominal Concentration		100	99	97.3	99.8

SD: standard deviation
CV: coefficient of variation

The mean values for the study ranged from 97.2 to 100 % of the nominal concentration.

The homogeneity and stability tests for the 1 mg/ml concentration level were conducted as part of this study. To bracket the 50 mg/ml concentration level, the homogeneity and stability tests were conducted at 100 mg/ml as part of another study ([M-053225-01-1], **study summary not included yet**). For the 100 mg/ml concentration, the mean, SD and CV % for homogeneity was 108, 2.0, and 1.9, respectively.

Table 5.6.1/01- 2: Distribution of prothioconazole in doses suspensions

Sample	Target concentration 1 mg/mL
1	0.98
2	0.97
3	0.95
Mean	0.97
SD	0.015
CV (%)	1.6
% of Nominal Concentration	97

SD: standard deviation
CV: coefficient of variation

Based on a CV of 1.6, prothioconazole dose suspension was homogeneously distributed.

Table 5.6.1/01- 3: Stability of prothioconazole in dose suspensions stored at refrigerator temperature

Day	1 mg/mL actual concentration	% of initial concentration
0	0.97	100
7	0.95	97.9
15	0.93	95.9
24	0.95	97.9
29	0.95	97.9
35	1.00	103
Mean	0.96	
SD	0.02	
CV (%)	2.1	
% of Nominal Concentration	96	

SD: standard deviation
CV: coefficient of variation

After 35 days of storage, there was no decline in concentration for the 1 mg/ml dose level. Prothioconazole was considered stable in dose suspension for a minimum of 35 days at refrigerator temperature. Likewise, there was no decline for a minimum of 35 days in the stability of prothioconazole in the 100 mg/ml dose level ([M-053225-01-1], *study summary not included yet*).

4. Mating procedure

Mating was accomplished by co-housing one female with one male for up to 7 consecutive days. During the mating phase vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated (designated gestation day 0) were placed in a plastic nesting cage. In order to evaluate those females which may have been inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all remaining females were placed in plastic nesting cages following the 7-day mating period.

C. METHODS

1. Observations

Parental animals: Animals were observed (cageside) for clinical signs twice daily during both the 4-week premating phase and the mating phase. Animals were observed at least once daily on weekends and holidays. During gestation and lactation females were observed as described above. Cageside observations characterized mortality, moribundity, behavioral changes, signs of difficult or prolonged delivery, and overt toxicity by viewing the animal in the cage. In the event a possible clinical sign was observed during the cageside evaluation, the animal may have been removed from the cage and a detailed assessment conducted. A detailed evaluation of clinical signs and a physical examination were conducted once per week.

Offspring: Pups were observed daily for clinical signs (cageside, as described for the adults) from birth until weaning. In the event a possible clinical sign was observed during the cageside evaluation, the pup may have been removed from the cage and a detailed assessment conducted.

2. Feed consumption and body weight

Parental animals: During the premating phase, body weights were measured daily (except weekends) and fresh feed was provided (and feed consumption measured) once/week for both males and females. During the mating period body weights were measured, and fresh feed was provided, as they were during the premating phase, however, as the animals were co-housed, no feed consumption was determined. Males continued to have their body weights measured daily (except weekends) after co-housing until sacrificed (fresh feed was provided once/week; however, no weights were taken). During gestation and lactation, dam body weight was measured daily, with the following exception: Body weight determinations were suspended from gestation day 21 through completion of delivery, with the exception of the few females weighed on day 21 prior to the procedure being amended. Fresh feed was provided (and feed consumption measured) once/week during gestation, and twice during the first week of lactation and then once/week for the remainder of the lactation phase.

Offspring: Pup body weights were recorded as soon as possible following parturition. Pup body weights were also recorded on lactation days 4,7,14, and 21.

3. Reproductive performance

Mating, fertility and gestation indices were determined for each dose group. For each dam the mean time to insemination as well as duration of gestation was recorded. The number of live and stillborn pups was recorded for each litter. Pups were sexed and their body weights recorded as soon as possible following parturition.

4. Termination/Gross pathology

Parental animals: Following the weaning of their respective litters (lactation day 21) each dam was terminated by carbon dioxide asphyxiation prior to the performance of a gross external examination. Prior to termination, the estrous cycle stage was determined for each dam. Terminal body weights were taken and the abdomen and thoracic cavities were opened and a gross internal examination was performed. The uterus was excised and the implantation sites, if present, were counted. The ovaries, kidneys, adrenals, thyroids, and liver were removed and weighed.

Females which were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed after gestation day 24. Females which were never observed as being inseminated and/or with an internal vaginal plug were sacrificed and necropsied at least 24 days after the completion of the mating phase if they did not deliver. Females were sacrificed, and a gross necropsy was performed on these animals as described above. Also examined in these females was an evaluation of the patency of the cervical/uterine os via flushing of the uterine horns with formalin.

Following the mating phase, male rats were sacrificed by carbon dioxide asphyxiation. The terminal body weight was recorded and gross external and internal necropsies were performed. Organs that were weighed included the liver, kidneys, adrenals, thyroids, and testes.

No tissues were saved from either the males or females.

Offspring: The size of each litter was adjusted on lactation day 4 to yield, as close as possible, four males and four females per litter. If the number of male or female pups was less than four, a partial adjustment was made (e.g. three females and five males). No adjustment was made for litters of fewer than eight pups. Adjustments were made by random selection of the pups using software provided by SAS. Culled pups were sacrificed by intracranial injection of 0.01-0.05cc Fatal Plus ([redacted] Ml), and were discarded.

The pups not culled on lactation day 4 were maintained with the dam until weaning (21-days of age). At weaning, pups were sacrificed and underwent a gross internal and external examination for any structural abnormalities or pathological changes, particularly as they may have related to the organs of the reproductive system.

Pups found dead or terminated in moribund condition were examined via a gross internal and external examination for possible defects and/or cause of death.

5. Statistics

The data were analyzed using applications provided by DATATOX Software (Instem Computer Systems), SAS Software (SAS Institute Inc.), or TASC (Toxicology Analysis System Customized, 1993).

Parameter	Statistical test
Parametric data (including body weight gain and feed consumption)	**Univariate Analysis of Variance (ANOVA)** (and in case of significant results Dunnett's t-Test)
Nonparametric data (e.g., number of estrous cycles, litter size, and number of implantation sites)	**Kruskall-Wallis test** (and, in case of significant differences Dunn's test)
Nonparametric dichotomous data (e.g. fertility and gestation indices)	**2 by N CHI² test**; in case of significant differences Fisher's exact test with Bonferroni correction
Frequency of gross lesions	Examined visually, in the event of questionable distribution examination by statistical analysis using the **Chi-square** and **Fisher's exact tests**. Comparisons were made at both the 0.05 and 0.01 levels of significance

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above.

B. MORTALITY AND CLINICAL SIGNS

1. Clinical signs in parental animals

During premating and mating the only treatment-related clinical sign noted was that of urine stain observed in one male and three females of the 500 mg/kg dose group. No other dose-related clinical observations were noted for either males or females. During gestation two females were observed with urine stain in the 500 mg/kg dose group. No other potentially test compound related clinical signs were noted. No test compound-related clinical signs were observed during lactation. In the light of consistent findings obtained in other rat toxicity studies, the urine stain is interpreted as a clear sign for severe disturbance of kidney function and systemic water / electrolyte homeostasis, which appears to be the characteristic toxicity of prothioconazole. Specifically, a pilot maternal toxicity dose range finding study in pregnant rats (conducted in the same test laboratory and rat strain, and using gavage application as in the present study) showed at 1000 mg/kg bw/d clearly increased water consumption, dehydration (causing 25 % mortality) and at 500 mg/kg bw/d clearly increased water consumption and dehydration (but no mortality). Therefore, 500 mg/kg bw/d in the present study is considered as a clearly parentally toxic dose.

Table 5.6.1/01- 4: Summary of clinical signs in parental animals

Prothioconazole (mg/kg bw/d)		Males					Females				
		0	**10**	**100**	**250**	**500**	**0**	**10**	**100**	**250**	**500**
No of animals examined	Pre-Mating	10	10	10	10	10	10	10	10	10	10
Urine stain		0	0	0	0	**1**	0	0	0	0	**3**
No of animals examined	Gestation						10	10	8	9	8
Urine stain							0	0	0	0	**2**
No of animals examined	Lactation						10	10	8	9	8
-		No compound-related clinical signs observed									

** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

2. Viability and clinical signs in offspring

There were no test compound-related clinical signs observed in the pups.

C. FEED CONSUMPTION AND BODY WEIGHT

1. Parental feed intake, body weight and body weight gain

There were no statistically significant effects on body weight or feed consumption in either the males or females during the premating phase of the study or in females during gestation and lactation. In the light of the clear decrease of male body weights observed in the main reproductive toxicity study at 750 mg/kg bw/d in P-generation males, it is plausible to assume a respective trend also in the present study in males treated with 500 mg/kg bw/d.

Table 5.6.1/01- 5: Feed consumption (g) of parental animals

	Generation	**Prothioconazole (mg/kg bw/d)**							
		0	**10**		**100**		**250**		**500**	
			(%)[a]		*(%)*[a]		*(%)*[a]		*(%)*[a]
Males									
Day 0-7	Premating	74.6	72.1	*(-3)*	72.1	*(-3)*	74.1	*(-1)*	70.1	*(-6)*
Day 7-14		71.4	69.5	*(-3)*	68.2	*(-4)*	71.2	*(±0)*	70.2	*(-2)*
Day 14-21		66.2	64.6	*(-2)*	64.4	*(-3)*	72.1	*(+9)*	70.6	*(+7)*
Day 21-28		64.8	62.9	*(-3)*	64.0	*(-1)*	67.0	*(+3)*	67.2	*(+4)*
Females										
Day 0-7	Premating	80.2	83.1	*(+4)*	79.5	*(-1)*	87.1	*(+9)*	80.9	*(+1)*
Day 7-14		82.5	79.7	*(-3)*	80.8	*(-2)*	84.8	*(+3)*	86.9	*(+5)*
Day 14-21		82	80.5	*(-2)*	82.6	*(+1)*	86.9	*(+6)*	85.3	*(+4)*
Day 21-28		83.9	77.5	*(-8)*	79.3	*(-5)*	84.5	*(+1)*	82.0	*(-2)*
GD 0-6	Gestation	75.6	74.6	*(-1)*	75.9	*(±0)*	78.2	*(+3)*	78.0	*(+3)*
GD 6-13		77.8	80.6	*(+4)*	78.8	*(+1)*	79.9	*(+3)*	78.6	*(+1)*
GD 13-20		79.3	77.4	*(-2)*	78.7	*(-1)*	77.0	*(-3)*	79.9	*(+1)*

	Generation	Prothioconazole (mg/kg bw/d)									
		0	10		100		250		500	
				(%)[a]		*(%)[a]*		*(%)[a]*		*(%)[a]*
LD 0-7	Lactation	125.9	131.0	*(+4)*	130.5	*(+4)*	126.9	*(+1)*	122.9	*(-2)*
LD 7-14		184.9	169.1	*(-9)*	191.1	*(+3)*	191.6	*(+4)*	179.8	*(-3)*
LD 14-21		207.9	204.2	*(-2)*	204.5	*(-2)*	220.8	*(+6)*	210.6	*(+1)*

[a] *% difference to control* GD gestation day LD lactation day
* statistically significant difference from control p<0.05
** statistically significant difference from control p<0.01
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/01- 6: Body weights (g) of parental animals

	Generation	Prothioconazole (mg/kg bw/d)									
		0	10		100		250		500	
				(%)[a]		*(%)[a]*		*(%)[a]*		*(%)[a]*
Males											
Day 0	Pre- and postmating	307.9	309.9	*(+1)*	312.2	*(+1)*	308.5	*(±0)*	310.0	*(+1)*
Day 8		327.9	330.3	*(+1)*	332.2	*(+1)*	334.3	*(+2)*	322.6	*(-2)*
Day 15		342.7	346.2	*(+1)*	348.5	*(+2)*	352.4	*(+3)*	**333.0**	***(-3)***
Day 22		356.3	361.3	*(+1)*	342.4	*(+2)*	370.3	*(+4)*	**343.7**	***(-4)***
Day 29		361.5	368.6	*(+2)*	371.3	*(+3)*	375.4	*(+4)*	**349.8**	***(-3)***
Day 36		376.3	382.2	*(+2)*	382.6	*(+2)*	390.9	*(+4)*	**363.7**	***(-3)***
Day 43		390.1	395.7	*(+1)*	395.8	*(+1)*	403.5	*(+3)*	**377.5**	***(-3)***
Females										
Day 0	Premating	196.5	193.9	*(-1)*	190.8	*(-3)*	194.8	*(-1)*	193.1	*(-2)*
Day 8		201.0	201.1	*(±0)*	197.4	*(-2)*	204.4	*(+2)*	201.3	*(±0)*
Day 15		208.7	206.0	*(-1)*	203.3	*(-3)*	211.6	*(+1)*	206.2	*(-1)*
Day 22		214.4	212.2	*(-1)*	207.6	*(-3)*	214.5	*(±0)*	217.2	*(+1)*
Day 29		228.8	220.9	*(-3)*	211.3	*(-8)*	223.3	*(-2)*	220.1	*(+1)*
GD 0	Gestation	215.5	215.9	*(±0)*	210.3	*(-2)*	216.8	*(+1)*	217.8	*(+1)*
GD 6		234.5	232.3	*(-1)*	228.6	*(-3)*	236.7	*(+1)*	232.2	*(-1)*
GD 13		255.2	254.8	*(±0)*	247.1	*(-3)*	253.4	*(-1)*	255.5	*(±0)*
GD 20		312.6	307.9	*(-1)*	294.8	*(-6)*	306.7	*(-2)*	305.4	*(-2)*
LD 0	Lactation	239.7	240.2	*(±0)*	235.4	*(-2)*	241.5	*(+1)*	230.9	*(-4)*
LD 4		250.1	254.2	*(+2)*	249.1	*(±0)*	251.2	*(±0)*	248.8	*(-1)*
LD 7		247.8	260.7	*(+5)*	246.2	*(-1)*	253.1	*(+2)*	240.8	*(-3)*
LD 14		273.1	277	*(+1)*	270.1	*(-1)*	278.6	*(+2)*	266.7	*(-2)*
LD 21		263.1	271.3	*(+3)*	267.1	*(+2)*	281.2	*(+7)*	280.9	*(+7)*

[a] *% difference to control* GD: gestation day LD: lactation day
* statistically significant difference from control p<0.05
** statistically significant difference from control p<0.01
Findings considered plausibly related to treatment with prothioconazole are written in **bold letters**

2. Offspring body weight and body weight gain

The study author concluded that there were no test compound-related effects on pup body weight. However, in the light of the body weight effects observed in the main reproductive toxicity study at 750 mg/kg bw/d in F1 pups (birth weight not affected, body weight decrease starting at day 4 and getting stronger beyond day 4), it is plausible to assume a respective trend also in the present study at 500 mg/kg bw/d (but not at 250 mg/kg bw/d and below) (see Table 5.6.1/01- 8).

D. REPRODUCTIVE EVALUATIONS

The mating index was 100 % for the control, 10, 100 and 250 mg/kg bw/d females and 80 % at the high dose. The fertility index was 100, 100, 80, 90, and 100 % for the control and at 10, 100, 250 and 500 mg/kg bw/d, respectively. The gestation index was 100% for all dose levels. There were no statistically significant effects on days to insemination, gestation length, or the median number of implants.

Table 5.6.1/01- 7: Reproductive data

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**10**	**100**	**250**	**500**
P-Generation					
No. mated / no. paired	10 / 10	10 / 10	10 / 10	10 / 10	8 / 10
No. delivering a litter	10	10	8	9	8
No. with implants	10	10	8	9	8
Mating index	100	100	100	100	80.0
Fertility index	100	100	80.0	90.0	100.0
Gestation index	100	100	100	100	100
Mean time to insemination (days)	1.6	1.7	1.6	1.7	2.6
Mean duration of gestation (days)	22.2	22.1	22.1	22.0	22.1
Mean no. implants	10.9	10.7	10.0	10.9	10.1

Mating index = no. inseminated / no. paired x 100; Fertility index = no. pregnant / no. inseminated x 100
Gestation index = no. with live pups/ no. pregnant x 100
* $p < 0.05$ (Kruskal-Wallis and Dunn's Test)
Findings considered related to treatment with prothioconazole are written in **bold letters**

There were no test compound-related effects on any other litter parameters.

Table 5.6.1/01- 8; Summary of litter data

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**10**	**100**	**250**	**500**
No. litters	10	10	8	9	8
Total no. pups born	107	99	76	96	78
Total no. pups missing	0	0	1	2	1
Total no. pups dying	0	1	1	1	0
Total no. pups cannibalized	0	0	0	0	0
Mean litter size	10.7	9.9	9.5	10.7	9.8

Parameter	Prothioconazole (mg/kg bw/d)				
	0	10	100	250	500
Mean pup weight, combined (g):					
- day 0	5.9	5.8 *(-2%)*	5.6 *(-5%)*	5.8 *(-2%)*	5.7 *(-3%)*
- day 4 (pre-cull)	9.5	9.4 *(-1%)*	9.3 *(-2%)*	8.8 *(-7%)*	**8.7** ***(-8%)***
- day 7	13.4	13.9 *(+4%)*	13.3 *(-1%)*	12.8 *(-4%)*	**12.0** ***(-10%)***
- day 14	24.9	25.8 *(+4%)*	25.0 *(±0%)*	24.0 *(-4%)*	**22.7** ***(-9%)***
- day 21	40.5	40.5 *(+3%)*	38.1 *(-3%)*	38.7 *(-2%)*	**36.6** ***(-7%)***
Mean male pup weight (g):					
- day 0	6.1	6.0 *(-2%)*	5.9 *(-3%)*	6.0 *(-2%)*	5.8 *(-5%)*
- day 4 (pre-cull)	9.6	9.6 *(±0%)*	9.6 *(±0%)*	8.9 *(-7%)*	**8.8** ***(-8%)***
- day 7	13.6	14.1 *(+4%)*	13.8 *(+1%)*	12.9 *(-5%)*	**12.1** ***(-11%)***
- day 14	25.4	26.2 *(+3%)*	25.6 *(+1%)*	24.2 *(-5%)*	**22.9** ***(-10%)***
- day 21	40.5	41.4 *(+2%)*	39.1 *(-3%)*	38.9 *(-4%)*	**37.2** ***(-8%)***
Mean female pup weight (g):					
- day 0	5.8	5.6 *(-3%)*	5.5 *(-4%)*	5.6 *(-3%)*	5.6 *(-3%)*
- day 4 (pre-cull)	9.5	9.3 *(-2%)*	9.1 *(-4%)*	8.7 *(-8%)*	**8.5** ***(-11%)***
- day 7	13.2	13.8 *(+5%)*	13.0 *(-2%)*	12.7 *(-4%)*	**11.8** ***(-11%)***
- day 14	24.5	26.0 *(+6%)*	24.7 *(+1%)*	23.8 *(-3%)*	**22.5** ***(-8%)***
- day 21	38.3	40.1 *(+5%)*	37.6 *(-2%)*	38.5 *(+1%)*	**35.9** ***(-6%)***
Sex ratio at birth (% males)	54.5	55.0	39.3	48.8	49.5
No. still-born pups	0	0	0	0	0
Mean no. viable pups at:					
- birth	11	10	10	11	10
- day 4 (pre-cull)	11	10	9	10	10
- day 4 (post-cull)	8	7	8	8	7
- day 21	8	7	8	8	7
Live birth index	100.0	100.0	100.0	100.0	100.0
Viability index	100.0	100.0	96.9	97.9	97.5

Parameter	Prothioconazole (mg/kg bw/d)				
	0	10	100	250	500
Lactation index	100.0	98.8	100.0	98.6	100.0
Birth index	96.1	88.3	94.5	96.3	95.3

* p < 0.05; ** p < 0.01 (Dunnett's test)
Live birth index = no. live-born pups per litter / total no. pups per litter x 100
Viability index = No. live pups on day 4 pre-cull per litter / no. live pups born per litter x 100
Lactation index = No. live pups on day 21 per litter / no. live pups on day 4 post-cull per litter x 100
Birth index = total no. pups born per litter / total no. implantation sites per dam x 100
Findings considered plausibly related to treatment with prothioconazole are written in **bold letters**

E. POSTMORTEM EVALUATIONS

1. Necropsy examinations - adults

There were no test compound-related parental necropsy findings. Except for a possibly minimally decreased terminal body weight in high dose males, no effects on terminal body weight or the absolute or relative organ weights were noted at any exposure level. Estrous cycles were determined prior to termination; however, due to the absence of any effects on uterine or ovary weights these results are not reported.

Table 5.6.1/01- 9: Absolute and relative organ weights of parental animals (Day 43)

		Prothioconazole (mg/kg bw/d)				
		0	10	100	250	500
Males						
Terminal bodyweight (g) *(% difference to control)*		387.8	393.6 *(+2)*	396.1 *(+2)*	401.6 *(+4)*	**373.5** ***(-4)***
Adrenal	(g)	0.072	0.067	0.073	0.064	0.064
	(%)	0.019	0.017	0.018	0.016	0.017
Kidney	(g)	2.858	2.906	2.986	3.226	3.024
	(%)	0.739	0.740	0.756	0.802	0.811
Liver	(g)	17.102	17.129	16.658	18.194	17.539
	(%)	4.438	4.352	4.210	4.515	4.698
Testes	(g)	3.705	3.588	3.630	3.871	3.615
	(%)	0.959	0.916	0.924	0.967	0.970
Thyroid	(g)	0.020	0.019	0.021	0.019	0.019
	(%)	0.0056	0.0048	0.0052	0.0047	0.0050
Females						
Terminal bodyweight (g) (% difference to control)		253.0	261.1 *(+3)*	248.3 *(-2)*	265.8 *(+5)*	259.5 *(+3)*
Adrenal	(g)	0.99	0.106	0.097	0.100	0.087
	(%)	0.039	0.041	0.039	0.038	0.034
Kidney	(g)	2.233	3.873	2.196	2.383	2.293
	(%)	0.883	1.477	0.885	0.896	0.883
Liver	(g)	13.500	14.090	13.138	14.544	14.967
	(%)	5.317	5.352	5.262	5.468	5.747

		Prothioconazole (mg/kg bw/d)				
		0	**10**	**100**	**250**	**500**
Ovaries	(g)	0.124	0.141	0.136	0.145	0.144
	(%)	0.049	0.054	0.055	0.055	0.055
Thyroid	(g)	0.014	0.016	0.015	0.014	0.015
	(%)	0.0057	0.0060	0.0060	0.0051	0.0059

* p≤0.05, , ** p≤0.01 (for absolute organ weights: Ancova + Dunnetts tests; for terminal body weight and relative organ weights: Anova + Dunnett's tests or Kruskal-Wallis Anova + Mann-Whitney u-tests)
Findings considered plausibly related to treatment with prothioconazole are written in **bold letters**

2. Necropsy examinations - offspring

There were no test compound-related necropsy findings observed in the pups.

III. CONCLUSION

Since the present range finding study included only a limited group size, the study is only of limited value to establish a reprotoxicological profile including the setting of NOAELs.

Nevertheless, it is plausible to assume that clear parental toxicity was indicated at 500 mg/kg bw/d by the observed urine stain, which is interpreted as a clear sign for severe disturbance of kidney function and systemic water / electrolyte homeostasis. Minimally decreased paternal body weights and slightly decreased pup body weights were also observed at this dose level. Reproductive parameters were not affected up to the highest dose tested.

Thus, possible NOAELs have been established at 250 mg/kg bw/d (parental and offspring toxicity) and at 500 mg/kg bw/d (reproductive toxicity).

Report: KCA 5.6.1/02 [redacted]0; 2001; M-036206-01-1
Title: A two-generation reproductive toxicity study with JAU 6476 in the Wistar rat
Report No.: 110500
Document No.: M-036206-01-1
Guideline(s): OPPTS 870.3800 (1998); OECD 416 (1983); Health Canada PMRA DACO No. 4.5.1 (1988); JMAFF 59 NohSan No. 4200 (1985); Guideline 91/414/EEC (1995)
Guideline deviation(s): none
GLP/GEP: **yes**

Deviations: Deviations from the current OECD (2001) guideline:
Thyroids were not weighed in adult animals and morphological examination of sperm samples for P-generation was not performed. Brain, spleen, thymus, abnormal tissues, and any target organs were preserved for possible histopathological examination of the F2 pups only. However this does not affect the overall acceptability of the study.

Executive summary:

In a 1999-2001 GLP study, groups of 30/sex Wistar rats were treated with prothioconazole (batch no. 6233/0031, purity 98.1-98.8 %) by daily gavage dosing from pre-mating through to weaning of F1 generation pups. Selected F1 progeny were then similarly treated until weaning of F2 generation pups. The nominal dose levels used were 0, 10, 100 and 750 mg/kg bw/d (actual dose levels determined by

analysis were 0, 9.7, 95.6 and 726 mg/kg bw/d) in 0.5 % aqueous methylcellulose/ Tween 80. The pre-mating periods (P and F1) were 10 weeks, the mating periods were 2 weeks, and dosing continued throughout mating, gestation and lactation up to day 21 post partum. Daily administration of the test compound to the F1-generation pups began on day 22 post partum until weaning of F2-generation pups. Observations included bodyweights, feed consumption, clinical signs and litter parameters. Oestrous cycle was assessed over a three week pre-mating period in all females (P and F1 generations). Day of vaginal opening and preputial separation were recorded in F1 animals. Anogenital distance was measured on the day of birth for all F2 progeny. All animals received a gross necropsy at termination and the following organs were weighed: liver, testes/ovaries, reproductive organs, pituitary, brain, thymus, adrenal glands, kidneys and spleen. Terminal oestrous cycle stage was assessed. Uteri were examined for implantation sites. One testis and one epididymis from all P and F1 males were taken for sperm enumeration, morphology (F1 generation only) and motility. A quantitative evaluation of the ovaries for pre-antral follicles, antral follicles and corpora lutea was performed on 10 females per group (controls and high dose for P-generation, all groups for the F1-generation). From F1 and F2 pups, brain, spleen and thymus were weighed from 1 pup/sex/litter. The following organs and tissues from control and high dose adults (P and F1) were examined microscopically: testes/ovaries, reproductive organs, gross lesions, adrenal glands, liver, pituitary, and accessory sex organs. Reproductive organs of animals suspected of reduced fertility were also examined microscopically.

There was evidence of parental general systemic toxicity at the intermediate and high dose levels. Effects at 100 mg/kg bw/d included slightly lower bodyweights in males (F1), decreased thymus weights in females (P and F1) and increased liver weights in both sexes (P and F1). At 750 mg/kg bw/d similar but more marked effects were recorded, along with clinical observations such as urine stain, dehydration and salivation prior to dosing, reduced efficiency of food utilisation (both sexes), lower bodyweight gains during gestation, increased kidney weights (males) and histopathological findings in the liver (hepatocytomegaly in both sexes) and kidneys (multifocal cortical nephrosis in both sexes). The parental (particularly the maternal) toxicity at 750 mg/kg bw/d is considered to be very high, even sublethal, based primarily on the kidney dysfunction and resulting dehydration. In the same Wistar rat substrain, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25 % mortality or, at 750 mg/kg bw/d, could not be fully compensated by a drastically increased (up to > 170 % of control) water consumption (as determined in the pilot developmental toxicity study or the new main developmental toxicity study). Since the present two-generation study was conducted with gavage dosing in the same Wistar rat substrain as the new main developmental toxicity study, it is plausible to assume that the observed drastically increased water intake in the new main developmental toxicity study at 750 mg/kg bw/d does also reflect the situation in this two-generation study (in which water consumption was not measured) at 750 mg/kg bw/d. Severe disturbance of the kidney function and systemic water / electrolyte homeostasis is a consistent finding in all toxicity studies in the rat and appears to be the characteristic toxicity of prothioconazole with cases of death at 500 mg/kg bw/d in a 90-day study and, at 750 mg/kg bw/d in the 1-year study, and a high mortality rate in the 2-year study at 500-750 mg/kg bw/d, all conducted with Wistar rats (different substrain).

The following effects were recorded on reproductive organs/parameters. In F1 males, total sperm counts were lower at all dose levels. However, there was no corresponding effect on epididymal sperm counts, no dose-response relationship, no similar effect on testicular and epididymal sperm counts in the P-generation, no treatment-related effects on any other sperm parameters (i.e., on sperm morphology and motility), no histopathological findings in the testes and finally there was no effect on the reproductive outcome in this two-generation reproductive toxicity study. It is therefore considered that these lower sperm counts were not an effect of treatment. In females at the high dose only, oestrous cycling was affected, which was attributed to the aforementioned very strong general systemic maternal toxicity at this very high dose. The effect was not apparent at lower dose levels. At the high dose of 750 mg/kg bw/d there were slight effects on time to insemination (increased), number of implantation sites (reduced), litter number (reduced) and duration of gestation (increased). Each of these effects was seen in females from both generations (time to insemination in F1 females only). All these effects are attributed to the very strong general systemic parental toxicity at this very high dose. There were no

effects on mating, fertility or gestation indices. In a quantitative evaluation of the ovaries the number of pre-antral follicles, and to a lesser extent the number of antral follicles, was increased at all dose levels in the F1-generation but without any dose-response relationship. By contrast, pre-antral and antral follicles were not affected in the P generation. Furthermore, all values of pre-antral follicles in the F1-generation were well within the range of historical control data. Therefore the F1-generation differences on pre-antral follicles between the concurrent control and the prothioconazole-treated groups are considered to be incidental and not treatment-related. There was no treatment-related effect on the number of corpora lutea.

There were no adverse effects on pup viability, but pup weight gain was reduced at the high dose level (750 mg/kg bw/d) resulting in lower terminal pup weights. There were no gross necropsy findings in pups. Spleen weights were lower in both generations of pups at the high dose level only. Day of vaginal opening and preputial separation were recorded in F1 animals. There was no toxicologically significant effect on time to vaginal opening. In F1 pups of the high dose group (750 mg/kg bw/d) preputial separation was slightly delayed, which is considered secondary to the clearly retarded growth. At the respective individual day of preputial separation, the high dose pups had reached a practically identical body weight as the controls. Anogenital distance (AGD) at birth was measured in F2-generation pups. At 750 mg/kg bw/d there was a tendency towards a higher AGD which is attributed to the incidentally slightly higher birth weight of these pups.

The NOAEL for parental toxicity is 10 mg/kg bw/d, based on slight bodyweight effects and organ weight changes at 100 mg/kg bw/d.

Reproductive performance was not affected by treatment. The NOAEL for reproductive effects was 100 mg/kg bw/d, based on affected oestrous cycling, slight reductions in implantation sites and litter size, increased time to insemination and minimally increased duration of gestation at 750 mg/kg bw/d. These mild effects were recorded at a dose level which also gave rise to marked general systemic parental toxicity; hence a specific effect on the reproductive systems is not indicated.

The only effects on pups were recorded at the high dose level and included reduced pup weight gain, reduced pup spleen weights and delayed preputial separation. These effects were recorded at a dosage giving rise to marked general parental toxicity hence a specific developmental toxicity effect is not indicated. The NOAEL for effects on offspring is 100 mg/kg bw/d.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476
Description:	Beige powder
Batch No.:	6233/0031
Purity:	98.1-98.8 %
CAS No.:	178928-70-6
Stability of the test compound:	Guaranteed for at least for study duration
2. Vehicle:	0.5 % (w/v) methylcellulose and 0.4 % (v/v) Tween 80 (MCT)
3. Test animals:	
Species:	Rat
Strain:	Wistar Hannover, Crl:(Glx/BRL/Han)IGS BR
Sex:	Males and females (nulliparous and non-pregnant)
Age:	Approx. 9-10-weeks
Weight at dosing:	Males: 230.8-323.2 g

	Females: 155.9-204.5 g
Source:	[redacted], USA
Acclimation period:	One week
Diet:	Purina Mills Certified Rodent Diet 5002 (meal), St. Louis, MO, USA, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Animals were individually housed (except during the mating phase and as noted below for the F1-pups) in suspended stainless steel wire-mesh cages. During the gestation and lactation phases, individual dams and their litters, as well as following the weaning of F1-pups until they were able to survive individually, were housed in polycarbonate cages with corn cob bedding.
Environmental conditions:	
Temperature:	18-26 °C
Humidity:	30-70 %
Air changes:	At least 10 air changes/hour
Photo period:	12 h of fluorescent light

B. STUDY DESIGN

1. In-life dates: February 10, 1999 - November 11, 1999

2. Animal assignment and treatment

Following one week of acclimation, male and female rats were assigned to either a control or one of three chemically-treated groups (30 rats/sex/group) using a weight stratification-based computer program obtained from INSTEM Computer Systems (Stone, Staffordshire, UK).

The test substance was administered via oral gavage at nominal dosages of 0, 10, 100, or 750 mg prothioconazole/kg bw/d. The rats were treated continuously (seven days/week) from pre-mating through to weaning with the exception that females were not dosed from day 21 of gestation until the next dosing period following the completion of delivery.

Following the weaning of the F1-generation pups (21-days of age) approximately 30 pups/sex/group were randomly selected as parents of the F2-generation using SAS software (SAS Institute Inc., Gary, North Carolina). Only one male and one female were selected per litter. Daily administration of the test compound to the F1-generation pups began on lactation day 22 until weaning of F2-generation pups.

Dose selection:

Doses for this study were mainly based on the preliminary results of a pilot reproduction study ([redacted], 1999, 5.6.1/01 [M-018760-01-1]), in which male and female Wistar rats were administered, via oral gavage, either 0, 10, 100, 250, or 500 mg prothioconazole/kg bw/d. Parental toxicity was indicated at 500 mg/kg bw/d by observed urine stain, which is interpreted as a clear sign for severe disturbance of kidney function and systemic water / electrolyte homeostasis. Minimally decreased paternal body weights and slightly decreased pup body weights were also observed at this dose level.

3. Test substance preparation and analysis

Stock dosing suspensions were prepared prior to the first day of dosing and as needed throughout the study by adding prothioconazole technical to an aqueous 0.5 % (w/v) methylcellulose and 0.4 % (v/v) Tween 80 (MCT) suspension. Following preparation, the concentration of the test compound at each dose level was determined and the stock suspensions refrigerated ([redacted] and [redacted], 1998 [M-091268-01-1]). On the days of dosing, the stock suspensions were thoroughly mixed and an aliquot of each was taken, from which the appropriate animals were dosed. The stock formulations were then

returned to the refrigerator. Any unused portion of the daily aliquot was discarded. During the study, new stock dosing suspensions were prepared as needed, and the concentration of the test compound verified each time. Homogeneity and stability of the test substance in the MCT vehicle at the storage conditions was verified prior to study initiation ([redacted], 1999 (5.6.1/01) and [redacted], 2001 [M-053225-01-1], **study summary not included yet**).

The concentration of prothioconazole in the various dosing suspensions was analytically verified at least every four weeks during the in-life phase of this study. Mean analytically verified doses for each group were 0, 9.7, 95.6, and 726 mg/kg bw/d, with values ranging from 87.4-114 %, 86.3-104 %, and 88.2-103 % of the corresponding nominal doses of 10, 100, and 750 mg/kg bw/d, respectively. Prothioconazole was not detected in the control vehicle. All analyses (i.e., homogeneity, stability, and concentration verifications) for this study were conducted in principle according to methodology described previously ([redacted] and [redacted], 1998, [M-091268-01-1]).

4. Mating procedure

Mating was accomplished by co-housing one female with one male for up to 14 consecutive days. Approximately four animals from each dose group were cohoused daily beginning on the first day of the mating phase and continuing until all animals were cohoused. During the mating phase vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated (designated gestation day 0) were placed in a polycarbonate nesting cage. In order to evaluate those females which may have been inseminated without exhibiting sperm in the vaginal smear or an internal vaginal plug, all remaining females were placed in polycarbonate nesting cages following the 14-day mating period.

C. METHODS

1. Observations

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

Offspring: Both F1 and F2-pups were observed daily for clinical signs (cageside, as described for the adults) from birth until the start of the premating phase (F1-pups) and until weaning (F2-pups). A detailed clinical observation and a physical examination was performed on the days the pups were weighed. Following weaning of the last litter, the F1-pups retained to produce the next generation were observed as previously described for the adults as well as observed for vaginal opening and preputial separation.

2. Body weight and feed consumption

Parental animals: Body weights were collected Monday-Friday for males from initiation of the study until sacrifice. Body weights for females were collected Monday-Friday during premating and mating and daily throughout gestation and lactation with the exception of gestation day 21 when dosing was ceased until the next dosing period following completion of delivery. Dosing performed on Saturday and Sunday was based on Friday's body weight. Fresh feed was provided (and feed consumption measured) once/week for both males and females during the premating period. During the mating period and until sacrifice fresh feed was provided once/week, but not measured. During gestation, fresh feed was provided (and feed consumption measured) on days 0, 6, 13, and 20 and during lactation, fresh feed was provided (and feed consumption measured) on days 0, 4, 7, 14, and 21, with the exception of week one when feed consumption was measured twice (days 0-4 and 4-7).

Offspring: Pup body weights were recorded on lactation days 0, 4, 7, 14, and 21, and when vaginal opening or preputial separation were observed. Fresh feed was provided at least once/week for the weanlings, from lactation day 21 until the start of pre-mating. Measurement of feed consumption and body weights for the F1-generation began during the 10-week premating phase as previously described for the P-generation.

3. Oestrous cycling

The oestrous cycle (determined by examining daily vaginal smears over a three-week period prior to mating) was characterized for all P- and F1-generation females. Additionally, the oestrous cycle stage was determined for all females just prior to termination.

4. Reproductive performance

Mating, fertility and gestation indices were determined for each dose group. For each dam the mean time to insemination as well as duration of gestation was recorded. The number of live and stillborn pups (both F1- and F2-generations) was recorded for each litter. Pups were sexed and their body weights recorded as soon as possible following parturition (lactation day 0).

5. Developmental milestones

Following weaning of the last litter, the F1-pups retained to produce the next generation were observed for vaginal opening and preputial separation. Additionally, due to an effect observed on preputial separation in the F1-generation pups retained for the next generation, the anogenital distance was measured on lactation day 0 for all F2-pups.

6. Postmortem examination

Parental animals: Following the weaning of their respective litters (lactation day 21) each dam (both P- and F1-generations) was vaginally lavaged for terminal estrous cycle and then terminated by carbon dioxide asphyxiation prior to the performance of a gross external examination. Males were sacrificed following the mating phase (sacrifice between day 99 and day 107).

Terminal body weights of males and females were taken and the abdomen and thoracic cavities were opened, a gross internal examination was performed, and the uterus was excised and the implantation sites, if present, were counted. The ovaries, testes, epididymides (total weight for both, and cauda weight for the side not being utilized for sperm analysis), seminal vesicles (with coagulating glands and their fluids), prostate, uterus (with oviducts and cervix), brain, pituitary, thymus, liver, kidneys, adrenal glands, and spleen were removed, weighed, and fixed in 10 % buffered formalin. Gross lesions and vagina were also collected and fixed in 10 % buffered formalin. The ovaries as well as one testicle (the side not utilized for sperm analysis) were collected and fixed in Bouin's fixative.

Females which were sperm positive and/or had an internal vaginal plug but did not deliver were sacrificed after gestation day 24 by carbon dioxide asphyxiation. Females which were never observed as being inseminated and/or with an internal vaginal plug were sacrificed and necropsied at least 24 days after the completion of the mating phase if they did not deliver. A gross necropsy was performed on these animals as described above, including a term body weight, term estrous cycle, organ weights and organ preservation. Also examined in these females was the patency of the cervical/uterine os (via flushing of the uterine horns with saline).

For all P- and F1-generation males at termination, sperm was collected from one testes and one epididymis for enumeration of homogenization-resistant spermatids and cauda epididymal sperm reserves, respectively. In addition, an evaluation of the morphology and motility was performed on sperm sampled from the distal portion of the vas deferens. Sperm motility and counts were conducted using the Integrated Visual Operating System (IVOS, Hamilton-Thorne Research, 1998).

Offspring: The size of each litter was adjusted on lactation day 4 to yield, as close as possible, four males and four females per litter. No adjustment was made for litters of fewer than eight pups.

Adjustments were made by random selection of the pups. Culled pups were sacrificed, grossly abnormal pups underwent a gross internal and external examination, and all culled pups were discarded.

The F1 and F2-pups not culled on lactation day 4 were maintained with the dam until weaning (21-days of age). At 21-days of age a sufficient number of F1-pups/sex/litter were maintained to produce the next generation. F1-pups not selected to become parents of the next generation and all F2-pups were sacrificed and examined macroscopically for any structural abnormalities or pathological changes, particularly as they may relate to the organs of the reproductive system. From the pups sacrificed on day 21, the brain, spleen, and thymus was weighed from one male and one female/litter if available. Any treatment related abnormal tissues, or target organs (when known), were preserved for possible microscopic examination.

For the F2-generation pups, due to anticipated changes in the OECD guidelines, the brain, spleen, thymus, and any abnormal tissue was preserved in 10 % buffered formalin. Pups found dead or terminated in moribund condition were examined for possible defects and/or cause of death.

4. Histology

The following tissues from adult animals (P- and F1-generations) were examined microscopically: cervix, epididymis (caput, corpus, and cauda), gross lesions, adrenal glands, liver, ovaries, pituitary, prostate, testicles, seminal vesicles coagulating gland, uterus, oviducts, and vagina.

A quantitative evaluation of the ovaries for pre-antral follicles, antral follicles and corpora lutea was conducted on 10 randomly assigned F1 dams per dose level as well as 10 randomly assigned P-generation dams in the control and high level (dams which died prior to mating were not included in this randomization). Evaluation of the ovarian follicles is not required for the P-generation but was performed due to abnormalities observed on estrous cycling in this generation.

Histopathological evaluations were conducted on the control and highest-dose groups (P and F1). Reproductive organs of animals suspected of reduced fertility were also examined microscopically. If histopathological findings were attributed to treatment, the low and mid dose levels were evaluated. Representative sections of all tissues collected were processed, embedded in paraffin, sectioned, mounted, stained with hematoxylin and eosin (H&E), and examined under a light microscope by a veterinary pathologist.

5. Statistics

The data were analyzed using applications provided by DATATOX Software (Instem Computer Systems), SAS Software (SAS Institute Inc.), or TASC (Toxicology Analysis System Customized, 1993).

Parameter	Statistical test
Parametric data (including body weight gain and feed consumption)	**Univariate Analysis of Variance (ANOVA)** (and in case of significant results Dunnett's t-Test)
Nonparametric data (e.g., number of estrous cycles, litter size, and number of implantation sites)	**Kruskall-Wallis test** (and in case of significant differences Dunn's test)
Nonparametric dichotomous data (e.g. fertility and gestation indices)	**2 by N CHI^2 test**; in case of significant differences Fisher's exact test with Bonferroni correction
Frequency of gross lesions	Examined visually; in the event of questionable distribution examination by statistical analysis using the **Chi-square** and **Fisher's exact tests**. Comparisons were made at both the 0.05 and 0.01 levels of significance

Parameter	Statistical test
Sperm motility and anogenital distance	A trend test was conducted with SAS® PROC GLM using linear contrasts. If the trend test with 4 groups was significant ($p<0.05$), the high dose group was deemed significantly different from control and the trend test was repeated with the 3 remaining groups (control, low, and mid dose). The analysis process continued until the trend test was not statistically significant.
Sperm count	The control and high dose group were compared with a **two sample t-test.**
Sperm morphology	A comparison for homogeneity of variances was conducted with a **folded F statistic** from SAS® PROC TTEST. If statistically significant at the 0.01 level, the control and high dose group were compared with the Cochran and Cox p-value approximation to the two sample t-test. Otherwise, the control and high dose group were compared with a two sample t-test.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B.3 above.

B. MORTALITY AND CLINICAL SIGNS

1. Clinical signs in parental animals

Clinical observations attributed to compound were urine stain, dehydration and salivation prior to dosing, observed in the 750 mg/kg bw/d males and females of both generations. There were no compound-related clinical signs observed in the 10 or 100 mg/kg bw/d dose groups in either generation.

Table 5.6.1/02- 1: Summary of clinical signs in P- and F1-Generation parental animals

Prothioconazole (mg/kg bw/d)		Males				Females			
		0	**10**	**100**	**750**	**0**	**10**	**100**	**750**
P-Generation									
No of animals examined	Pre-Mating	30	30	30	30	30	30	30	30
Salivation prior to dosing		0	0	0	**4**	0	0	0	**5**
Urine stain		1	0	0	**4**	0	0	0	**17****
Dehydration		0	0	0	**2**	-	-	-	-
No of animals examined	Gestation	-	-	-	-	24	30	28	28
Urine stain						0	0	0	**7***
No of animals examined	Lactation	-	-	-	-	24	30	29	28
Salivation prior to dosing						0	0	0	1

Prothioconazole (mg/kg bw/d)		**Males**				**Females**			
		0	**10**	**100**	**750**	**0**	**10**	**100**	**750**
F1-Generation									
No of animals examined	Pre-Mating	30	30	30	30	30	30	30	30
Salivation prior to dosing		-	-	-	-	0	0	0	**3**
Urine stain		0	0	0	**4**	0	0	0	**4**
Dehydration		0	0	0	**1**	0	0	0	**1**
No of animals examined	Gestation	-	-	-	-	27	24	28	26
Urine stain						0	0	0	**1**
No of animals examined	Lactation	-	-	-	-	27	24	28	26
		No salivation prior to dosing and/or urine stain observed							

** significantly different from control, $p \leq 0.01$
- finding not recorded/observed in this generation
Findings considered related to treatment with prothioconazole are written in **bold letters**

2. Viability and clinical signs in offspring

There were no compound-related clinical observations on any pup prior to weaning in any dose level in either generation.

Clinical observations attributed to compound were urine stain and salivation prior to dosing in the F1-postweaned pups of the 750 mg/kg bw/d dose group. No other compound-related findings were observed in any other dose group.

Table 5.6.1/02- 2: Summary of clinical signs in F1 and F2 pups

	Prothioconazole (mg/kg bw/d)			
	0	**10**	**100**	**750**
F1 pups - postweaning				
No of pups/litters examined	256/24	331/30	320/29	280/28
Salivation prior to dosing	1/1	1/1	0/0	**15/11****
Urine stain	0/0	0/0	0/0	**3/1**

** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

C. FEED CONSUMPTION AND BODY WEIGHT

1. Parental feed intake, body weight and body weight gain

Bodyweight gains in high dose P-generation males during the pre-mating period were reduced and body weights were significantly lower than controls (up to -7 %) (see Figure 5.6.1/02- 1, Figure 5.6.1/02- 3, Table 5.6.1/02- 5 and Table 5.6.1/02- 7). Feed consumption was increased in these animals (up to +19 % increase, see Table 5.6.1/02- 3) hence there was decreased efficiency of feed utilisation. There were no similar effects in P-generation females. In high dose F1-generation animals, bodyweights were initially lower than controls (by 17 % in males and 7 % in females) and this difference was maintained during the F1 pre-mating period (Figure 5.6.1/02- 2, Figure 5.6.1/02- 4, Table 5.6.1/02- 6). Since feed consumption in these animals was increased (up to +28 %) over this period (Table 5.6.1/02- 4), decreased efficiency of feed utilization occurred again. Bodyweights of F1 males at 100 mg/kg bw/d were also significantly lower than controls (up to -8 %) but without accompanying effect on feed

consumption. Amongst high dose females of both generations there was also a slight decrease in bodyweight gain during gestation (Table 5.6.1/02- 7 and Table 5.6.1/02- 8) and a slight decrease in feed consumption during lactation (Table 5.6.1/02- 3 and Table 5.6.1/02- 4). There were no other notable effects on feed consumption or bodyweights.

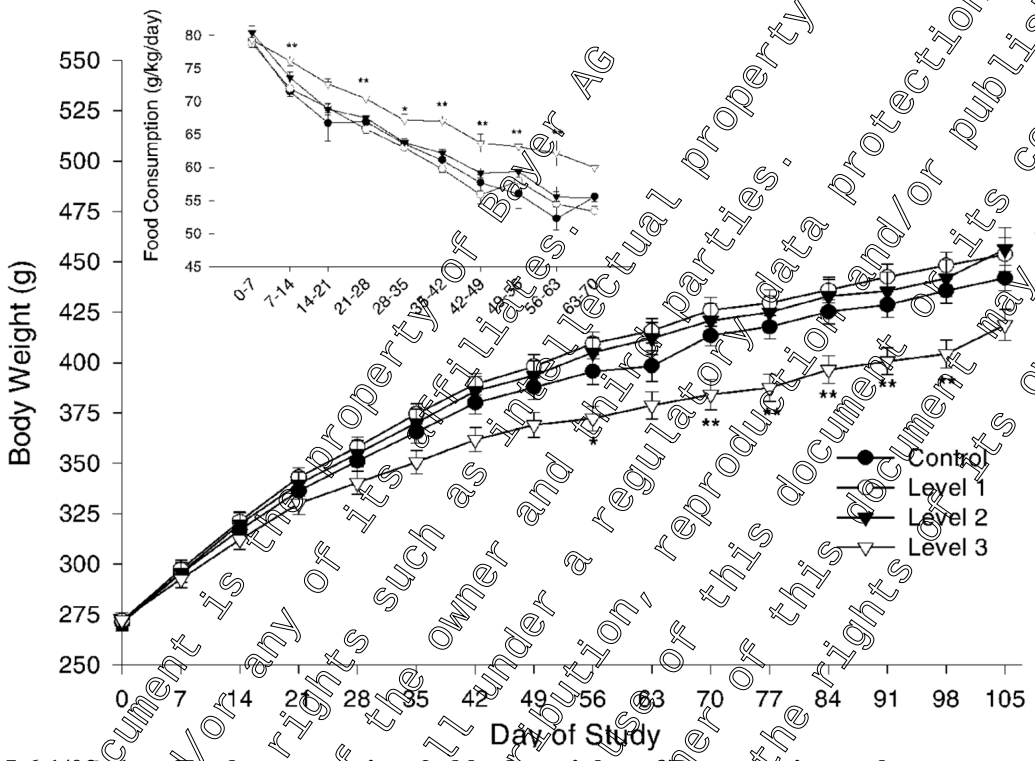


Figure 5.6.1/02- 1: Feed consumption and body weights of P-generation males
(* $p \leq 0.05$, ** $p \leq 0.01$)

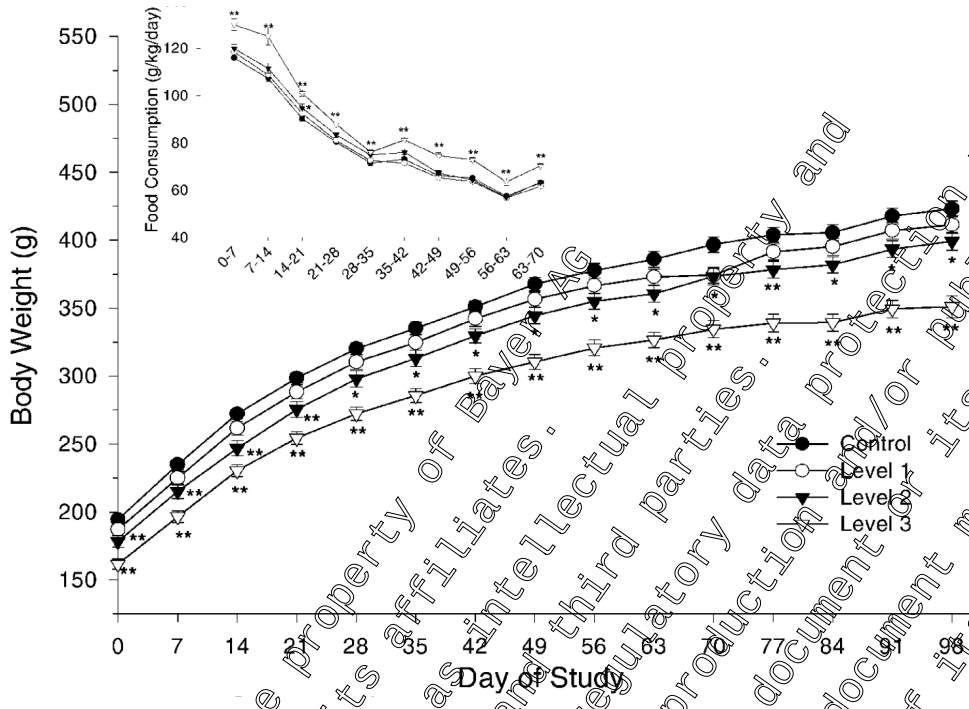


Figure 5.6.1/02- 2: Feed consumption and body weights of F1-generation males
(* $p \leq 0.05$, ** $p \leq 0.01$)

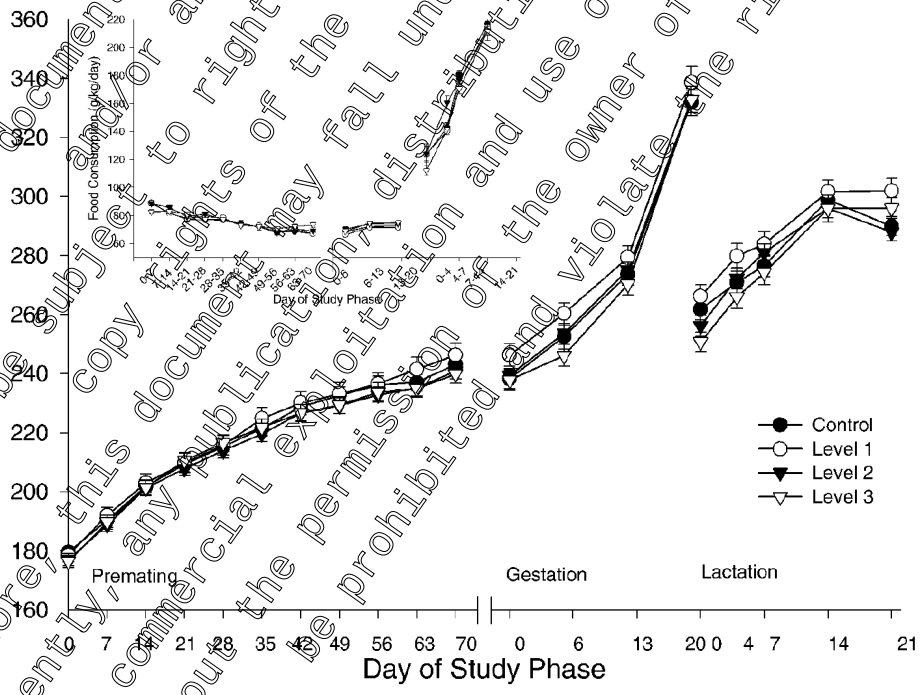


Figure 5.6.1/02- 3: Feed consumption and body weights of P-generation females
(* $p \leq 0.05$, ** $p \leq 0.01$)

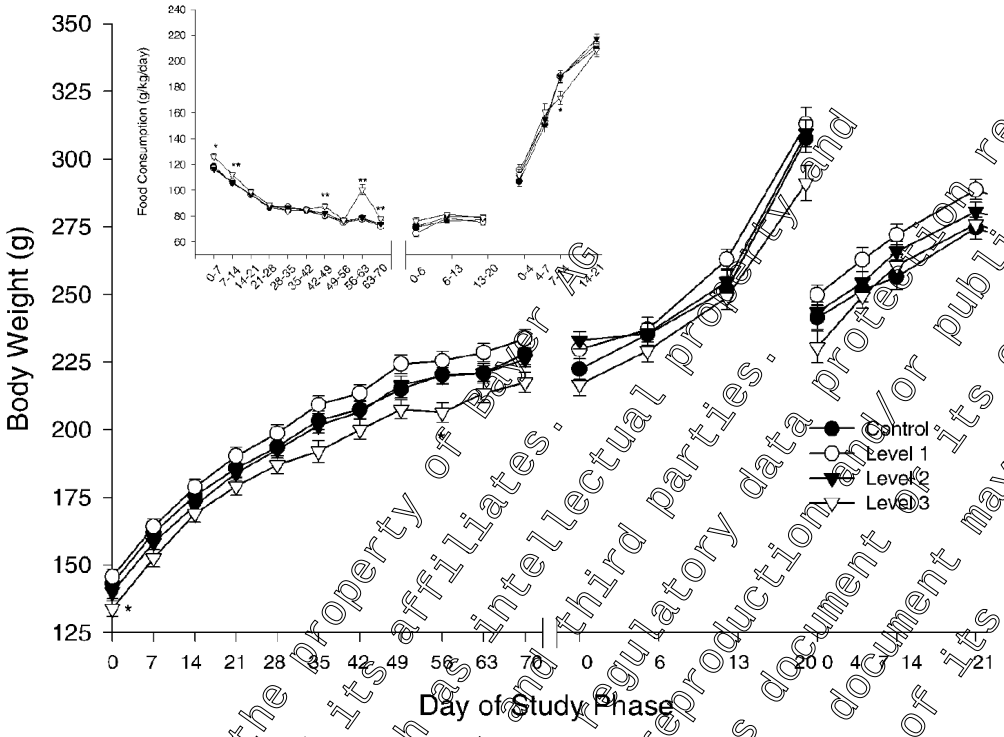


Figure 5.6.1/02- 4: Feed consumption and body weights of F1-generation females (*: $p \leq 0.05$, **: $p \leq 0.01$)

Table 5.6.1/02- 3: Feed consumption (g) of P-generation

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750	(%)[a]
Males							
Day 0-7	P-generation - premating	79.2	78.8	*(-1)*	80.4	*(+2)*	79.3	*(±0)*
Day 7-14		71.5	71.9	*(+1)*	73.6	*(+3)*	**76.1****	***(+6)***
Day 14-21		66.7	69	*(+3*	68.8	*(+3)*	**72.6**	***(+9)***
Day 21-28		66.9	65.8	*(-2)*	67.5	*(+1)*	**70.4****	***(+5)***
Day 28-35		63.6	63	*(-1)*	63.8	*(±0)*	**67.1***	***(+6)***
Day 35-42		62.1	59.8	*(-2)*	62.2	*(+2)*	**67.0****	***(+10)***
Day 42-49		57.7	55.9	*(-3)*	59.2	*(+3)*	**63.7****	***(+10)***
Day 49-56		56	58	*(+4)*	59.4	*(+6)*	**63.2****	***(+13)***
Day 56-63		52.3	54.5	*(+4)*	55.6	*(+6)*	**62.2****	***(+19)***
Day 63-70		55.6	53.4	*(-4)*	55.4	*(±0)*	**60.0**	***(+8)***
Females								
Day 0-7	P-generation - pre-mating	89	88.7	*(±0)*	88.4	*(-1)*	82.6*	*(-7)*
Day 7-14		81.8	81.7	*(±0)*	84.8	*(+4)*	82.8	*(+1)*
Day 14-21		77.4	77.9	*(+1)*	79.3	*(+2)*	80.9	*(+5)*
Day 21-28		77.1	79.6	*(+3)*	80.1	*(+4)*	77.5	*(+1)*
Day 28-35		77.0	78.3	*(+2)*	77.4	*(+1)*	77.6	*(+1)*

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750 (%)[a]
Day 35-42		74.1	73.4	*(-1)*	74.5	*(+1)*	73.2 *(-1)*
Day 42-49		72.4	72.3	*(±0)*	72.2	*(±0)*	72.4 *(±0)*
Day 49-56		68.1	68.8	*(+1)*	68.0	*(±0)*	70.8 *(+4)*
Day 56-63		68.8	70.4	*(+2)*	70.5	*(+2)*	72.4 *(+5)*
Day 63-70		67.1	67.6	*(±0)*	69.2	*(+3)*	**73.5**** *(+10)*
Gestation day 0-6	P-generation - gestation	68.8	66.5	*(-3)*	70.4	*(+2)*	69.0 *(±0)*
Gestation day 6-13		72.3	71.5	*(-1)*	74.2	*(+3)*	74.6 *(+3)*
Gestation day 13-20		72.9	71.5	*(-2)*	74.0	*(+2)*	75.1 *(+3)*
Lactation day 0-4	P-generation - lactation	123.4	123.8	*(±0)*	124.0	*(±0)*	**111.9** *(-9)*
Lactation day 4-7		142.9	140.3	*(-2)*	160.5*	*(+12)*	141.7 *(-1)*
Lactation day 7-14		180.8	175.3	*(-3)*	177.1	*(-2)*	**170.7** *(-6)*
Lactation day 14-21		216.9	214.3	*(-1)*	211.8	*(-2)*	211.6 *(-2)*

[a] *% difference to control*
* statistically significant difference from control $p<0.05$
** statistically significant difference from control $p<0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/02- 4: Feed consumption (g) of F1-generation

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750 (%)[a]
Males							
Day 0-7	F1-generation - premating	116.0	118.4	*(+2)*	119.9	*(+3)*	**129.9**** *(+12)*
Day 7-14		107.3	108.8	*(+1)*	111.6	*(+4)*	**125.0**** *(+16)*
Day 14-21		90.2	92.5	*(+3)*	94.8*	*(+5)*	**101.0**** *(+12)*
Day 21-28		80.3	81.0	*(+1)*	83.5	*(+4)*	**88.0**** *(+10)*
Day 28-35		71.4	72.5	*(+2)*	74.8	*(+5)*	**76.0**** *(+6)*
Day 35-42		73.1	71.9	*(-2)*	76.1	*(+4)*	**81.2**** *(+11)*
Day 42-49		66.7	65.2	*(-1)*	67.2	*(+2)*	**74.5**** *(+13)*
Day 49-56		65.1	63.6	*(-2)*	64.2	*(-1)*	**72.9**** *(+12)*
Day 56-63		57.5	56.7	*(-1)*	56.9	*(-1)*	**63.4*** *(+10)*
Day 63-70		63.1	61.6	*(-2)*	63.3	*(±0)*	**70.3**** *(+11)*
Females							
Day 0-7	F1-generation - pre-mating	118.6	117.3	*(-1)*	116.7	*(-2)*	**126.3*** *(+6)*
Day 7-14		105.9	106.7	*(+1)*	106.1	*(±0)*	**112.3**** *(+6)*
Day 14-21		96.8	96.7	*(±0)*	96.6	*(±0)*	98.8 *(+2)*
Day 21-28		86.5	87.4	*(+1)*	87.2	*(+1)*	88.9 *(+3)*
Day 28-35		84.4	87.2	*(+3)*	86.2	*(+2)*	83.9 *(-1)*
Day 35-42		84.4	85.0	*(+1)*	85.0	*(+1)*	85.1 *(+1)*

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750 (%)[a]
Day 42-49		80.1	80.8	*(+1)*	82.4	*(+3)*	**87.8**** ***(+10)***
Day 49-56		76.5	75.0	*(-2)*	76.1	*(-1)*	76.9 *(+1)*
Day 56-63		78.5	77.8	*(-1)*	78.7	*(±0)*	**100.5**** ***(+28)***
Day 63-70		73.2	72.2	*(-1)*	74.2	*(+1)*	**78.2**** ***(+7)***
Gestation day 0-6	F1-generation - gestation	71.4	66.6	*(-7)*	72	*(+1)*	76.4 *(+7)*
Gestation day 6-13		76.4	78.7	*(+3)*	79.2	*(+4)*	81.4 *(+7)*
Gestation day 13-20		77.4	75.3	*(-3)*	79.6	*(+3)*	78.7 *(+2)*
Lactation day 0-4	F1-generation - lactation	107	116	*(+8)*	110.4	*(+3)*	112 *(+5)*
Lactation day 4-7		147.3	150.8	*(+2)*	149.5	*(+1)*	160.7 *(+9)*
Lactation day 7-14		188.2	188.2	*(±0)*	184.3	*(-2)*	**171.6*** ***(-9)***
Lactation day 14-21		210.1	213.3	*(+2)*	217.2	*(+3)*	208.3 *(-1)*

[a] *% difference to control*
* statistically significant difference from control p<0.05
** statistically significant difference from control p<0.01
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/02- 5: Body weights (g) of P-generation

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750 (%)[a]
Males							
Day 0	P-generation - pre- and postmating	270.8	271.9	*(±0)*	271.7	*(±0)*	272.2 *(+1)*
Day 7		295.4	297.4	*(+1)*	295.7	*(±0)*	292.6 *(-1)*
Day 14		316.9	321.2	*(+1)*	319.8	*(+1)*	312.0 *(-2)*
Day 21		336.2	343.1	*(+2)*	339.7	*(+1)*	329.9 *(-2)*
Day 28		351.2	358.6	*(+2)*	354.9	*(+1)*	340.4 *(-3)*
Day 35		363.5	374.6	*(+2)*	370.1	*(+1)*	350.6 *(-4)*
Day 42		380.1	389.4	*(+2)*	386.0	*(+2)*	**361.8** ***(-5)***
Day 49		387.8	398.2	*(+3)*	393.6	*(+1)*	**369.1** ***(-5)***
Day 56		395.6	409.5	*(+4)*	404.9	*(+2)*	**372.3** ***(-6)***
Day 63		398.3	415.7	*(+4)*	411.9	*(+3)*	**378.8** ***(-5)***
Day 70		413.3	426.0	*(+3)*	420.8	*(+2)*	**384.1**** ***(-7)***
Day 77		417.6	429.6	*(+3)*	424.9	*(+2)*	**387.5**** ***(-7)***
Day 84		425.2	436	*(+3)*	432.8	*(+2)*	**396.6**** ***(-7)***
Day 91		428.7	442.5	*(+3)*	435.4	*(+2)*	**400.7**** ***(-7)***
Day 98		435.7	448.2	*(+3)*	441.7	*(+1)*	**404.2**** ***(-7)***
Females							
Day 0	P-generation - pre-mating	179.5	178.5	*(-1)*	177.0	*(-1)*	176.4 *(-2)*
Day 7		190.4	192.0	*(+1)*	188.7	*(-1)*	189.7 *(±0)*

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10		100	750	
				(%)[a]			(%)[a]
Day 14		201.8	203.0	*(+1)*	201.2	201.4	*(±0)*
Day 21		209.3	209.8	*(±0)*	207.8	210.6	*(+1)*
Day 28		215.3	215.7	*(±0)*	213.9	216.5*	*(+1)*
Day 35		221.4	224.8	*(+2)*	219.6	221.8	*(±0)*
Day 42		228.8	230.1	*(+1)*	226.7	226.7	*(-1)*
Day 49		232.7	233.2	*(±0)*	229.2	229.4	*(-1)*
Day 56		236.1	236.6	*(±0)*	232.9	233.8	*(-1)*
Day 63		237.0	241.3	*(+2)*	235.2	235.0	*(-1)*
Day 70		243.1	246.3	*(+1)*	240.8	239.8	*(-1)*
Gestation day 0	P-generation - gestation	238.3	246.3	*(+3)*	239.4	237.8	*(±0)*
Gestation day 6		252.6	260.4	*(+3)*	253.7	246.1	*(-3)*
Gestation day 13		273.7	279.4	*(+2)*	275.2	270.3	*(-1)*
Gestation day 20		332.1	338.7	*(+2)*	332.9	322.9	*(-3)*
Lactation day 0	P-generation - lactation	261.8	266.2	*(+2)*	256.2	250.7	*(-4)*
Lactation day 7		276.6	283.9	*(+3)*	281.0	274.2	*(-1)*
Lactation day 14		298.9	301.6	*(+1)*	296.0	295.9	*(-1)*
Lactation day 21		290.0	301.9	*(+4)*	288.0	296.1	*(+2)*

[a] *% difference to control*
* statistically significant difference from control $p<0.05$
** statistically significant difference from control $p<0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/02- 6: Body weights (g) of F1-generation

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10		100	750	
				(%)[a]			(%)[a]
Males							
Day 0	F1-generation - pre- and postmating	194.3	187.1	*(-4)*	**178.2***	**161.6****	***(-17)***
Day 7		235.0	225.0	*(-4)*	**215.2****	**196.6****	***(-16)***
Day 14		272.2	261.7	*(-4)*	**249.6****	**230.4****	***(-15)***
Day 21		298.5	288.3	*(-3)*	**275.4****	**254.3****	***(-15)***
Day 28		320.2	310.4	*(-3)*	**297.9***	**272.3****	***(-15)***
Day 35		335.2	324.6	*(-3)*	**313.2***	**285.7****	***(-15)***
Day 42		351.1	342.3	*(-3)*	**329.7***	**299.9****	***(-15)***
Day 49		367.8	356.5	*(-3)*	**344.4***	**310.5****	***(-16)***
Day 56		377.7	366.6	*(-3)*	**355.1***	**320.8****	***(-15)***
Day 63		386.0	373.1	*(-3)*	**360.7***	**326.6****	***(-15)***
Day 70		396.7	384.3	*(-3)*	**373.4***	**334.5****	***(-16)***
Day 77		403.9	391.5	*(-3)*	**378.4****	**339.1****	***(-16)***

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750 (%)[a]
Day 84		405.5	395.2	*(-3)*	**382.0***	***(-6)***	**339.4**** ***(-16)***
Day 91		417.7	407.1	*(-3)*	**393.5***	***(-6)***	**349.4**** ***(-16)***
Day 98		423.1	411.4	*(-3)*	**399.2***	***(-6)***	**351.1**** ***(-17)***
Females							
Day 0	F1-generation - pre-mating	143.2	145.6	*(+2)*	140.0	*(-2)*	**133.8*** ***(-7)***
Day 7		161.9	164.2	*(+1)*	158.3	*(-2)*	**152.5** ***(-6)***
Day 14		175.7	178.8	*(+2)*	172.0	*(-2)*	**169.0** ***(-4)***
Day 21		185.7	190.3	*(+2)*	184.1	*(-1)*	**179.1** ***(-4)***
Day 28		193.6	198.7	*(+3)*	192.5	*(-1)*	**187.4** ***(-3)***
Day 35		203.4	209.2	*(+3)*	201.5	*(-1)*	**192.0** ***(-6)***
Day 42		207.4	213.5	*(+3)*	206.5	*(±0)*	**200.0** ***(-4)***
Day 49		214.9	224.3	*(+4)*	216.6	*(+1)*	**207.5** ***(-3)***
Day 56		220.4	225.5	*(+2)*	219.8	*(±0)*	**206.3*** ***(-6)***
Day 63		220.9	228.5	*(+3)*	220.8	*(±0)*	213.5 *(-3)*
Day 70		228.0	233.6	*(+2)*	226.0	*(-1)*	217.4 *(-5)*
Gestation day 0	F1-generation - gestation	224.4	229.7	*(+2)*	223.0	*(-1)*	216.7 *(-3)*
Gestation day 6		235.2	237.0	*(+1)*	235.7	*(±0)*	229.2 *(-3)*
Gestation day 13		251.9	263.0	*(+4)*	254.7	*(+1)*	249.0 *(-1)*
Gestation day 20		307.6	313.0	*(+2)*	309.5	*(+1)*	**291.1** ***(-5)***
Lactation day 0	F1-generation - lactation	241.5	249.8	*(+3)*	243.5	*(+1)*	**230.5** ***(-5)***
Lactation day 7		256.5	272.0*	*(+6)*	265.9	*(+4)*	260.6 *(+2)*
Lactation day 14		274.5	288.7	*(+5)*	280.5	*(+2)*	275.9 *(+1)*
Lactation day 21		264.9	278.5	*(+5)*	273.3	*(+3)*	266.8 *(+1)*

[a] *% difference to control*
* statistically significant difference from control $p<0.05$
** statistically significant difference from control $p<0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/02- 7: Body weight gain (g) of P-generation

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10	(%)[a]	100	(%)[a]	750 (%)[a]
Males							
Day 0 – 70	P-generation - pre- and postmating	142.5	154.1	*(+8)*	149.1	*(+5)*	**111.9** ***(-21)***
Day 0 – 98		164.9	176.3	*(+7)*	170.0	*(+3)*	**132.0** ***(-20)***
Females							
Day 0 – 70	P-generation - pre-mating	63.6	67.8	*(+7)*	63.8	*(±0)*	63.4 *(±0)*

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10		100		750	
				(%)[a]		(%)[a]		(%)[a]
GD 0 – 6	P-generation - gestation	14.3	14.1	*(-1)*	14.3	*(±0)*	**8.3**	***(-42)***
GD 6 - 13		21.1	19	*(-10)*	21.5	*(+2)*	24.2	*(+15)*
GD 13 – 20		58.4	59.3	*(+2)*	57.7	*(-1)*	52.6	*(-10)*
GD 0 – 20		93.8	92.4	*(-1)*	93.3	*(±0)*	85.1	*(-9)*
LD 0 – 7	P-generation - lactation	14.8	17.7	*(+20)*	24.8	*(+68)*	23.5	*(+59)*
LD 7 - 14		22.3	17.4	*(-21)*	15	*(-33)*	21.7	*(-3)*
LD 14 – 21		-8.9	0.3	*(+103)*	-8	*(+10)*	0.2	*(+102)*
LD 0 – 21		28.2	35.7	*(+27)*	31.8	*(+13)*	45.4	*(+61)*

[a] *% difference to control*
* statistically significant difference from control $p<0.05$
** statistically significant difference from control $p<0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/02- 8: Body weight gain (g) of F1-generation

	Generation	Prothioconazole (mg/kg bw/d)					
		0	10		100		750	
				(%)[a]		(%)[a]		(%)[a]
Males								
Day 0 – 71	F1-generation - pre- and postmating	202.4	197.2	*(-3)*	195.2	*(-4)*	**172.9**	***(-15)***
Day 0 – 98		228.8	224.3	*(-2)*	221	*(-3)*	**189.5**	***(-17)***
Females								
Day 0 – 70	F1-generation - pre-mating	84.8	88.0	*(+4)*	86.0	*(+1)*	83.6	*(-1)*
GD 0 – 6	F1-generation - gestation	10.8	7.3	*(-32)*	12.7	*(+18)*	12.5	*(+16)*
GD 6 - 13		16.7	26.0	*(+56)*	19.0	*(+14)*	19.8	*(+19)*
GD 13 – 20		55.7	50.0	*(-10)*	54.8	*(-2)*	42.1	*(-24)*
GD 0 – 20		83.2	83.3	*(±0)*	86.5	*(+4)*	74.4	*(-11)*
LD 0 – 7	F1-generation - lactation	15	22.2	*(+48)*	22.4	*(+49)*	30.1	*(+101)*
LD 7 - 14		18	16.7	*(-7)*	14.6	*(-19)*	15.3	*(-15)*
LD 14 – 21		-9.6	-10.2	*(-6)*	-7.2	*(+25)*	-9.1	*(+5)*
LD 0 – 21		23.4	28.7	*(+23)*	29.8	*(+27)*	36.3	*(+55)*

[a] *% difference to control*
* statistically significant difference from control $p<0.05$
** statistically significant difference from control $p<0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

Parental toxicity

The parental (particularly, the maternal) toxicity at 750 mg/kg bw/d is considered to be very high, even sublethal, based primarily on kidney dysfunction and resulting dehydration. To fully understand the nature and extent of parental toxicity in the present study, it needs to be evaluated also in the light of relevant findings observed in other rat studies:

- In the same Wistar rat substrain, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25 % mortality or, at 750 mg/kg bw/d, could not be fully compensated by a drastically increased (up to > 170 % of control) water consumption (as determined in the pilot developmental toxicity study or the new main developmental toxicity study (see Figure 5.6.1/02- 5 and 5.6.2/03 [M-067839-01-1]). Since the present two-generation study was conducted with gavage dosing in the same Wistar rat substrain as the new main developmental toxicity study (see below), it is plausible to assume that the observed drastically increased water intake in the new main developmental toxicity study at 750 mg/kg bw/d (as depicted in the following graph) does also reflect the situation in the two-generation study (in which water consumption was not measured) at 750 mg/kg bw/d.

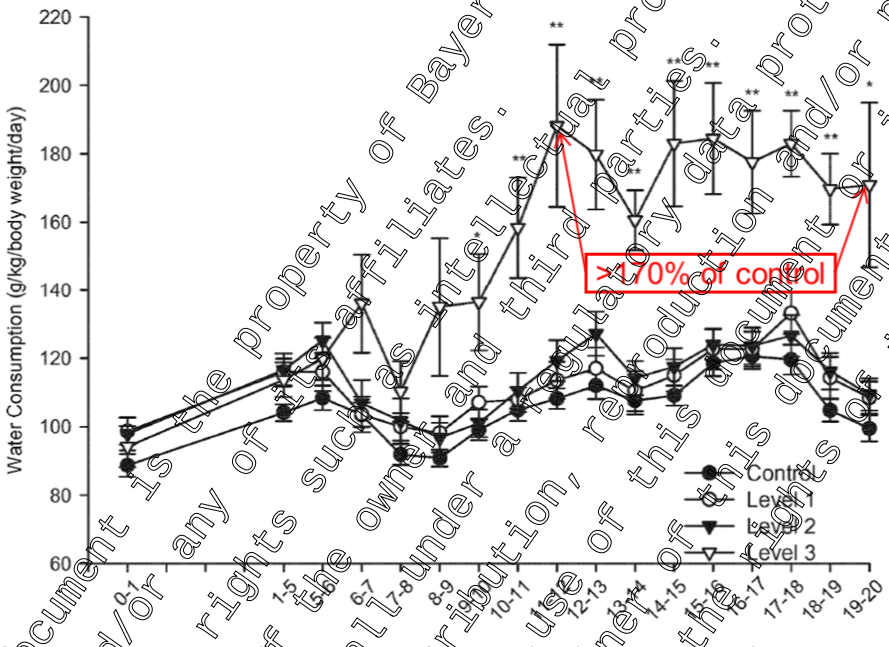


Figure 5.6.1/02- 5: Mean gestational water consumption in the new main developmental toxicity study, conducted in the same Wistar rat substrain as the present two-generation study (* $p \leq 0.05$, ** $p \leq 0.01$)

- In another Wistar rat substrain, strongly to drastically increased water consumption was observed in pregnant dams at ≥ 500 mg/kg bw/d due to the disturbed water homeostasis. At these dose levels dams also showed increased urine excretion. The urine stain observed in the present study at 750 mg/kg bw/d possibly also relates to increased urine excretion (see 5.6.2/01, [M-012279-01-1]).
- Furthermore, markedly increased water intake, kidney damage and isolated deaths (possibly related to kidney failure) were observed in the subchronic rat study (500 mg/kg bw/d, one female in week 13; see 5.3.1/01, [M-012338-01-1]**study summary not included yet**) and in the 1-year rat study (750 mg/kg bw/d, two males in weeks 40 and 51; one female in week 37; see 5.5/01, [M-030441-01-1]**study summary not included yet**).
- A high mortality rate, which was obviously related to kidney failure, was observed in both sexes in the 2-year rat study at doses of 500-750 mg/kg bw/d; see 5.5/02 [M-084962-01-1] *(*study summary not included yet*)*.

D. REPRODUCTIVE EVALUATIONS

1. Oestrous cycling

A treatment-related decrease in the number of oestrous cycles and concomitant increase in the cycle length occurred in both generations at 750 mg/kg bw/d; this effect was less pronounced in the F1-generation than in the P-generation (Table 5.6.1/02- 9). The effects on cycling were attributed to the very strong general systemic maternal toxicity at this very high dose (see also aforementioned explanations on maternal kidney dysfunction / dehydration / lethality as also observed in the other toxicological studies in pregnant and non-pregnant rats). The effect was not apparent at lower dose levels.

Table 5.6.1/02- 9: Estrous cycling of P- and F1-generation animals

Parameter	Prothioconazole (mg/kg bw/d)			
	0	**10**	**100**	**750**
P-Generation				
Mean no. oestrous cycles/14 days	3.4	3.2	3.2	**2.7***
Mean oestrous cycle duration (days)	4.3	4.2	4.4	**5.1***
F1-Generation				
Mean no. oestrous cycles/14 days	3.6	3.4	3.5	**3.1***
Mean oestrous cycle duration (days)	4.4	4.5	4.4	**4.7**

* significantly different from control ($p < 0.05$ Kruskal-Wallis and Dunn's Test)
Findings considered related to treatment with prothioconazole are written in **bold letter**

2. Reproductive parameters and litter data

There were no treatment-related effects at any dose level on the mating, fertility and gestation indices. However, at 750 mg/kg bw/d a non-statistically significant increase in the number of days to insemination occurred in the F1-generation and reduced mean numbers of implantation sites and reduced mean litter size occurred in both generations (Table 5.6.1/02- 10). A very slight statistically non-significant increase in the duration of gestation in both generations was evident at 750 mg/kg bw/d. All these effects are attributed to the very strong general systemic parental toxicity at this very high dose. There were no adverse effects on reproductive parameters in either generation at dose levels up to 100 mg/kg bw/d.

Table 5.6.1/02- 10: Reproductive data of P- and F1-generation animals

Parameter	Prothioconazole (mg/kg bw/d)				Historical control data [a]
	0	**10**	**100**	**750**	
P-Generation					
No. mated / no. paired	27 / 30	30 / 30	30 / 30	30 / 30	197/200
No. delivering a litter	24	30	29	28	173
No. with implants	24	30	29	28	176
Mating index	90.0	100	100	100	90.0-100.0
Fertility index	88.9	100	96.7	96.7	86.2-96.6
Gestation index	100	100	100	96.6	96.0-100.0
Mean time to insemination (days)	2.6	3.4	2.5	2.9	1.2-3.5

Parameter	Prothioconazole (mg/kg bw/d)				Historical control data [a]
	0	10	100	750	
Mean duration of gestation (days)	21.9	21.9	22.1	**22.3**	21.6-22.1
Mean no. implants	11.8	11.6	12.2	**10.8**	9.6-13.3
Mean litter size	10.8	11.1	11.4	**10.0**	9.4-11.8
F1-Generation					
No. mated / no. paired	30 / 30	30 / 30	30 / 30	29 / 30	149/150
No. delivering a litter	27	26	28	26	132
No. with implants	27	26	28	26	133
Mating index	100	100	100	96.7	96.7-100.0
Fertility index	90.0	86.7	96.7	93.1	76.9-96.7
Gestation index	100	100	96.6	92.6	95.5-100.0
Mean time to insemination (days)	2.4	3.0	3.0	**3.8**	2.2-3.4
Mean duration of gestation (days)	22.0	22.0	22.2	**22.4**	21.8-22.2
Mean no. implants	10.7	11.0	11.1	**9.3**	10.7-11.5
Mean litter size	10.2	10.5	9.7	**8.2**	9.9-10.8

Mating index = no. inseminated / no. paired x 100; Fertility index = no. pregnant / no. inseminated x 100
Gestation index = no. with live pups/ no. pregnant x 100
[a] Historical control range from 7 studies in Wistar rats performed 1998-2001 (presented in original report)
* $p < 0.05$ (Kruskal-Wallis and Dunn's Test)
Findings considered related to treatment with prothioconazole are written in **bold letters**

Pup body weight gain was significantly retarded at 750 mg/kg bw/d in both generations from either day 4 or day 7 post partum (Table 5.6.1/02-11). However, there was no effect of treatment on pup viability (stillborn pups, post-implantation losses, pup deaths in the neonatal period or later in the lactation period were all similar to controls - data presented in the table below as live birth, viability, lactation and birth indices). There was no effect on pup growth or viability at lower dose levels.

Table 5.6.1/02- 11: Summary of litter data (P and F1 generation)

Parameter	Prothioconazole (mg/kg bw/d)			
	0	10	100	750
P-Generation adults = F1 pups				
No. litters	24	30	29	28
Total no. pups born	258	334	331	281
Total no. pups missing	2	2	2	8
Total no. pups dying	3	1	5	4
Total no. pups cannibalized	0	0	0	0
Mean litter size	10.8	11.1	11.4	10.0

Parameter	Prothioconazole (mg/kg bw/d)			
	0	10	100	750
Mean pup weight, combined (g):				
- day 0	5.9	5.7 *(-3%)*	5.8 *(-2%)*	5.9 *(±0%)*
- day 4 (pre-cull)	9.5	9.4 *(-1%)*	9.2 *(-3%)*	**8.6*** ***(-9%)***
- day 7	14.6	15.1 *(+3%)*	14.4 *(-1%)*	**12.5**** ***(-14%)***
- day 14	29.2	29.2 *(±0%)*	28.2 *(-3%)*	**24.2**** ***(-17%)***
- day 21	44.6	45.5 *(+2%)*	42.8 *(-4%)*	**38.2**** ***(-14%)***
Mean male pup weight (g):				
- day 0	6.0	5.9 *(-2%)*	6.0 *(±0%)*	6.1 *(+2%)*
- day 4 (pre-cull)	9.7	9.7 *(±0%)*	9.4 *(-3%)*	**8.7*** ***(-10%)***
- day 7	15.0	15.3 *(+2%)*	14.7 *(-2%)*	**12.6**** ***(-16%)***
- day 14	29.8	30.4 *(+2%)*	28.7 *(-4%)*	**24.6**** ***(-17%)***
- day 21	45.8	46.5 *(+2%)*	43.8 *(-4%)*	**38.7**** ***(-16%)***
Mean female pup weight (g):				
- day 0	5.7	5.6 *(-2%)*	5.6 *(-2%)*	5.7 *(±0%)*
- day 4 (pre-cull)	9.3	9.2 *(-1%)*	9.0 *(-3%)*	**8.4*** ***(-10%)***
- day 7	14.4	14.8 *(+3%)*	14.1 *(-2%)*	**12.2**** ***(-15%)***
- day 14	28.9	29.3 *(+1%)*	27.8 *(-4%)*	**23.8**** ***(-18%)***
- day 21	43.5	44.5 *(+2%)*	41.8 *(-4%)*	**37.5**** ***(-14%)***
Sex ratio at birth (% males)	48.7	44.8	47.6	53.1
No. still-born pups	2	3	11	1
Mean no. viable pups at:				
- birth	11	11	11	10
- day 4 (pre-cull)	11	11	11	10
- day 4 (post-cull)	8	8	8	8
- day 21	8	8	8	8
Live birth index	99.1	99.1	97.1	99.7
Viability index	99.4	99.8	95.6	96.1
Lactation index	98.4	98.3	99.2	98.4
Birth index	90.7	95.3	93.4	91.9
F1-Generation adults – F2 pups				
No. litters	27	26	28	25
Total no. pups born	276	274	271	212
Total no. pups missing	2	4	8	4
Total no. pups dying	3	0	4	0
Total no. pups cannibalized	2	0	0	1
Mean litter size	10.2	10.5	9.7	8.2
Mean M + F pup weight (g) on:				
- day 0	5.8	5.8 *(±0%)*	6.0 *(+3%)*	6.1 *(+5%)*
- day 4 (pre-cull)	9.4	9.5 *(+1%)*	9.7 *(+3%)*	9.5 *(+1%)*
- day 7	14.5	14.8 *(+2%)*	14.8 *(+2%)*	**13.7** ***(-6%)***
- day 14	28.9	29.8 *(+3%)*	29.0 *(±0%)*	**25.5**** ***(-12%)***
- day 21	43.4	44.7 *(+3%)*	44.3 *(+2%)*	**40.0*** ***(-8%)***

Parameter	Prothioconazole (mg/kg bw/d)			
	0	10	100	750
Mean male pup weight (g):				
- day 0	5.9	5.9 *(±0%)*	6.2 *(+5%)*	6.2 *(+5%)*
- day 4 (pre-cull)	9.6	9.7 *(+1%)*	10.0 *(+4%)*	9.6 *(±0%)*
- day 7	14.7	15.1 *(+3%)*	15.2 *(+3%)*	**13.8 *(-6%)***
- day 14	29.3	30.2 *(+3%)*	29.5 *(+1%)*	**25.6** *(-13%)***
- day 21	44.4	45.7 *(+3%)*	45.3 *(+2%)*	**40.4* *(-9%)***
Mean female pup weight (g):				
- day 0	5.7	5.5 *(-4%)*	5.8 *(+2%)*	6.0 *(+5%)*
- day 4 (pre-cull)	9.3	9.2 *(-1%)*	9.4 *(+1%)*	9.4 *(+1%)*
- day 7	14.3	14.4 *(+1%)*	14.4 *(+1%)*	**13.6 *(-5%)***
- day 14	28.5	29.3 *(+3%)*	28.5 *(±0%)*	**25.3** *(-11%)***
- day 21	42.6	43.5 *(+2%)*	43.2 *(+1%)*	**39.6 *(-7%)***
Sex ratio at birth (% males)	48.6	56.2	53.2	48.4
No. still-born pups	2	0	2	3
Mean no. viable pups at:				
- birth	10	10	10	8
- day 4 (pre-cull)	10	10	9	8
- day 4 (post-cull)	8	8	8	7
- day 21	8	8	8	7
Live birth index	99.4	99.3	99.2	96.2
Viability index	98.3	98.5	96.3	97.6
Lactation index	99.0	99.6	99.6	100
Birth index	93.4	94.2	87.2	87.5

* $p < 0.05$; ** $p < 0.01$ (Dunnett's test)
Live birth index = no. live-born pups per litter / total no. pups per litter x 100
Viability index = No. live pups on day 4 pre-cull per litter / no. live pups born per litter x 100
Lactation index = No. live pups on day 21 per litter / no. live pups on day 4 post-cull per litter x 100
Birth index = total no. pups born per litter / total no. implantation sites per dam x 100
Findings considered related to treatment with prothioconazole are written in **bold letters**

E. DEVELOPMENTAL MILESTONES

The time to preputial separation and vaginal opening were measured in the first generation (F1 pups).

Vaginal opening

Time to vaginal opening was slightly shorter in high dose females (Table 5.6.1/02- 12), but since this difference was not statistically significant and the value was within the historical control range, this change is not considered toxicologically significant.

Table 5.6.1/02- 12: Vaginal opening in F1 pups

Parameter	Prothioconazole (mg/kg bw/d)				Historical control data
	0	10	100	750	
Vaginal opening (days)	35.1	34.5	35.7	33.8	33.1-38.2 [a]

[a] Historical control range from 5 studies in Wistar rats performed 1998-2001 (presented in original report)
Findings considered related to treatment with prothioconazole are written in **bold letters**

Preputial separation

In F1 pups of the high dose group (750 mg/kg bw/d), the mean age at preputial separation was slightly (5.7 %) but statistically significantly increased (to post partum (PP) day 46.5), compared to controls (PP day 44.0) (Table 5.6.1/02- 13).

Table 5.6.1/02- 13: Preputial separation in F1 pups

Parameter	Prothioconazole (mg/kg bw/d)				Historical control data
	0	10	100	750	
Preputial separation (days *(% difference to control)*)	44.0	44.1 *(+0)*	45.1 *(+2.5)*	**46.5** *(+5.7)***	41.3-45.9 [a]

* $p < 0.05$; ** $p < 0.01$ (Dunnett's test)
[a] Historical control range from 7 studies in Wistar rats performed 1998-2001 (presented in original report)
Findings considered related to treatment with prothioconazole are written in **bold letters**

As outlined below, this slight delay in preputial separation is considered secondary to the clearly retarded growth.

Corresponding with the tabulated data presented in Table 5.6.1/02- 11 (Summary of litter data), the following graphical presentation of the mean F1 pup body weights (both sexes combined) from birth to PP day 21 demonstrates a statistically significant decrease in the high dose group starting on PP day 4:

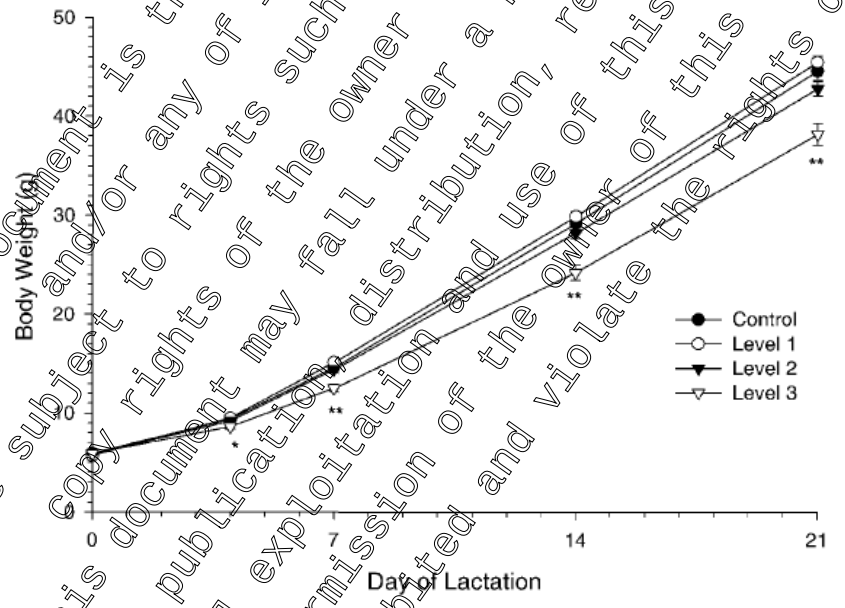


Figure 5.6.1/02- 6: Mean body weight of F1 pups during lactation

As already mentioned above (see Table 5.6.1/02- 11), the body weight of male F1 high dose pups on PP day 21 (the day of weaning) was decreased by 16 %, compared to controls.

On PP day 44 (the mean day of preputial separation in F1 control pups), the mean body weight of male F1 high dose pups was decreased by 13 %, compared to controls (Table 5.6.1/02- 14).

Table 5.6.1/02- 14: Body weight of F1 pups on PP day 44

Parameter	Prothioconazole (mg/kg bw/d)			
	0	10	100	750
Mean body weight of males on PP day 44 (g *(% difference to control)*)	169.8	172.0 *(+1)*	161.3 *(-5)*	**147.1** *(-13)***

* p < 0.05; ** p < 0.01 (Dunnett's test)
Findings considered related to treatment with prothioconazole are written in **bold letters**

Data which would allow establishing a direct link between individual pup weight at the respective day of preputial separation was not presented in the original study report. Therefore a new compilation of the individual pup body weight at the respective day of preputial separation was generated by the original study director as a direct download from the electronic raw data of this GLP-monitored study (presented in M-524357-01-1). Based on the newly compiled data, the following graph illustrates for the control and the high dose group (750 mg/kg bw/d) the relation between the day of preputial separation and the respective body weight at the day of preputial separation (for individual animals and as the respective group mean value).

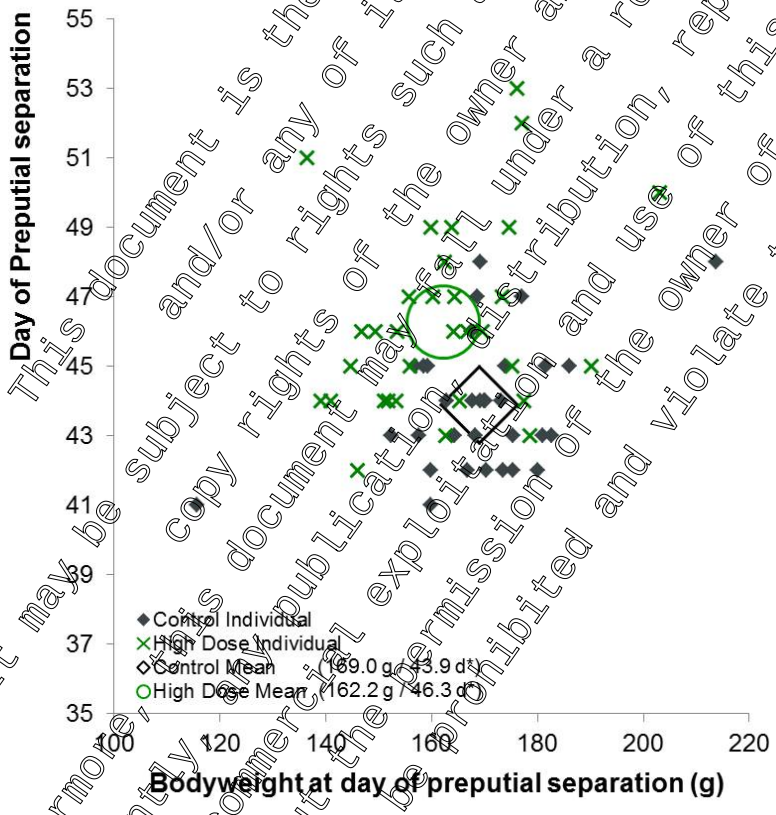


Figure 5.6.1/02- 7: Relation between the day of preputial separation and the respective body weight at the day of preputial separation in F1 pups. (* Group mean values (calculated from individual data) differ slightly from those stated in the study report (calculated from mean litter data))

These data show that:

- all individual high dose body weights at the respective individual day of preputial separation are within the range of the respective control values
- the "clowd" of high dose individual values has an intersection with the respective "clowd" of control values but has also a tendency towards a higher age
- also the mean high dose value has a tendency towards a higher age (+5.7 %) whereas the mean body weight is practically identical (-4.0 %; 162.2 g versus 169.0 g) in the high dose compared to controls

Thus, the high dose of 750 mg/kg bw/d caused in male F1 pups:

- a slightly (+2.5 days / +5.7 %) increased age at preputial separation
- no effect on birth weight
- a clearly (-16 %) decreased body weight on PP day 21 (day of weaning)
- a clearly (-13 %) decreased body weight on PP day 44 (day of preputial separation in controls)
- a practically identical (-4.0 %) body weight at the individual day of preputial separation, compared to controls

Based on these data, it is concluded that the slight delay in preputial separation of F1 pups at the high dose is secondary to the clearly retarded growth (which is related to general toxicity). At the respective individual day of preputial separation, the high dose pups had reached a practically identical body weight as the controls. This data constellation is clearly different from that to be expected for an endocrine-mediated effect – in that case a delay in preputial separation would be connected with a higher body weight at the day of preputial separation due to continuous growth of the pup over time.

This assessment is in line with the published negative correlation between body weight and age at day of preputial separation. [redacted]. (2004) describe that a 50 % feed restriction in pregnant rats from gestation day 7 through lactation caused in their male progeny a 47 % lower body weight at postnatal day 21 and a 6.6 day delay of age at preputial separation[2]. [redacted]. (2014) state that there is broad overall evidence, that in untreated controls the onset of male puberty (age at day of preputial separation) is negatively correlated with male pup body weight[3]. The authors analyzed the control data set of 23 reproductive toxicity studies and concluded that body weight reductions of approximately 10–19 % at the time of puberty onset may represent an important transition point above which body weight can have a large impact on mean age at puberty onset (i.e., delays of several days).

Anogenital distance at birth

In response to the delay in preputial separation time in the F1-generation, anogenital distance (AGD) at birth was measured in F2-generation pups. The anogenital distance of F2-pups at birth was slightly but significantly greater in both sexes at 750 mg/kg bw/d and in males at 100 mg/kg bw/d (Table 5.6.1/02-15). This finding, which for all males was within the historical control range, is attributed to the incidentally slightly higher birth weight of these pups.

Table 5.6.1/02- 15: Anogenital distance at birth in F2 pups

	Prothioconazole (mg/kg bw/d)				Historical control data[a]
	0	10	100	750	
AGD (mm) at birth					
- males	4.20	4.20	4.33*	4.36*	3.76-4.39

[redacted]., 2004. The effects of feed restriction during in utero and postnatal development in rats. *Toxicological Sciences* **82**(1): 237-249. [M-244954-01-1]

[3] [redacted]., 2014. Anti-androgenicity can only be evaluated using a weight of evidence approach. *Regulatory Toxicology and Pharmacology*; **68**(2014):175-192. [M-494773-01-1]

	Prothioconazole (mg/kg bw/d)						Historical control data[a]
	0	10		100		750	
- females	2.24	2.26		2.27		2.35*	1.91-2.24
Body weight (g) at birth *(% difference to control)*							
- males	5.9	5.9	*(±0%)*	6.2	*(+5%)*	6.2	*(+5%)*
- females	5.7	5.5	*(-4%)*	5.8	*(+2%)*	6	*(+5%)*

* $p < 0.05$; ** $p < 0.01$ (Dunnett's test)
[a] Historical control range from 10 studies in Wistar rats performed 1998-2001 (presented in original report)
Findings considered related to treatment with prothioconazole are written in **bold letters**

The following graphs illustrate the relation between the anogenital distance at birth and the respective body weight at birth for individual animals and as the respective group mean value for the control and the high dose group (750 mg/kg bw/d).

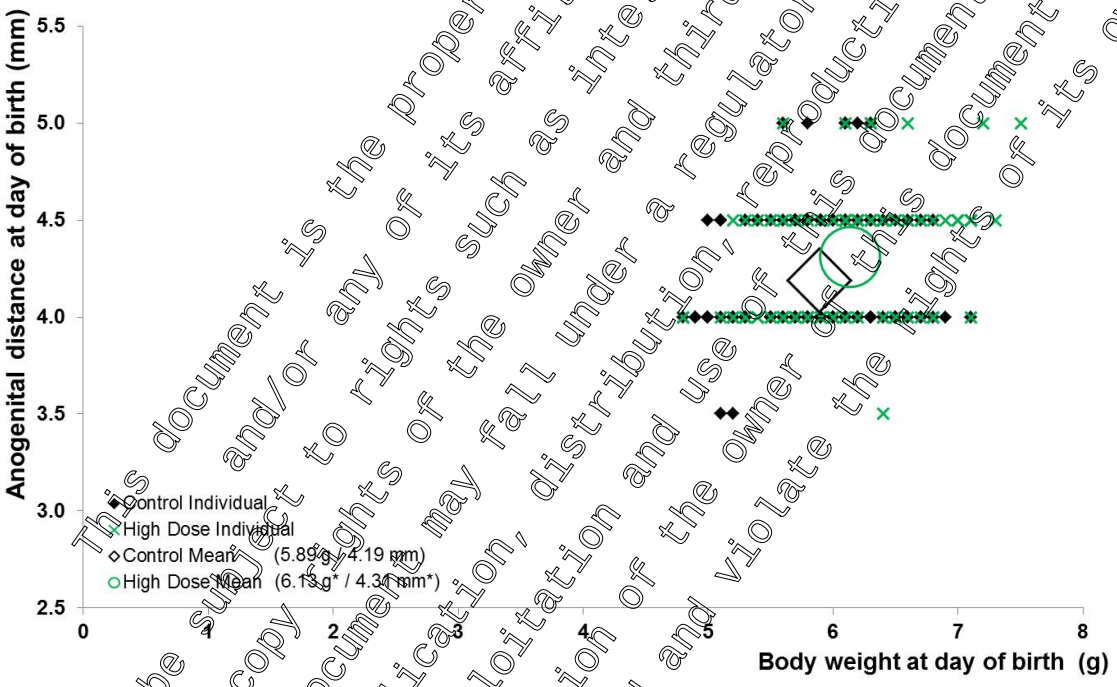


Figure 5.6.1/02- 8: Male F2 pups - anogenital distance at day of birth versus body weight at day of birth. (* Group mean values (calculated from individual data) differ slightly from those stated in the study report (calculated from mean litter data))

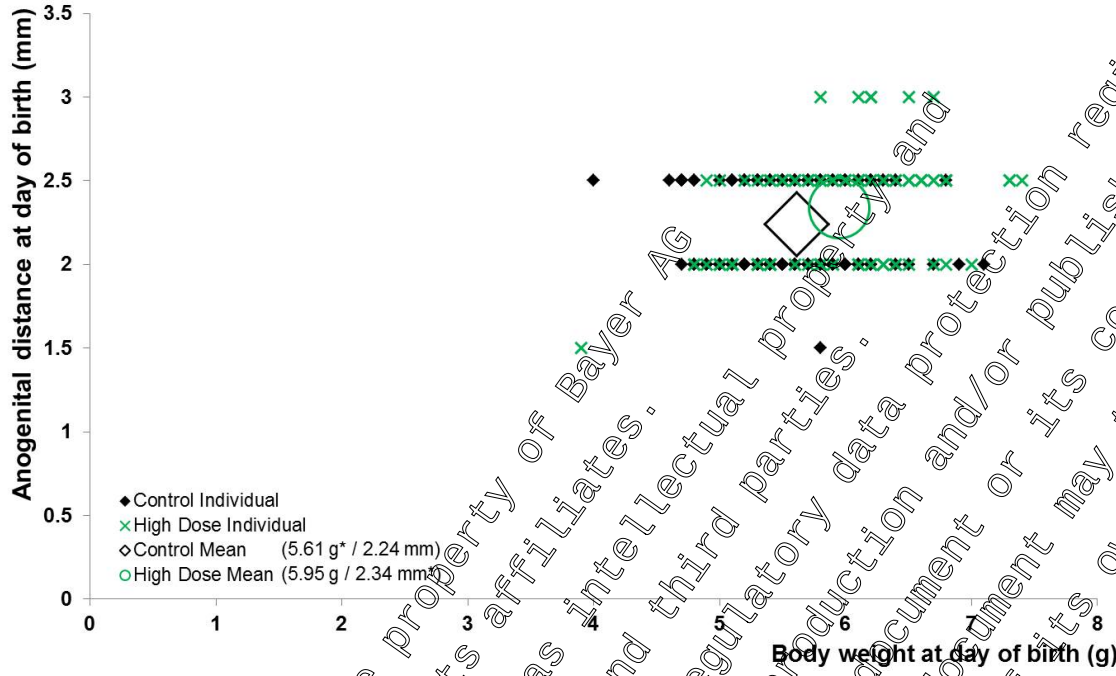


Figure 5.6.1/02- 9: Female F2 pups - anogenital distance at day of birth versus body weight at day of birth. (* Group mean values (calculated from individual data) differ slightly from those stated in the study report (calculated from mean litter data))

At 750 mg/kg bw/d there is a tendency towards a higher AGD (+2.8 % or +4.5 % for males or females, respectively) as well as a tendency towards a higher body weight (+4.1 % or +6.1 % for males or females, respectively) compared to controls. An endocrine-mediated difference in AGD at day of birth would be expected to occur at an unchanged body weight (and would be most likely restricted to one gender). Since this is not the case and the mean high dose body weight at the day of birth is slightly above control, for both sexes the slight increase in AGD at the high dose is considered secondary to the incidentally slightly higher body weight.

Furthermore, if the AGD is normalized with the cube root of body weight (as suggested by [redacted] 1999[4]), there is no relevant difference between control and high dose groups – neither in the scatter of individual values, nor in the mean values for both males and females (Figure 5.6.1/02- 10 and Figure 5.6.1/02- 11). This confirms that there is no treatment-related effect and explicitly no endocrine-mediated effect of prothioconazole on AGD.

[4] [redacted] 1999. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. *Reproductive Toxicology*, **13**(1999):383-390.

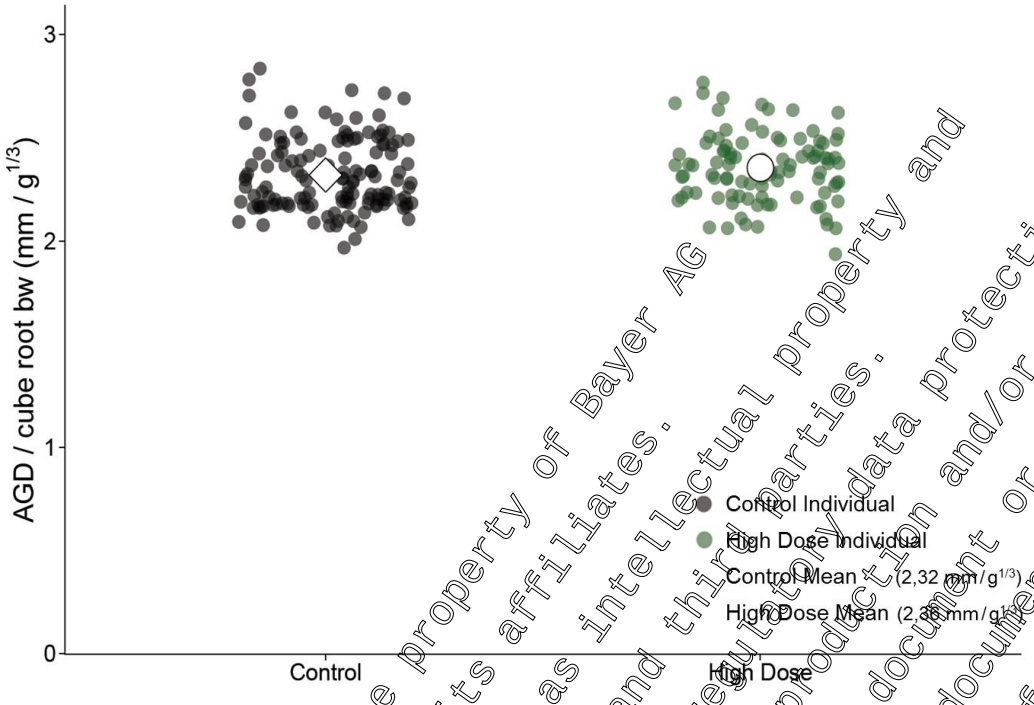


Figure 5.6.1/02- 10: Ratio AGD / cube root of body weight – male F2 pups at day of birth

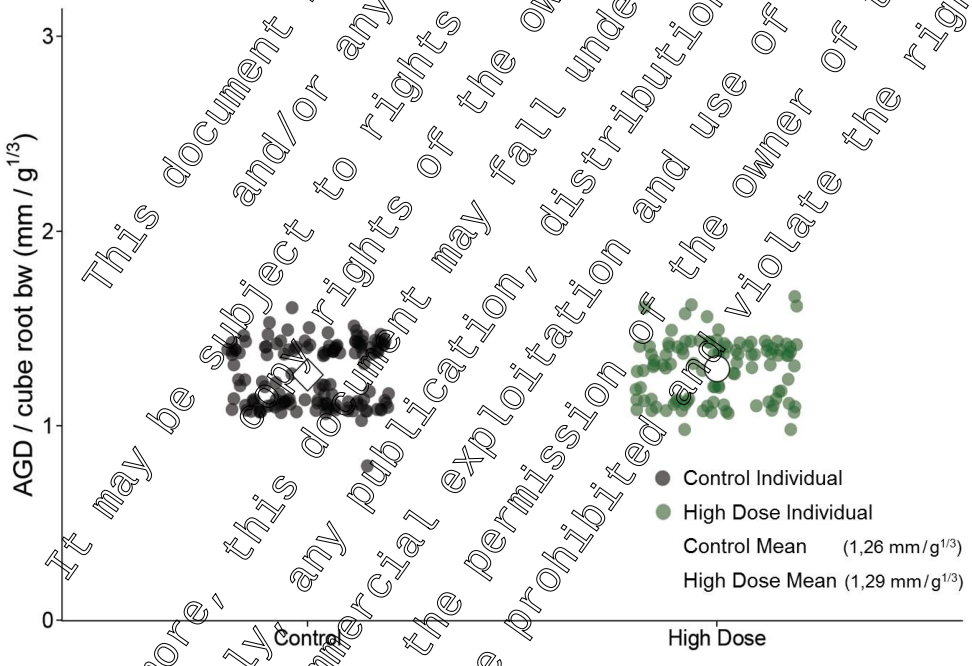


Figure 5.6.1/02- 11: Ratio AGD / cube root of body weight – female F2 pups at day of birth

F. POSTMORTEM EVALUATIONS

1. Sperm analysis (P- and F1-generation)

Sperm morphology and total sperm count were initially evaluated in the control and high-dose groups. Due to statistically significant differences observed on testicular sperm count in the F1-generation, the F1 low and mid dose groups were also analyzed.

Testicular sperm counts in all treated groups in the F1-generation were significantly lower than controls (Table 5.6.1/02- 16). This does not represent a treatment-related effect for the following reasons:

- the absence of dose-response relationship
- the absence of a similar effect on epididymal sperm counts
- the absence of a similar effect on testicular and epididymal sperm counts in the P-generation
- the absence of treatment-related effects recorded on any other sperm parameters (i.e., on sperm morphology and motility)
- the absence of histopathological findings in the testes
- the lack of effect on the reproductive outcome in this two-generation reproductive toxicity study

Furthermore, testicular sperm counts were evaluated in the context of new historical control data (HCD) from the same rat strain and test laboratory as the present study ([redacted], 2015 [M-525951-01-1]). The testicular sperm counts were reported in the present study and in two of the studies of the HCD, which were conducted during the same time period as the present study (1999-2000), as the "total number of sperm in 20 fields examined". Thereafter, this parameter was reported as the "number of sperm per gram testis". However, based on the original raw data from the present study, the original study director recalculated the parameter for the present study also as "number of sperm per gram testis". This recalculation allows a direct comparison between the present study and the historical control data of the studies conducted between 2001 and 2004.

This comparison demonstrates that the historical control values from three further studies are close to or even below the values from the prothioconazole low-, mid- and high-dose groups. This indicates, together with the absence of any dose-response in the prothioconazole low- to high-dose groups, that the prothioconazole concurrent control value was incidentally high. The differences in testicular sperm counts observed in the F1-generation do therefore not demonstrate a treatment-related effect of prothioconazole.

Table 5.6.1/02- 16: Summary of sperm analysis in P and F1 generation animals

Sperm parameter	Prothioconazole (mg/kg bw/d)				Historical control data
	0	10	100	750	
P-Generation					
Motile sperm (%)	87.50	85.00	84.82	83.79	85.14-87.50 [a]
Progressively motile sperm (%)	63.86	60.21	60.82	59.90	55.79-67.6 [a]
Total sperm count – epididymis	163.6	-	-	164.2	115.7-163.6 [a]
Total sperm count – testis (total number in 20 fields)	114.8	-	-	105.3	83.9-114.8 [a]
Morphology - % normal[#]	-	-	-	-	
Morphology - % abnormal[#]	-	-	-	-	
Morphology - % detached[#]	-	-	-	-	

Sperm parameter	Prothioconazole (mg/kg bw/d)				Historical control data
	0	10	100	750	
F1-Generation					
Motile sperm (%)	83.82	84.26	81.15	84.16	83.82-85.7 [a]
Progressively motile sperm (%)	48.00	51.26	50.70	52.00	48.0-52.6 [a]
Total sperm count - epididymis	134.9	-	-	146.4	100.0-134.9 [a]
Total sperm count – testis (total number in 20 fields)	99.6	86.4*	79.8*	85.2*	96.7-121.3 [b]
Total sperm count – testis (sperm/gram)[##]	92.8	81.1	76.8	81.8	64.0-81.3 [c]
Morphology - % normal	97.0	-	-	96.6	92.7-97.0 [a]
Morphology - % abnormal	1.6	-	-	2.0	1.3-2.3 [a]
Morphology - % detached	1.4	-	-	1.4	1.0-6.0 [a]

- not determined; * $p<0.05$ (two sample t-test); value for sperm counts is number of sperm heads counted in 20 microscope fields (automated analysis system)

[#] Morphological examination of sperm samples for the P-generation was not performed due to 'evaporation of the samples'. However, it is unlikely that there would have been compound-related effects on morphology in the P-generation since there were no sperm morphology effects observed in the F1-generation and no compound-related effects on any other sperm parameters in either generation.

[##] recalculated based on the original study raw data

[a] Historical control data 1998-2001 (3 studies, included in original report)

[b] Historical control data 1999-2000 (2 studies) (M-525951-01-1).

[c] Historical control data 2001-2004 (3 studies) (M-525951-01-1).

Findings considered related to treatment with prothioconazole are written in **bold letters**

2. Quantitative ovary evaluation (P- and F1-generation)

In response to the aforementioned abnormalities recorded in oestrous cycles at 750 mg/kg bw/d, which are attributed to the very strong general systemic maternal toxicity at this dose, a quantitative evaluation of the ovaries for pre-antral follicles, antral follicles and corpora lutea was conducted on 10 females per group (controls and high dose for P-generation, all groups for the F1-generation). Females were assigned for this evaluation at random from each group. The number of pre-antral follicles, and to a lesser extent the number of antral follicles, was increased at all dose levels in the F1-generation but without any dose-response relationship (Table 5.6.1.02- 17). By contrast, pre-antral follicles were lower than controls and antral follicles were not affected in the P-generation. Furthermore, all values of pre-antral follicles in the F1-generation were well within the range of historical control data retrieved from the same rat strain and test laboratory as the present study ([redacted], 2015 [M-525951-01-1]). Therefore the F1-generation differences on pre-antral follicles between the concurrent control and the prothioconazole-treated groups are considered to be incidental and not treatment-related.

The number of corpora lutea was significantly lower in the P-generation at 750 mg/kg bw/d, but was not affected in the F1-generation (counts were variable and did not show any relationship to dosage). Based on the inconsistency of these results and based on the overall unaffected reproductive outcome, these changes are not considered to represent a treatment-related effect.

Table 5.6.1/02- 17: Summary of quantitative ovary evaluation (P- and F1-generation)

Ovary parameter	Prothioconazole (mg/kg bw/d)				Historical control data
	0	10	100	750	
P-generation					
Pre-antral follicles	126.8	-	-	99.4	
Antral follicles	95.1	-	-	100.1	
Corpora lutea	62.4	-	-	36.1*	-
F1-generation					
Pre-antral follicles	55.2	76.2	70.5	71.8*	35.9-81.6[a]
Antral follicles	42.5	52.9	54.9	54.0	-
Corpora lutea	28.5	22.2	33.6	22.6	-

- not determined; * p<0.05 (Dunnett's test)

[a] Historical control data from 1999-2004 (5 studies) performed in the same laboratory as the present study (M-525951-01-1).

3. Further necropsy examinations - adults

There were no notable gross necropsy findings in adults.

Terminal body weights were significantly decreased from control in P-generation 750 mg/kg bw/d males and in F1-generation 100 and 750 mg/kg bw/d bw/d males.

Absolute and relative kidney weights were increased in both P and F1 750 mg/kg bw/d males. Similarly, absolute and relative liver weights were increased in 100 and 750 mg/kg bw/d P males, in 750 mg/kg bw/d F1 males, in 750 mg/kg bw/d P females and in 100 and 750 mg/kg bw/d F1 females. Absolute and relative thymus weights were decreased in 100 and 750 mg/kg P females. Absolute and relative thymus weights were also decreased in 750 mg/kg bw/d P and F1 males, but this is related to the strong body weight decrease. Relative pituitary weights were significantly decreased from control in 10 and 750 mg/kg bw/d P females only. Other pituitary weights were not different from control in either sex in either generation. For this reason as well as a lack of dose response, large overlap of one standard deviation ranges and extremely small actual differences between groups (on the order of five ten thousandths of one percent of body weight), this change is considered a chance event. Other changes noted in the tables are considered either chance events or due to the effect of terminal body weights.

Table 5.6.1/02- 18: Absolute and relative organ weights of P-generation adults

Prothioconazole (mg/kg bw/d)		Males				Females			
		0	10	100	750	0	10	100	750
P-Generation									
Terminal bodyweight (g) *(% difference to control)*		441.0	452.0 *(+3)*	448.3 *(+2)*	**402.8*** ***(-9)***	287.2	300.9* *(+5)*	289.6 *(+1)*	289.8 *(+1)*
Adrenal	(g)	0.064	0.066	0.060	0.060	0.101	0.101	0.103	0.095
	(%)	0.015	0.015	0.013	0.015	0.035	0.034	0.035	0.033
Brain	(g)	2.093	2.085	2.074	2.039	1.938	1.937	1.939	1.908
	(%)	0.477	0.464	0.467	0.510*	0.678	0.646	0.672	0.661
Kidney	(g)	2.981	2.907	3.050	**3.126***	2.404	2.540	2.515	2.477
	(%)	0.676	0.645*	0.683	**0.778***	0.835	0.844	0.870	0.853

Prothioconazole (mg/kg bw/d)		Males 0	10	100	750	Females 0	10	100	750
Liver	(g)	15.451	15.946	**16.779***	**17.282***	15.406	17.083	15.943	**18.308****
	(%)	3.509	3.526	**3.750***	**4.297***	5.329	5.677	5.496	**6.306***
Testis/Ovary	(g)	3.631	3.654	3.559	3.554	0.103	0.103	0.107	0.098
	(%)	0.826	0.812	0.798	0.890	0.036	0.034	0.037	0.034
Spleen	(g)	0.714	0.729	0.720	0.675	0.598	0.635	0.586	0.563
	(%)	0.162	0.161	0.162	0.168	0.209	0.211	0.203	0.195
Uterus	(g)	-	-	-	-	0.655	0.560	0.676	0.487
	(%)					0.230	0.187*	0.236	0.168*
Epididymidis	(g)	1.396	1.514	1.481	1.383	-	-	-	-
	(%)	0.317	0.337	0.332	0.345*				
Prostate	(g)	0.959	1.053	1.047	0.958	-		-	
	(%)	0.218	0.233	0.235	0.240				
Cauda Epididymidis (g)		0.320	0.323	0.320	0.314	-			-
	(%)	0.078	0.072	0.072	0.078				
Seminal vesicle	(g)	1.308	1.313	1.487*	1.373	-	-	-	-
	(%)	0.296	0.292	0.334*	0.342*				
Pituitary	(g)	0.010	0.010	0.010	0.009	0.014	0.013	0.013	0.013
	(%)	0.0023	0.0021	0.0022	0.0024	0.0050	0.0043*	0.0046	0.0045*
Thymus	(g)	0.491	0.542	0.466	**0.382**	0.286	0.251	**0.232***	**0.206****
	(%)	0.112	0.119	0.104	**0.095***	0.101	0.084	**0.081***	**0.072***

* p≤0.05, , ** p≤0.01 (for absolute organ weights: Ancova + Dunnetts tests; for terminal body weight and relative organ weights: Anova + Dunnett's tests or Kruskal-Wallis Anova + Mann-Whitney u-tests)
Findings considered related to treatment with prothioconazole are written in **bold letters**

Table 5.6.1/02- 19: Absolute and relative organ weights of F1-generation adults

Prothioconazole (mg/kg bw/d)		Males 0	10	100	750	Females 0	10	100	750
F1-Generation adults									
Terminal bodyweight (g) *(%difference to control)*		428.5	413.8 *(-3)*	**404.3*** *(-6)*	**351.5*** *(-18)*	261.5	272.5 *(+4)*	269.2 *(+3)*	254.3 *(-3)*
Adrenal	(g)	0.064	0.062	0.057	0.058	0.095	0.100	0.102	0.086
	(%)	0.015	0.015	0.014	0.017*	0.036	0.037	0.038	0.034
Brain	(g)	2.030	2.055	2.028	1.968	1.910	1.923	1.911	1.851*
	(%)	0.476	0.501	0.504*	0.565*	0.736	0.711	0.713	0.742
Kidney	(g)	2.835	2.740	2.756	**2.818***	2.266	2.362	2.372	2.169
	(%)	0.662	0.663	0.681	**0.803***	0.867	0.867	0.883	0.854
Liver	(g)	14.926	14.662	14.578*	**15.199***	14.126	14.576	**15.901**	**15.688**
	(%)	3.479	3.525	3.605	**4.317***	5.389	5.326	**5.898**	**6.137***

Prothioconazole (mg/kg bw/d)		Males				Females			
		0	10	100	750	0	10	100	750
Testis/Ovary	(g)	3.662	3.613	3.565	3.560	0.094	0.099	0.106	0.098
	(%)	0.858	0.879	0.884	1.020*	0.036	0.037	0.039	0.040
Spleen	(g)	0.686	0.667	0.658	0.604	0.563	0.578	0.582	0.540
	(%)	0.160	0.161	0.163	0.172*	0.216	0.212	0.217	0.211
Uterus	(g)	-	-	-	-	0.553	0.523	0.552	0.562
	(%)					0.212	0.193	0.205	0.242
Epididymidis	(g)	1.343	1.339	1.298	1.306	-	-	-	-
	(%)	0.315	0.326	0.322	0.375*				
Prostate	(g)	0.800	0.800	0.808	0.785	-	-	-	-
	(%)	0.186	0.195	0.200	0.224*				
Cauda Epididymidis	(g)	0.292	0.285	0.277	0.291	-	-	-	
	(%)	0.068	0.069	0.069	0.083*				
Seminal vesicle	(g)	1.143	1.111	1.234*	1.178*	-	-	-	-
	(%)	0.267	0.269	0.305*	0.335*				
Pituitary	(g)	0.010	0.010	0.009	0.008	0.012	0.013	0.013	0.012
	(%)	0.0023	0.0023	0.0021	0.0024	0.0046	0.0048	0.0050	0.0049
Thymus	(g)	0.508	0.481	0.478	**0.325**	0.249	0.245	0.231	0.205
	(%)	0.118	0.116	0.119	**0.092***	0.097	0.091	0.086	0.082

* p≤0.05, ** p≤0.01 (for absolute organ weights: Ancova + Dunnetts tests; for terminal body weight and relative organ weights: Anova + Dunnett's tests or Kruskal-Wallis Anova + Mann-Whitney-u-tests)
Findings considered related to treatment with prothioconazole are written in **bold letters**

Histopathological examinations revealed increased incidences of hepatocytomegaly and of multifocal cortical nephrosis in males and in females of both generations at 750 mg/kg bw/d.

Table 5.6.1/02- 20: Notable findings of histopathological examinations of P- and F1-generation adults

Prothioconazole (mg/kg bw/d)	Males				Females			
	0	10	100	750	0	10	100	750
P-Generation adults								
Kidney, # tissues examined	30	1	30	30	30	0	30	30
- nephrosis	-	-	-	**27***	-	-	-	**4**
(mean severity)				**(1.8)**				**(1.0)**
Liver, # tissues examined	30	0	30	30	30	0	30	30
- hepatocytomegaly	1	-	5	**28***	-	-	-	**4**
(mean severity)	(1.0)		(1.0)	**(2.6)**				**(2.0)**
F1-Generation adults								
Kidney, # tissues examined	30	2	30	30	30	1	30	30
- nephrosis	-	-	-	**30***	1	-	-	**6**
(mean severity)				**(1.9)**				**(1.0)**

Prothioconazole (mg/kg bw/d)	Males				Females			
	0	10	100	750	0	10	100	750
Liver, # tissues examined	30	0	30	30	30	0	30	30
- hepatocytomegaly	3	-	8	**27***	1		4	**20***
(mean severity)	(1.7)		(1.0)	**(2.9)**	(1.0)		(1.3)	**(1.6)**

( ) average severity of animals with lesion: 1 (minimal) to 5 (severe).
* significantly different from control (p≤0.05)
\- no incidence

4. Further necropsy examinations - offspring

F1 pups not selected to become parents of the next generation and all F2 pups were sacrificed on lactation day 21 and examined macroscopically. Brain, spleen, and thymic tissues were weighed from one male and one female per litter, to the extent possible.

Terminal body weights were significantly decreased from control in 750 mg/kg bw/d pups of both sexes.

There were no notable gross necropsy findings in pups. In male and female pups (both F1 and F2), reduced spleen weights were recorded at 750 mg/kg bw/d. Absolute brain weights from both generations were normal, but relative weight in 750 mg/kg bw/d pups of both sexes, both generations, were significantly increased over control. This was an effect of the decreased body weight noted above and was not treatment related. Thymus weights were not remarkable in the F1 pups. Absolute thymus weights were significantly decreased in the 750 mg/kg F2 male pups only. Relative thymus weights were not different from control. As the absolute change was not confirmed by relative changes or by changes in the females, it is not considered treatment related.

There were no notable histopathological changes observed in pups at any dose level.

Table 5.6.1/02- 21: Absolute and relative organ weights of F1- and F2-generation pups

Prothioconazole (mg/kg bw/d)		Males				Females			
		0	10	100	750	0	10	100	750
F1-pups									
Mean bodyweight on lactation day 21 (g) *(% difference to control)*		45.8	46.5 *(+2)*	43.8 *(-4)*	**38.7**** ***(-16)***	43.5	44.5 *(+2)*	41.8 *(-4)*	**37.5**** ***(-14)***
Brain	(g)	1.447	1.473	1.447	1.418	1.407	1.422	1.410	1.371
	(%)	3.188	3.174	3.370	3.723**	3.216	3.194	3.384	3.659**
Thymus	(g)	0.210	0.211	0.208	0.189	0.208	0.220	0.208	0.190
	(%)	0.459	0.451	0.479	0.487	0.472	0.493	0.496	0.498
Spleen	(g)	0.217	0.232	0.204	**0.168****	0.211	0.214	0.195	**0.163****
	(%)	0.471	0.495	0.467	**0.433**	0.478	0.478	0.464	**0.426***
F2-pups									
Terminal bodyweight (g) *(% difference to control)*		44.4	45.7 *(+3)*	45.3 *(+2)*	**40.4*** ***(-9)***	42.6	43.5 *(+2)*	43.2 *(+1)*	**39.6** ***(-7)***
Brain	(g)	1.468	1.498	1.496	1.457	1.415	1.428	1.445	1.420
	(%)	3.329	3.339	3.373	3.686**	3.333	3.283	3.391	3.604*
Thymus	(g)	0.209	0.204	0.215	0.181*	0.209	0.202	0.206	0.187
	(%)	0.469	0.449	0.481	0.450	0.486	0.459	0.480	0.469

Prothioconazole (mg/kg bw/d)		Males				Females			
		0	10	100	750	0	10	100	750
Spleen	(g)	0.216	0.220	0.208	**0.175****	0.223	0.213	0.215	**0.174****
	(%)	0.484	0.481	0.462	**0.431**	0.520	0.485	0.500	**0.432****

* p≤0.05, , ** p≤0.01 (Dunnett's test)
Findings considered related to treatment with prothioconazole are written in **bold letters**

III. CONCLUSION

There was evidence of parental general systemic toxicity at the intermediate and high dose levels. Effects at 100 mg/kg bw/d included slightly lower bodyweight gains in males (F1), decreased thymus weights in females (P and F1) and increased liver weights in both sexes (P and F1). At 750 mg/kg bw/d similar but more marked effects were recorded, along with clinical observations such as urine stain, dehydration and salivation prior to dosing, reduced efficiency of food utilisation (both sexes), lower bodyweight gains during gestation, increased kidney weights (males) and histopathological findings in the liver (hepatocytomegaly in both sexes) and kidneys (multifocal cortical nephrosis in both sexes). The parental (particularly, the maternal) toxicity at 750 mg/kg bw/d is considered to be very high, even sublethal, based primarily on the kidney dysfunction and resulting dehydration. Based on slight bodyweight effects and organ weight changes at 100 mg/kg bw/d the NOAEL for parental toxicity is 10 mg/kg bw/d.

Reproductive performance was not affected by treatment. The NOAEL for reproductive effects was 100 mg/kg bw/d, based on affected oestrous cycling, slight reductions in implantation sites and litter size, increased time to insemination and minimally increased duration of gestation at 750 mg/kg bw/d. These mild effects were recorded at a dose level which also gave rise to marked general systemic parental toxicity, hence a specific effect on the reproductive systems is not indicated.

The only effects on pups were recorded at the high dose level of 750 mg/kg bw/d and included retarded pup weight gain, reduced pup spleen weights and slightly delayed preputial separation, which is considered secondary to the clearly retarded growth. These effects on pups were recorded at a dosage giving rise to marked general parental toxicity as mentioned above, hence a specific developmental toxicity effect is not indicated. The NOAEL for effects on offspring is 100 mg/kg bw/d.

CA 5.6.2 Developmental toxicity studies

Report:	KCA 5.6.2/02 [redacted].; 1997; M-012279-01-1
Title:	JAU 6476 - Developmental toxicity study in rats after oral administration
Report No.:	25827
Document No.:	M-012279-01-1
Guideline(s):	OECD 414 (1981); US-EPA Series 83-3, (1984); JMAFF (1984); US-EPA 712-C-96-207, OPPTS 870.3700 (1996); Directive 88/302/EEC (1988)
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.6.2/02. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.6.2/01** (also in numbering of tables/figures).

Deviations:	Deviations from the current OECD guideline (2001): Feed consumption was recorded in five-day intervals instead of three-day intervals. However this does not affect the overall acceptability of the study.

Executive summary:

Prothioconazole (batch: NLL 6096-4, purity: 99.5–99.8 %) in 0.5 % aqueous carboxymethylcellulose was administered to groups of 26 inseminated female Wistar (Hsd Cpb:WU) rats by gavage during gestation days 6 – 19 at daily dose levels of 0, 80, 500 and 1000 mg/kg bw/d. The study was compliant with or exceeded the requirements of the contemporary OECD Guideline 414 (1981). Dosing was extended from day 15 to day 19 in anticipation of changes to US-EPA Guidelines for this type of study (which brings the dosing pattern in this study in line with the 2001 OECD Guideline 414).

Maternal effects included increased urine excretion at 500 and 1000 mg/kg bw/d, strongly increased water consumption (up to 131 % of control) at 500 mg/kg bw/d and drastically increased water consumption (up to 175 % of control) at 1000 mg/kg bw/d throughout the treatment period. Feed consumption was decreased at 1000 mg/kg bw/d and decreased bodyweight gains were recorded at 500 and 1000 mg/kg bw/d, with animals at 1000 mg/kg bw/d showing transient bodyweight loss. Clinical laboratory tests revealed at 500 mg/kg bw/d and above slightly decreased AST, slightly increased cholesterol, and slightly decreased T4. At 1000 mg/kg bw/d, ALT and ALP were slightly increased. At necropsy, relative liver weights were slightly increased at 1000 mg/kg bw/d. It can be concluded that 1000 mg/kg bw/d induced very strong (sublethal) maternal toxicity related to kidney dysfunction and resulting dehydration with clear effects already starting at 500 mg/kg bw/d. Severe disturbance of the kidney function and systemic water / electrolyte homeostasis is a consistent finding in all toxicity studies in the rat and appears to be the characteristic toxicity of prothioconazole with cases of death at 1000 mg/kg bw/d in pregnant dams of another Wistar substrain, at 500 mg/kg bw/d in a 90-day study, at 750 mg/kg bw/d in the 1-year study, and a high mortality rate in the 2-year study at 500-750 mg/kg bw/d, all conducted with rats of the same Wistar substrain as in the present study.

Developmental effects occurred at 1000 mg/kg bw/d and included engorged placentae, retarded fetal development (decreased fetal weight, incomplete ossification, dilatation of the renal pelvis), and increased incidences of the common spontaneous variation rudimentary 14th lumbar ribs and of the (in the present rat strain) common spontaneous malformation microphthalmia. All these developmental effects are seen as secondary unspecific consequences of the observed very strong (sublethal) maternal toxicity and not as indications for a direct, specific developmental or teratogenic potential of prothioconazole. This assessment is supported by the following facts:

- In the present study, the severity of maternal toxicity at 1000 mg/kg bw/d correlates positively with the degree of fetal toxicity (body weight decrease) and with the occurrence of microphthalmia.

- For the same rat strain (in which, as already mentioned, microphthalmia is a common spontaneous malformation) and test laboratory as used in the present study, an INHALATIVE developmental toxicity study with exposure to a sensory irritating compound caused, secondarily to refrectory induced maternal hypoventilation / hypoxia, a retarded fetal development together with an increased incidence of the common spontaneous malformation microphthalmia (at even higher fetal / litter incidences than in the present study). The test substance used in this study did not cause any microphthalmia at an approx. 10-fold higher ORAL dose, excluding a specific teratogenic potential of this compound.
- A follow-up study to the present study (using sophisticated objective fetal eye measurements) was conducted as an authority requirement in the original Annex I listing process and demonstrated the absence of microphthalmia in a different Wistar rat substrain (in which microphthalmia is NOT a common spontaneous malformation). This result verifies the original hypothesis of an indirect unspecific effect (secondary to maternal toxicity) by demonstrating the absence of a direct specific effect (the rat strain was sensitive to the development of ocular malformations including microphthalmia caused by a positive control substance).

Incidences of rudimentary 14th ribs were statistically significantly increased in all treated groups compared to concurrent controls. In the historical control context, concurrent control incidences were unusually low (second-lowest in 53 studies conducted in the same rat strain and test laboratory). Incidences of treated groups were within historical control ranges up to and including 500 mg/kg bw/d and exceeded historical control ranges only slightly at 1000 mg/kg bw/d. Thus, the study director of the present study set the NOAEL for this finding at 500 mg/kg bw/d. This plausible NOAEL would also be supported by a retrospective comparison of the respective maternal and developmental results from the present study with those of the aforementioned follow-up study. However, as a very conservative approach, in the present study a NOAEL for supernumerary 14th ribs is set at 80 mg/kg bw/d. This is supported by respective benchmark dose calculations resulting in $BMDL_{10}$ values of >300 mg/kg bw/d.

Based on these results and considerations in can be concluded:

The NOAEL for maternal toxicity is 80 mg/kg bw/d, based on strong toxicity at 500 mg/kg bw/d and very strong (sublethal) toxicity at 1000 mg/kg bw/d. Severe disturbance of kidney function and systemic water / electrolyte homeostasis appears to be the characteristic toxicity of prothioconazole

A plausible NOAEL for developmental toxicity could be set at 500 mg/kg bw/d.

As a very conservative approach, the NOAEL for developmental toxicity is set at 80 mg/kg bw/d, based on incidences of rudimentary 14th ribs which were increased compared to concurrent controls at 500 mg/kg bw/d, but are still within the historical control range. Respective benchmark dose calculations confirm $BMDL_{10}$ values of >300 mg/kg bw/d.

At 1000 mg/kg bw/d an increased incidence of engorged placentas, retarded fetal development (reduced fetal weights, delayed ossification and renal pelvis dilatation) and increased incidences of the in the present rat strain common spontaneous malformation microphthalmia were observed.

All observed developmental effects are considered as unspecific enhancements secondary to strong (at 500 mg/kg bw/d) or very strong (sublethal) (at 1000 mg/kg bw/d) maternal toxicity. Therefore, these findings are no indications for a specific or direct developmental toxic or teratogenic potential of prothioconazole. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, such a constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. Specifically, the Guidance states: *"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species."*

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476
Description:	Solid, white powder
Batch No.:	NLL 6096-4
Purity:	99.5 – 99.8 %
CAS No.:	Not reported
Stability of the test compound:	The stability was guaranteed for the duration of the study.
2. Vehicle:	0.5 % carboxymethylcellulose suspension in demineralized water
3. Test animals:	
Species:	Rat
Strain:	Wistar (Hsd Cpb:WU)
Sex:	Males and females (nulliparous and not pregnant) (Males were only used for mating)
Weight at dosing:	199 – 258 g (females)
Source:	[redacted], Germany
Acclimation period:	At least 7 days before mating
Diet:	Standard rat diet Altromin® 1324 ([redacted], Germany), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in Type II Makrolon® cages on low-dust wood shavings supplied [redacted], Germany. The males were kept individually in Type III Makrolon® cages.
Environmental conditions:	
Temperature:	22.0 ± 3.0°C
Humidity:	Approx. 50 %
Air changes:	At least 10 times per hour
Photo period:	Artificial illumination, 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: January 17, 1996 - March 19, 1996

2. Animal assignment and treatment

Mating and start of gestation

The animals were mated by placing two females overnight into a Type III cage together with one male rat. If sperm was detected in the vaginal smear taken on the morning following mating, this day was regarded as day 0 of gestation.

Dose levels, experimental group and rationale for choice of dose levels

The male animals were used for mating only and were not treated. After insemination was ascertained, 26 females each were allocated to four experimental groups according to a computer-generated randomization plan.

The animals were treated daily from day 6 to 19 *post coitum* (p.c.). The animals were given the administration formulations orally by gavage. Gavage was selected since oral exposure is a likely route of exposure for humans.

The following doses (related to the test compound) were administered:

Table 5.6.2/01- 1: Study design

	Control	Group 1	Group 2	Group 3
Number of dams	26	26	26	26
Concentration (mg/L)	0	8	50	100
Dose (mg/kg body weight)	0	80	500	1000

The animals of all experimental groups received a uniform volume of 10 ml/kg body weight. The animals of the control group received vehicle only (0.5 % carboxymethylcellulose, CMC) at the same volume.

The dose levels used were selected based on a previously conducted developmental toxicity dose range finding study (BAYER AG, Study No. T2058009, [M-012330-01-1]) with the following dose levels: 0/100/300/1000 mg/kg bw/d.

3. Test substance preparation and analysis

For treatment of the animals, administration formulations were prepared using a 0.5 % carboxy-methylcellulose suspension in demineralized water, a vehicle which has no effect on the parameters investigated. The administration formulations were stored for the duration of their use at room temperature.

Investigations on the stability of the active ingredient in samples of 1.0 mg/ml and 100.0 mg/ml (dose volume 10 ml/kg bw) covering the range of concentrations used in this study revealed no significant deviations after 7-day storage from the content determined on the day of preparation (Table 5.6.2/01-2). The homogeneity of the administration formulations at the 1.0 mg/ml and the 100.0 mg/ml concentration also complied (Table 5.6.2/01- 3).

Table 5.6.2/01- 2: Analysis of preparations for stability (in (%) of target concentration and actual weight units)

Time	Target concentration (mg/mL) 1		100	
0 h (=start)	**86.1 %**	(0.86 mg/mL)	**85.5 %**	(85.51 mg/mL)
Day 7	**107.6 %**[a]	(0.93 mg/L)	**114.0 %**[a]	(97.45 mg/mL)

[a]: calculation (%) of target concentrations based on the analytical result on 0 h.

Table 5.6.2/01- 3: Analysis of preparations for homogeneity

Sampling location	Target concentration (mg/mL) 1	100
top; 1st inj.	0.86	87.95
top; 2nd inj.	0.84	87.02
middle; 1st inj.	0.87	78.97
middle; 2nd inj.	0.86	77.86

Sampling location	Target concentration (mg/mL) 1	100
bottom; 1st inj.	0.88	91.10
bottom; 2nd inj.	0.87	90.18
Mean:	**0.86 mg/mL** c_V **= 1.6 %**	**85.51 mg/mL** c_V **= 6.7 %**

A content check of the formulations of all concentrations was carried out during the in life period of the study in week 2 and week 5 after initiation of treatment of the first animals. The results revealed no significant deviation (±20 %) of the active ingredient content from the nominal value in the formulations in any of the three treatment groups.

Table 5.6.2/01- 4: Analysis of preparations for test-item content (in (%) of target concentration and actual weight units)

Sampling location	8 mg/mL	50 mg/mL	100 mg/mL
Week 2	**96.5 %** (7.72 mg/mL)	**86.7 %** (43.33 mg/mL)	**102.3 %** (102.29 mg/mL)
Week 5	**110.7 %** (8.86 mg/mL)	**98.4 %** (49.19 mg/mL)	**95.3 %** (95.25 mg/mL)

The values are means of two injections from two individual samples for each concentration.

4. Statistics

Animals without implantation sites were excluded from statistical evaluation. Animals with total resorption were not taken into account for calculation of group mean values of body weights, body weight gains, feed intakes, water intakes and organ weights.

The mean values in the tables calculated by computer are the rounded results of the calculations with non-rounded raw data.

Differences between the control and prothioconazole-treated groups were considered significant when $p < 0.05$. Statistical significance was tested using the following methods:

Statistical test	Parameter
Analysis of Variance (ANOVA) (and in case of significant results Dunnett's t-Test) as posthoc test for:	- feed consumption - water consumption - body weights and body weight gains - liver weights - uterus weights - corrected body weight gains - number of corpora lutea per dam - number of implantations per dam - number of live fetuses per dam and as % of implantantions per dam - placental weights - fetal weights
CHI² test (correction according to Yates) for:	- fertility rate - gestation rate - number of fetuses or litters with malformations

Statistical test	Parameter
2 by N CHI² test; in case of significant differences Fisher's exact test with Bonferroni correction for:	- number of implantations per group - number of preimplantation losses per group - number of postimplantation losses, early resorptions, late resorptions or dead fetuses per group - number of live fetuses per group in percent of implantations - number of male or female fetuses of fetuses with undeterminable sex per group - number of fetuses or litters with skeletal findings
Kruskall-Wallis test (and in case of significant differences Dunn's test) for:	- number of preimplantation losses per dam - number of postimplantation losses, early resorptions, late resorptions or dead fetuses per dam - number of male or female fetuses of fetuses with undeterminable sex per dam

For evaluation of the results of clinical chemistry the following tests were used:

Statistical test	Parameter
Analysis of Variance followed by **Dunnett's Test**:	- TRIGL (serum)
Adjusted Welsh Test:	- ALAT - APh - ASAT - CHOL - GLDH - TRIGL (liver)
Kruskal-Wallis Test followed by adjusted U Test:	- T3 - T4 - TSH

C. METHODS

1. Observations

From day 0 to 20 p.c. all animals were inspected twice daily - only once daily on weekends and holidays - and all findings were recorded. Attention was paid to disturbances in the general condition of the rats (appearance, behavior), and any alterations concerning their excretory products.

2. Feed and water consumption

The feed consumption of the animals on gestation days 0-6, 6-11, 11-16 and 16-20 was determined based on the differences in weight of feed provided and feed which remained unconsumed. Water consumption was determined during the same intervals (days 0-6, 6-11, 11-16 and 16-20) based on the differences in weight of the filled water bottles compared to the weight of the bottles with the water which remained unconsumed.

3. Body weight development

The body weights of the animals were determined on day 0 p.c. and daily from day 6 to 20 p.c. Corrected body weight gain was calculated by subtracting the weight of the uterus on day 20 p.c. from the body weight gain over the period from day 0 to day 20 p.c..

4. Clinical Laboratory Tests

The number of animals undergoing clinical laboratory tests is given in the following table.

Table 5.6.2/01- 5: Number of females assigned for clinical laboratory tests

	Prothioconazole (mg/kg bw/d)			
	0	**80**	**500**	**1000**
Total number of females with viable fetuses	26	26	22	24
Number of females for clinical laboratory tests	18	20	22	21

At cesarean section blood samples were collected by cardiac puncture and plasma or serum prepared for determination of the following enzymes, metabolites, proteins and hormones.

Clinical Chemistry		
Enzymes	*Metabolites and Proteins*	*Hormones*
Alanine aminotransferase (ALT)	Cholesterol (CHOL)	Thyroid stimulating hormone (TSH)
Aspartate aminotransferase (AST)	Triglycerides (TRIGL)	Triiodothyronin (T3)
Alkaline phosphatase (ALP)		Thyroxine (T4)
Glutamate dehydrogenase (GLDH)		

At cesarean section liver tissue was collected for the determination of triglycerides. The livers were frozen at approximately -20°C until analyses were performed.

5. Investigations at Cesarean Section

On gestation day (GD) 20, the dams were sacrificed using cardiotomy under deep carbon dioxide anesthesia. The following parameters were determined and assessed at cesarean section:

- Number of corpora lutea
- Number of implantations
- Uterus weight
- Individual weight and appearance of the placentas
- Number of (early/late) resorptions
- Number of live fetuses
- Sex of live fetuses
- Individual weights of fetuses
- External malformations and other findings deviating from normal
- Visceral malformations and other findings deviating from normal (evaluation of about half of the fetuses by razor blade sectioning according to the modified WILSON technique)
- Findings in abdominal, pelvic and thoracic organs as well as skeletal and cartilage findings by the DAWSON technique modified by the addition of cartilage staining (method described by Inouye, modified): evisceration, cartilage staining with alcian blue GX, clearing of the fetuses with diluted potassium hydroxide solution, staining of the skeletal system with alizarin red S and evaluation of the cartilage and skeletal system

Table 5.6.2/01- 6: Number of fetuses examined by Wilson and Dawson technique

	Prothioconazole (mg/kg bw/d)			
	0	80	500	1000
Total number of fetuses	290	292	270	282
Fetuses investigated according to				
mod. WILSON	138	137	128	135
mod. DAWSON	152	155	142	147

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSIS

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

No mortality occurred up to and including 1000 mg/kg bw/d.

2. Clinical signs of toxicity

Increased urine excretion and increased water consumption was observed at the two highest dose levels, starting as early as one day after initiation of treatment and lasting up to cesarean section.

Table 5.6.2/01- 7: Clinical observations during gestation

	Prothioconazole (mg/kg bw/d)			
	0	80	500	1000
Number of dams examined	26	26	26	26
Increased urination (n)	0	1	**14**	**21**
Visually increased water consumption (n)	0	0	**8**	**20**

Findings considered related to treatment with prothioconazole are written in **bold letters**

C. FEED AND WATER CONSUMPTION, BODY WEIGHT AND BODY WEIGHT GAIN

1. Feed and water consumption

As shown in Table 5.6.2/01- 8, feed consumption in females with viable fetuses was significantly decreased during day 6 to 11 p.c. in the 1000 mg/kg dose group. No other treatment-related effects were observed concerning feed consumption at other dose levels or time points evaluated.

Water consumption in females with viable fetuses was dose-dependently increased (statistically significant) during the entire treatment period at the 500 and 1000 mg/kg bw/d dose levels (consistent with the observed clinical signs). This effect is an unusual finding in developmental toxicity studies, was strongly expressed at 500 mg/kg bw/d (up to 131 % of control) and was drastically expressed at 1000 mg/kg bw/d (up to 175 % of control). No such effect was observed at 80 mg/kg bw/d.

Table 5.6.2/01- 8: Feed and water consumption during gestation

	Prothioconazole (mg/kg bw/d)							
	0	**80**		**500**		**1000**		
Mean Feed Consumption (g/animal/d) *(% difference to control)*								
Day 0 – 6	20.1	20.5	*(+2)*	19.8	*(-2)*	20.4	*(+2)*	
Day 6 - 11	18.6	19.3	*(+4)*	17.8	*(-4)*	**15.4****	***(-17)***	
Day 11 - 16	21.6	21.7	*(+1)*	21.3	*(-1)*	20.7	*(-4)*	
Day 16 - 20	22.7	22.5	*(-1)*	22.8	*(±0)*	23.1	*(+2)*	
Mean Water Consumption (g/animal/d) *(% difference to control)*								
Day 0 – 6	26.5	26.8	*(+1)*	26.7	*(+1)*	26.1	*(-2)*	
Day 6 - 11	27.0	27.3	*(+1)*	**33.2***	***(+23)***	**47.3****	***(+75)***	
Day 11 - 16	30.5	31.6	*(+4)*	**38.6****	***(+27)***	**49.9****	***(+64)***	
Day 16 - 20	33.3	34.2	*(+3)*	**43.6****	***(+31)***	**52.8****	***(+59)***	

* significantly different from control, $p \leq 0.05$
** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

2. Body weight and body weight gain

Transient body weight loss was observed during days 6 to 8 p.c. at 1000 mg/kg bw/d. Mean maternal body weight gain was significantly decreased during days 10 to 11 p.c. at 500 and 1000 mg/kg bw/d as well as during days 6 to 7 p.c. at 500 mg/kg bw/d.

Cumulative body weight gain (day 6-19 and 0-20 p.c.) was also slightly decreased in the 1000 mg/kg dose group (see Table 5.6.2/01- 9). Corrected body weight gain was statistically significantly and dose-dependently decreased up to -31 % at 500 and 1000 mg/kg bw/d.

No effect on body weight of the females was observed at 80 mg/kg bw/d.

Table 5.6.2/01- 9: Maternal body weight gain (g)

	Prothioconazole (mg/kg bw/d)			
	0	**80**	**500**	**1000**
Day 6 – 7	2.8	1.1	**0.5***	**-0.9***
Day 7 – 8	2.4	2.7	1.9	**-1.9****
Day 8 – 9	1.0	2.7	1.6	2.8
Day 9 – 10	4.2	3.3	3.5	3.4
Day 10 – 11	5.3	3.8	**3.1***	**3.3***
Day 11 – 12	3.0	3.5	3.5	4.6
Day 12 – 13	2.1	1.7	1.9	1.2
Day 13 - 14	4.3	4.6	4.3	2.8
Day 14 - 15	5.4	4.9	5.4	6.8
Day 15 - 16	7.3	7.8	7.4	7.7
Day 16 - 17	10.2	10.2	11.4	10.0
Day 17 – 18	13.3	11.7	13.5	11.6
Day 18 - 19	11.7	13.2	12.2	13.4
Day 19 - 20	6.6	6.2	7.3	7.7

	Prothioconazole (mg/kg bw/d)							
	0	**80**		**500**		**1000**		
Cumulative body weight gain (g) *(% difference to control)*								
Day 6 - 19	72.8	71.2	*(-2)*	70.4	*(-3)*	**64.8**	*(-11)*	
Day 0 - 20	102.0	101.7	*(±0)*	99.4	*(-3)*	**92.6**	*(-9)*	
Day 0 – 20 (corrected [a])	40.0	39.4	*(-2)*	**31.5***	***(-21)***	**27.6****	***(-31)***	

[a] for uterus weight
* significantly different from control, $p \leq 0.05$
** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

D. CLINICAL LABORATORY TESTS

Clinical chemistry results are presented in Table 5.6.2/01- 10. At 500 mg/kg bw/d and above, AST was slightly decreased, cholesterol was slightly increased (both without dose-response relationship) and T4 was slightly decreased. At 1000 mg/kg bw/d, ALT and ALP were slightly increased.

Table 5.6.2/01- 10: Summary of notable clinical chemistry results

Parameter	Prothioconazole (mg/kg bw/d)			
	0	**80**	**500**	**1000**
AST (U/L)	37.5	38.9	**32.8****	**34.0***
ALT (U/L)	40.7	43.0	42.7	**47.6****
ALP (U/L)	101	99	105	**134****
GLDH (U/L)	3.3	2.8	2.4	2.9
TRIGL (Blood) (mmol/L)	4.96	5.12	6.07	5.73
Cholesterol (mmol/L)	2.25	2.16	**2.67****	**2.61****
T4 (nmol/L)	28	26	**22***	**20****
T3 (nmol/L)	1.58	1.51	1.48	1.42
TSH (mcg/L)	3.17	3.43	3.71	3.53
TRIGL (Liver) (mcmol/g)	8.17	7.71	7.42	7.14

* significantly different from control, $p \leq 0.05$
** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

E. NECROPSY OBSERVATIONS

At necropsy, relative liver weights were significantly increased at 1000 mg/kg bw/d, but the magnitude of the increase was small (mean increase in relative weight 6 % higher than controls). The only notable gross necropsy finding was a single animal at 1000 mg/kg bw/d with material deposited in the ureter and urinary bladder along with multiple white areas in the kidneys (confirmed microscopically as urolithiasis with urothelial hyperplasia and hydronephrosis with pyelonephritis and transitional cell hyperplasia). Histopathological examination of the liver, thyroid, adrenals and gross abnormalities did not reveal any other notable findings.

Table 5.6.2/01- 11: Absolute and relative mean liver weights

Parameter	Prothioconazole (mg/kg bw/d)			
	0	**80**	**500**	**1000**
Liver weight (g)	11.6	11.9	11.7	12.2
Liver weight/ carcass weight ratio	0.0464	0.0470	0.0482	**0.0501****

** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

Maternal toxicity

At 500 mg/kg bw/d dams showed reduced bodyweight gains, and strongly increased water consumption (up to 131 % of control) and urination. At 1000 mg/kg bw/d effects aggravated with transient body weight loss and drastically increased water intake (up to 175 % of control), indicating kidney dysfunction and resulting dehydration. In the context of results from the rat subacute and subchronic toxicity studies, maternal toxicity was primarily related to severe disturbances of the kidney function and systemic water and electrolyte homeostasis. This is a consistent finding in all toxicity studies in the rat and appears to be the characteristic toxicity of prothioconazole. In two rat 28-day studies strongly increased water intake was observed at 1000 mg/kg bw/d. In a 90-day study conducted in Wistar rats of the same substrain as used in the present study (Hsd.Cpb:WU) at 500 mg/kg bw/d males and females exhibited strongly increased water intake, kidney damage and even a single case of death in one female in week 13 (possibly related to kidney failure). The renal "corticular tubular basophilia" observed in rat short term studies is assessed as a reactive regenerative response to a toxic insult. In the rat 1 year and oncogenicity studies, this lead to an increased incidence of "chronic progressive nephropathy" which even caused in the 1-year study an increased mortality at 750 mg/kg bw/d, and a high mortality rate in the 2-year study at 500-750 mg/kg bw/d, and also was the main endpoint for establishing the proposed ADI of 0.05 mg/kg bw (NOAEL: 5 mg/kg, LOAEL: 50 mg/kg). A further indicator that a strongly maternally toxic dose had been reached in the present study at 1000 mg/kg is the clear effect on body weights which already started at 500 mg/kg. In another Wistar rat substrain, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25 % mortality or, at 750 mg/kg bw/d, could not be fully compensated by a drastically increased (up to > 170 % of control) water consumption (as determined in the pilot developmental toxicity study or the second main developmental toxicity study (see 5.6.2/03, M-067839-01-1)).

In the present study water intake was also drastically increased (up to 175 % of control) in the high dose, thus it can be concluded that the severity of maternal toxicity is comparable in both developmental toxicity studies. The degree of maternal toxicity at the respective highest tested doses of both studies is considered very strong, even sublethal.

The occurrence of distinct maternal toxicity also has an impact on warranty of classification for reproductive effects. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, this constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. Specifically, the Guidance states: "*Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species.*"

F. CAESAREAN SECTION DATA

The pregnancy incidences and the mean numbers of corpora lutea and implantations were comparable across all test and control groups. Pre-implantation and post-implantation losses, live litter size, placental weight and fetal sex ratios were unaffected by treatment at all dose levels (Table 5.6.2/01- 12). The

incidence of engorged placentae was increased (without statistical significance), relative to the controls, at all dose levels. However the fetal incidences at 80 and 500 mg/kg bw/d were less than the upper incidence limit of 3.43 % in the historical control data, and there was no corresponding effect on placental weight in any group. An effect of treatment is considered to have occurred only at 1000 mg/kg bw/d since this incidence was outside the historical control range. Fetal weights of both sexes at 1000 mg/kg bw/d were slightly, but significantly reduced.

Table 5.6.2/01- 12: Summary of reproductive data

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**80**	**500**	**1000**	**HCD[a]**
No. pregnant / no. mated	26 / 26	26 / 26	22 / 26	25 / 26	-
No. dams with live fetuses	26	26	22	24[b]	138
Mean no. corpora lutea/dam	15.0	14.2	15.0	14.4	13.0 – 15.3
Mean no. implantation sites/dam	12.0	12.2	12.7	12.8	10.2 – 12.5
Pre-implantation loss (mean no. / dam)	3.0	2.0	2.3	1.7	1.5 – 3.9
Pre-implantation loss (% of corpora lutea)	20.1	14.1	15.4	11.6	11.1 – 25.1
Mean no. resorptions [c]/ dam	0.8	1.0	0.5	1.0	0.5 – 1.4
No. dead fetuses	0	0	0	0	-
Post-implantation loss (mean no. / dam)	0.8	1.0	0.5	1.0	-
Mean no. live fetuses/dam	11.2	11.2	12.3	11.3	9.5 – 11.8
Sex ratio (% males)	57.4	50.8	48.2	49.9	49.0 – 56.1
Mean fetal weight of both sexes combined (g)	3.63	3.57	3.57	**3.45****	3.60 – 3.84
Mean fetal weight of males (g)	3.70	3.64	3.69	**3.53***	**-**
Mean fetal weight of females (g)	3.53	3.49	3.46	**3.38***	**-**
Engorged placentae (% fetal incidence)	0.7	1.0	1.9	**4.3**	0.32 – 3.43
Placental weight (g) (± SD)	0.62 (± 0.052)	0.62 (± 0.069)	0.60 (± 0.075)	0.62 (± 0.082)	0.59 – 0.66

[a] Historical control data range 1993-1994 as included in original report (6 studies, 138 litters, 1470 fetuses)
[b] one dam had one resorbed implantation site only and was excluded from calculation of mean value
[c] only late resorptions occurred
* significantly different from control, $p \leq 0.05$ (Dunnett's test)
** significantly different from control, $p \leq 0.01$ (Dunnett's test)
Findings considered related to treatment with prothioconazole are written in **bold letters**

G. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

There was an apparent increase in the incidence (both fetal incidence and litter incidence) of microphthalmia in treated groups compared to the control, however the incidences at 80 and 500 mg/kg bw/d were not dose related (Table 5.6.2/01- 13) and covered by historical controls.

Table 5.6.2/01- 13: Incidence of microphthalmia (Fetal (litter) incidence in %)

Parameter	Prothioconazole (mg/kg bw/d)				
	0	80	500	1000	HCD[b]
External examination					
No. litters evaluated (external)	26	26	22	24	
No. fetuses evaluated (external)	290	292	270	282	-
Microphthalmia	0 (0.0)	0.3 (3.8)	0 (0.0)	**0.7 (4.2)**	
Eye rudiment flat	0 (0.0)	2.1 (11.5)	0.4 (4.5)	2.1 (20.8)	-
Visceral examination					
No. litters evaluated (visceral)	26	25	22	24	-
No. fetuses evaluated (visceral)	138	137	128	135	
Microphthalmia (Wilson's technique)	0 (0.0)	4.4* (16.0)	2.3 (13.6)	**8.1**** **(33.3)****	-
Microphthalmia, all fetuses [a]	0 (0.0)	2.4 (15.4)	1.1 (13.6)	**4.6 (33.3)**	0 – 1.95 (0 – 20)

* $p \leq 0.05$; ** $p \leq 0.01$.

[a] Total number of fetuses with microphthalmia (Fetuses with microphthalmia or eye rudiment flat detected at external examination were assigned to the subgroup for visceral examination. Cases of microphthalmia missed at external examination and assigned to skeletal evaluation would have been detected as "eyehole reduced in size" – but this was not the case. External, visceral and skeletal incidences of microphthalmia are combined to derive an "all fetuses" value (related to all (viscerally and skeletally) examined fetuses) for the purpose of comparison to the historical control data

[b] Historical control data (HCD) range from 1991-2001 (41 studies, same rat strain and test laboratory as in the present study)

Sources: original report, M-129256-02-1, M-576562-01-1, and M-577227-01-1

Findings considered related to treatment with prothioconazole are written in **bold letters**

In Wistar Hsd Cpb:WU rats microphthalmia occurs with a fetal incidence of 0 – 1.95 % and with litter incidences ranging from 0 – 20 %, and can therefore be regarded as a common spontaneous malformation in this rat strain.

The litter incidences of microphthalmia at 80 and 500 mg/kg bw/d were not dose-related and were within the historical control range for five studies including two studies each conducted in the previous and successive calendar years to this study (Figure 5.6.2/01- 1, Table 5.6.2/01- 14). Those studies also included control values from studies using the same vehicle as was used in the present study (CMC). The incidence of microphthalmia in the high dose group was outside the historical control range and there were also two fetuses with bilateral microphthalmia in the high dose group (considered more likely to indicate an effect of treatment), but there were no bilateral incidences of this finding in any other

group.

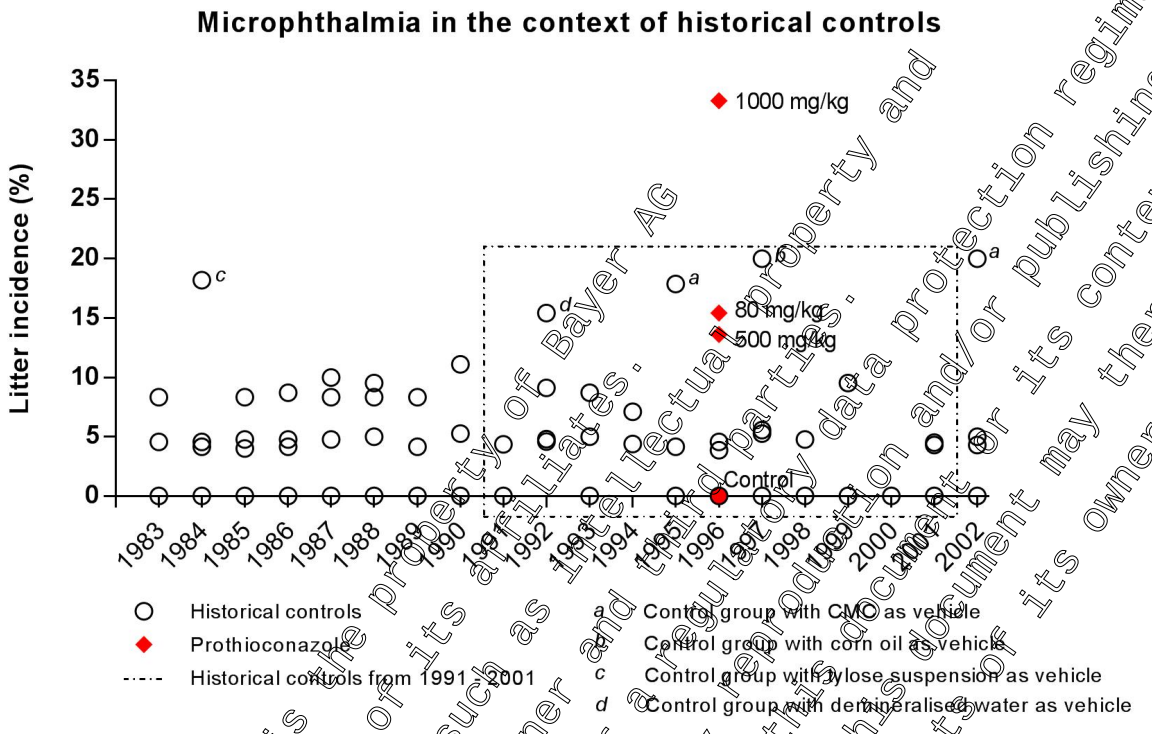


Figure 5.6.2/01- 1: Microphthalmia in the context of historical control data (rat strain Hsd Cpb:WU; same rat strain and test laboratory as in the present study). Total number of fetuses with microphthalmia is shown (external, visceral and skeletal incidences of microphthalmia are combined to derive an "all fetuses" value for the purpose of comparison to the historical control data (for detailed explanation see Table 5.6.2/01- 13)). The source of historical control data can be found in Table 5.6.2/01- 14.

Table 5.6.2/01- 14: Historical control data - microphthalmia (rat strain Hsd Cpb:WU; same rat strain and test laboratory as in the present study). Total number of fetuses with microphthalmia is shown (external, visceral and skeletal incidences of microphthalmia are combined to derive an "all fetuses" value for the purpose of comparison to the historical control data (for detailed explanation see Table 5.6.2/01- 13)).

Year	Study	No. of Fetuses investigated	Fetuses with Microphthalmia		No. of Litters investigated	Litters with Microphthalmia	
			No.	%		No.	%
1983	T6007810	218	1	0.46	22	1	4.55
	T2008626	114	1	0.88	12	1	8.33
7 other studies were conducted in 1983 which showed no microphthalmia in the control group							
1984	T5016716	254	1	0.39	24	1	4.17
	T9016807	173	1	0.58	22	1	4.55
	T8019035	205	4	1.95	22	4	18.18
6 other studies were conducted in 1984 which showed no microphthalmia in the control group							

Year	Study	No. of Fetuses investigated	Fetuses with Microphthalmia		No. of Litters investigated	Litters with Microphthalmia	
			No.	%		No.	%
1985	T5019339	231	1	0.43	21	1	4.76
	T5019825	122	1	0.82	16	1	8.33
	T0020125[+++]	271	1	0.37	25	1	4
	13 other studies were conducted in 1985 which showed no microphthalmia in the control group						
1986	T5022506	232	1	0.43	24	1	4.17
	T1023484	223	2	0.9	23	2	8.7
	T3024250	253	2	0.79	21	1	4.76
	4 other studies were conducted in 1986 which showed no microphthalmia in the control group						
1987	T6025171[+]	230	2	0.87	24	2	8.33
	T6023777	232	1	0.43	21	1	4.76
	T1027435	185	2	1.08	20	2	10
	4 other studies were conducted in 1987 which showed no microphthalmia in the control group						
1988	T2029650	200	1	0.5	21	1	5
	T1029424	211	2	0.95	24	2	8.33
	T0030368	209	3	1.44	22	2	9.52
	4 other studies were conducted in 1988 which showed no microphthalmia in the control group						
1989	T8030636	228	2	0.88	24	2	8.33
	T5033216	279	1	0.36	24	1	4.17
	4 other studies were conducted in 1989 which showed no microphthalmia in the control group						
1990	T0034599	80	1	1.25	9	1	11.11
1990	T7037368	170	1	0.59	19	1	5.26
	4 other studies were conducted in 1990 which showed no microphthalmia in the control group						
1991	T4040307	262	1	0.38	23	1	4.35
	4 other studies were conducted in 1991 which showed no microphthalmia in the control group						
1992	T9040474	213	2	0.94	22	2	9.1
	T3041008/A[++]	243	1	0.41	21	1	4.8
	T3041008/V[++]	263	2	0.76	22	2	9.1
	T4040348	231	1	0.43	22	1	4.6
	T9044173	149	2	1.34	13	2	15.4
apr 1993	T4050072	204	1	0.49	20	1	5.0
aug 1993	T7050318	256	5	1.95	23	2	8.7
	2 other studies were conducted in 1993 which showed no microphthalmia in the control group						
1994	T7055548	271	1	0.37	23	1	4.4
	T2058027	315	2	0.63	28	2	7.1
1995	T8058014	281	1	0.4	24	1	4.17
	T2055246	321	5	1.56	28	5	17.86
	1 other study was conducted in 1995 which showed no microphthalmia in the control group						
1996	T1054291	295	1	0.34	26	1	3.85
	T3055247	313	1	0.32	26	1	3.85
	T8054289	255	1	0.39	22	1	4.55
	2 other studies were conducted in 1996 which showed no microphthalmia in the control group including the present study (T2060240)						

Year	Study	No. of Fetuses investigated	Fetuses with Microphthalmia		No. of Litters investigated	Litters with Microphthalmia	
			No.	%		No.	%
1997	T0060860	224	4	1.79	20	4	20.0
	T3060250	217	1	0.46	19	1	5.26
	T8060255	224	1	0.45	18	1	5.56
	1 other study was conducted in 1997 which showed no microphthalmia in the control group						
1998	T7061370	246	1	0.41	21	1	4.76
	T9061390	240	1	0.42	21	1	4.76
	3 other studies were conducted in 1998 which showed no microphthalmia in the control group						
1999	T9061318	256	2	0.78	21	2	9.52
	2 other studies were conducted in 1999 which showed no microphthalmia in the control group						
2000	T5068551	232	0	0.0	20	0	0.0
2001	T1067765	283	1	0.4	22	1	4.5
	T6062800	275	1	0.4	23	1	4.3
	2 other studies were conducted in 2001 which showed no microphthalmia in the control group						
2002	T7062784	269	1	0.4	23	1	4.3
	T6071558	244	4	1.6	20	4	20.0
	T9062786	247	1	0.4	20	1	5.0
	1 other study was conducted in 2002 which showed no microphthalmia in the control group						

+ dermal application
++ intravenous application
+++ inhalation

Control data of 112 studies from 1983 – 2002; Sources: original report, M-129256-02-1, M-576562-01-1, and M-577227-01-1

Furthermore, microphthalmia showed often a high historical inter-group variability within a given study conducted in the same rat strain and laboratory as the present study (Table 5.6.2/01- 15). By arbitrarily exchanging the incidences of control and low-dose groups (studies T2055246 and T0060860) one could create a distribution pattern similar to that in the present study. Study T6071558 shows a perfect "dose-response" of declining incidences which of course does not indicate an anti-teratogenic effect. It is therefore considered crucial to assess the highly variable microphthalmia in the present study in the context of the historical control and variability data.

Table 5.6.2/01- 15: Examples for inter-group variability for microphthalmia

Year	Study	Litter incidence of microphthalmia (%)			
		Control	Low Dose	Mid Dose	High Dose
1995	T2055246	17.9	6.5	6.3	17.2
1996	Prothioconazole	0	15.4	13.6	33.3
1997	T0060860	20.0	0	4.2	27.8
2002	T6071558	20.0	12.5	4.8	0

Source: M-577227-01-1

Therefore, the microphthalmia incidences at 80 and 500 mg/kg bw/d are assessed as indicators for the high variability of this (in the present rat strain) common spontaneous malformation but not as a treatment-related effect. Only the increase at 1000 mg/kg bw/d is related to treatment but in the sense of an unspecific enhancement secondary to the very strong maternal toxicity based on kidney dysfunction

and resulting dehydration at this dose, and in the context of other unspecific signs of retarded fetal development (see below), and not related to a specific or direct teratogenic effect of the test substance.

For the same rat strain and test laboratory as in the present developmental toxicity study (Wistar Hsd Cpb:WU) the relation between maternal toxicity and an unspecific increased incidence of the common spontaneous malformation microphthalmia has been further investigated by [redacted] *et al.* (1996, [M-041671-02-1]): In an inhalative developmental toxicity study, exposure to the sensory irritating pyrethroid cyfluthrin (11.9 mg/m^3 (equivalent to 3.0 mg/kg bw/d)) caused a retarded development (reduced placental and fetal weights, retarded ossification) and an increased incidence of the common spontaneous malformation microphthalmia (fetal (litter) percent incidences: 5.4 (34.8) – compare prothioconazole at 1000 mg/kg bw/d: 4.6 (33.3)). Oral administration of cyfluthrin at up to 10-fold higher doses (30 mg/kg bw/d) did not provoke any developmental effects including microphthalmia. Therefore, the observed developmental effects in the inhalation study, including microphthalmia, are not considered substance-specific but rather occurred secondary to maternal toxicity. The irritating properties caused a reflectory induced maternal bradypnea (hypoventilation) / hypoxia with its physiological compensation mechanisms (hypothermia, respiratory alkalosis). This maternal imbalance caused in this rat strain (the same strain and laboratory as in the present study) a retarded fetal development and an increased incidence of the common spontaneous malformation microphthalmia. Oxygen enrichment of the inhaled air obviously partially compensated the bradypnea-related hypoxia and, thus, resulted in a reduction of the number of fetuses with microphthalmia at the same highest dose tested (fetal (litter) percent incidences: 2.9 (21.7) (see also summary 5.6.2/02). Therefore it is concluded that the increased incidence of the common spontaneous malformation including microphthalmia was triggered by disturbed maternal health. Also in humans, there is strong evidence for an unspecific mechanism (maternal hyperthermia) being a cause of fetal microphthalmia ([redacted] et al., 1998; M-394322-01-1)

In the present study the correlation between the degree of maternal toxicity caused by 1000 mg prothioconazole/kg bw/d and the occurrence of microphthalmia at this dose was substantiated by grouping the maternal toxicity results separately for those dams that produced pups with microphthalmia and for those that did not have any pups with microphthalmia (M-285563-01-1). The outcome of this evaluation is presented in Table 5.6.2/01- 16 below and shows that

- clear maternal toxicity was present at 1000 mg/kg; but between the two subgroups, maternal toxicity was clearly more pronounced in the group of dams that had fetuses with microphthalmia (group +MO) than in the group of dams that had no fetuses with microphthalmia (group -MO)
- fetal weight (as a correlating, unspecific developmental toxic effect secondary to maternal toxicity) was decreased at 1000 mg/kg; between the two subgroups, fetal weight was more decreased in fetuses from litters that included fetuses with microphthalmia (group +MO) than in group -MO

It can therefore be concluded that the severity of maternal toxicity correlates positively with the degree of fetal toxicity (body weight decrease) and with the occurrence of microphthalmia.

Table 5.6.2/01- 16: Mean values (and comparison to control values) for maternal toxicity (body weight gain, feed intake) and fetal weights in the first rat developmental toxicity study at 1000 mg/kg bw/d, grouped for dams that produced pups

with microphthalmia (+MO) and for dams that did not have any pups with microphthalmia (-MO), in comparison to control

Group	Maternal b.w. change (g)		Corrected mat. b.w. change d0-20 (g)	Feed intake d6-11 (g/animal/d)	Live fetal body weight (g)
	d6-8	d6-11			
1000 mg/kg +MO	**-5.4** (-204% of Control)	**+4.3** (28% of Control)	**+21.1** (53% of Control)	**13.4** (72% of Control)	**3.38** (93% of Control)
1000 mg/kg -MO	**-1.4** (-127% of Control)	**+8.1** (52% of Control)	**+30.8** (77% of Control)	**16.3** (88% of Control)	**3.48** (96% of Control)
Control	**+5.2**	**+15.5**	**+40.0**	**18.6**	**3.63**

The incidences of other external and visceral findings are shown in Table 5.6.2/01- 17. The only notable visceral finding other than microphthalmia was dilatation of the renal pelvis which was recorded at a high incidence in animals at 1000 mg/kg bw/d. This finding is considered to be secondary to retarded fetal development at this dosage, as indicated by decreased fetal weight and increased incidences of incomplete ossification (see Table 5.6.2/01- 18).

Table 5.6.2/01- 17: Summary of external and visceral findings (% fetal (litter) incidences for animals treated)

Parameter	Prothioconazole (mg/kg bw/d)				HCD
	0	80	500	1000	
External Examination					
No. fetuses (litters) evaluated in external examination	290 (26)	292 (26)	270 (25)	282 (24)	
Meningocele	0 (0.0)	0 (0.0)	0 (0.0)	0.4 (4.2)	*No data available*
Microphthalmia	0 (0.0)	0.3 (3.8)	0 (0.0)	0.7 (4.2)	*No data available*
Eye rudiment flat	0 (0.0)	2.1 (11.5)	0.4 (4.5)	2.1 (20.8)	*No data available*
Visceral Examination					
No. fetuses (litters) evaluated in visceral examination	138 (26)	137 (25)	128 (22)	135 (24)	
Microphthalmia (Wilsen's technique)	0 (0.0)	4.4* (16.0)	2.3 (13.6)	**8.1**** **(33.3)****	*No data available*
Microphthalmia (all fetuses) [c]	0 (0.0)	2.4 (15.4)	1.1 (13.6)	**4.6 (33.3)**	0 – 1.95 [b, c] (0 – 20)
Hydrocephalus	0.7 (3.8)	0 (0.0)	0.8 (4.5)	0 (0.0)	*No data available*
Bleeding in abdominal cavity	0.7 (3.8)	0.7 (4.0)	0 (0.0)	0 (0.0)	0.0-1.7 [a] (0.0-16.7)
Bleeding in liver	1.4 (7.7)	2.9 (12.0)	0 (0.0)	0 (0.0)	0.0-2.5 [a] (0.0-19.1)
Misplaced stomach	0 (0.0)	0 (0.0)	0 (0.0)	1.5 (4.2)	*No data available*

Parameter	Prothioconazole (mg/kg bw/d)				
	0	80	500	1000	HCD
Slight dilatation of renal pelvis	15.9 (57.7)	11.7 (44.0)	15.6 (50.0)	14.1 (50.0)	0.0-17.7 [a] (0.0-9.5)
Dilatation of renal pelvis	7.2 (26.9)	2.9 (12.0)	7.8 (22.7)	**17.0 (54.2)**	0.0-7.3 [a] (0.0-42.9)
Undescended testis	1.4 (7.7)	0.7 (4.0)	0.8 (4.5)	0 (0.0)	0.0-2.3 [a] (0.0-27.3)
Slight undescended testis	2.2 (11.5)	2.3 (12.0)	2.3 (13.6)	0.7 (4.2)	*No data available*

* $p < 0.05$; ** $p < 0.01$ (Fisher's exact test)

[a] Historical control data range from 1987-94, taken from report (40 studies with 793 litters and 8154 fetuses)

[b] Historical control data (HCD) range from 1991-2001 (41 studies, same rat strain and test laboratory as in the present study)

[c] Total number of fetuses with microphthalmia (Fetuses with microphthalmia or eye rudiment flat detected at external examination were assigned to the subgroup for visceral examination. Cases of microphthalmia missed at external examination and assigned to skeletal evaluation would have been detected as "eyehole reduced in size" – but this was not the case. External, visceral and skeletal incidences of microphthalmia are combined to derive an "all fetuses" value (related to all (viscerally and skeletally) examined fetuses) for the purpose of comparison to the historical control data

Findings considered related to treatment with prothioconazole are written in **bold letters.**

The incidences of skeletal / cartilaginous findings are shown in Table 5.6.2/01- 18Table 5.6.2/01- 17.

Incidences of rudimentary (punctiform and comma-shaped) supernumerary 14th lumbar ribs were statistically significantly increased in all treated groups compared to controls. Fetal and litter incidences were within historical control ranges up to and including 500 mg/kg bw/d and exceeded historical control ranges only slightly at 1000 mg/kg bw/d. Thus, the study director of the present study set the NOAEL for this finding at 500 mg/kg bw/d. This NOAEL would also be supported by a retrospective comparison of the respective maternal and developmental results from the present study with those of the special rat developmental toxicity study ([redacted] 2004). However, as a very conservative approach, in the present study a NOAEL for supernumerary 14th ribs is set at 80 mg/kg bw/d, based on the rationale provided on the following pages.

Increased incidences of incomplete ossification (distal and proximal phalanges, caudal vertebral bodies and 6th sternebral bone) were observed at 1000 mg/kg bw/d and indicate, together with the reduced fetal weights and renal pelvis dilatation, a retarded fetal development at this dose. The isolated decreased incidence of the 5th caudal vertebral body at 500 mg/kg bw/d is not considered to be of toxicological significance.

Table 5.6.2/01- 18: Summary of skeletal / cartilaginous tissue findings (% fetal (litter) incidences for animals treated)

Parameter	Prothioconazole (mg/kg bw/d)				
	0	80	500	1000	HCD [c]
Skeletal Examination					
No. fetuses (litters) evaluated	152 (26)	155 (26)	142 (22)	147 (24)	
Wavy ribs (variant)	15.1 (46.2)	6.5* (38.5)	4.2** (18.2)	2.0** (12.5)*	0.8-9.4 (4.3-30.0)
Rudimentary 14th rib (variant)	0.7 (3.8)	7.1* (42.3)**	**10.6*** **(54.5)****	**25.2**** **(62.5)****	0.0-24.4 [d] (0.0-57.1)
Dysplastic humerous (right)	1.3 (3.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0-0.9 (0.0-5.0)

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**80**	**500**	**1000**	**HCD[c]**
Dysplastic scapula[a]	3.9 (15.4)	1.3 (7.7)	0.7 (4.5)	0.7 (4.2)	*No data available*
Dysplastic scapula[b]	2.0 (7.7)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0-2.6 (0.0-7.7)
Dysplastic clavicle	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.7 (4.2)	*No data available*
Dysplastic femur (left or right)	0.7 (3.8)	1.9 (11.5)	0.0 (0.0)	0.0 (0.0)	0.0-0.9 (0.0-4.5)
Dysplastic pubic bone	0.0 (0.0)	0.0 (0.0)	0.7 (4.5)	0.7 (4.2)	*No data available*
Vertebral arch (7th cervical) misshapen	0.7 (3.8)	1.3 (7.7)	0.7 (4.5)	0.0 (0.0)	*No data available*
Vertebral arch (2nd thoracic) misshapen	0.7 (3.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	*No data available*
Left exoccipital bone misshapen	0.0 (0.0)	0.6 (3.8)	0.0 (0.0)	0.0 (0.0)	*No data available*
Cartilage defect on 3rd - 6th ribs	0.0 (0.0)	0.6 (3.8)	0.0 (0.0)	0.0 (0.0)	*No data available*
Total abnormal fetuses - no. (%)	13 (4.5)	16 (5.5)	7 (2.6)	19 (6.7)	
Total abnormal litters - no. (%)	8 (30.8)	11 (42.3)	6 (27.3)	11 (45.8)	
Distal phalanges – Digit(s)					
5th digit, right – unossified	2.6 (15.4)	5.2 (23.1)	4.9 (27.3)	**10.2* (37.5)**	0.0-69.9 (0.0-100.0)
5th digit, left – unossified	3.9 (19.2)	10.4 (30.8)	9.9 (27.3)	**14.3** (45.8)**	0.0-70.8 (0.0-100.0)
Proximal phalanges – Digit(s)					
3rd digit, right – unossified	70.4 (96.2)	78.1 (92.3)	73.9 (95.5)	**86.4**** (100.0)	43.1-80.5 (70.8-100.0)
3rd digit, left – unossified	76.3 (100.0)	81.3 (96.2)	79.6 (100.0)	**92.5**** (100.0)	51.2-85.0 (72.2-100.0)
4th digit, right – unossified	74.3 (96.2)	81.3 (96.2)	79.6 (95.5)	**90.5**** (100.0)	46.3-83.2 (79.2-100.0)
4th digit, left – unossified	82.2 (100.0)	85.8 (100.0)	82.4 (95.5)	**95.9**** (100.0)	56.1-88.5 (75.0-100.0)

Parameter	Prothioconazole (mg/kg bw/d)				
	0	80	500	1000	HCD[c]
Distal phalanges – Toe(s)					
1st toe, right – unossified	7.5 (26.9)	5.5 (23.1)	4.3 (27.3)	**19.3*** **(66.7*)**	0.0-57.5 (0.0-100.0)
4th toe, right – incompletely ossified	28.5 (69.2)	38.3 (73.1)	26.8 (77.3)	36.1 **(100.0*)**	10.7-76.8 (39.1-100.0)
4th toe, left – unossified	7.3 (23.1)	9.7 (46.2)	4.2 (22.7)	**17.0*** (50.0)	1.3-48.7 (7.7-57.3)
5th toe, right – unossified	12.0 (34.6)	20.6 (69.2)	17.6 (54.5)	**33.3**** **(83.3**)**	2.3-77.9 (8.7-100.0)
5th toe, left – unossified	14.6 (42.3)	21.9 (65.4)	15.6 (50.0)	**31.5**** **(79.2*)**	1.3-80.5 (7.7-100.0)
Sternebra(e)					
6th sternebra – incompletely ossified	16.4 (42.3)	18.1 (46.2)	19.0 (45.5)	**36.7**** **(79.2)**	2.4-32.1 (12.5-59.1)
Caudal vertebral body(ies)					
4th caudal vertebral body – present	100.0 (100)	96.1 (100)	96.5 (100)	**89.8**** (100)	92.0-98.7 (100.0)
5th caudal vertebral body – present	48.7 (92.3)	42.6 (84.6)	26.1** (72.7)	**17.7**** **(58.3*)**	39.8-78.0 (80.0-100.0)

* $p < 0.05$; ** $p < 0.01$ (Fisher's exact test); [a] unilateral, [b] bilateral
[c] Historical control data range 1992-94 (9 studies with 196 litters and 2149 fetuses) (Source: appendix of original report)
[d] Historical control data range 1991-2001 (40 studies) (Sources: original report, M-129256-02-1, M-576707-01-1, M-577830-01-1)
Findings considered related to treatment with prothioconazole are written in **bold letters**

For the following reasons, only the increased incidences of rudimentary 14th ribs at 500 and 1000 mg/kg bw/d (and not the respective incidences at 80 mg/kg bw/d) are regarded to indicate a treatment related effect. Rudimentary 14th ribs are a very common spontaneous variation in untreated rats. No fully formed 14th ribs (considered as a malformation) were observed at any dose group. The control incidences for rudimentary 14th ribs were unusually low in the present study, compared to historical control data (Table 5.6.2/01- 19,Figure 5.6.2/01- 2). Rudimentary 14th ribs occurred in controls from 52 / 53 studies (1991 – 2003, same rat strain and test laboratory as in the present study); control values in the present study were the lowest in the 52 studies. The fetal and litter incidences of this finding at 80 and at 500 mg/kg bw/d are within the range of the historical control data and exceeded the historical control range only slightly at 1000 mg/kg bw/d.

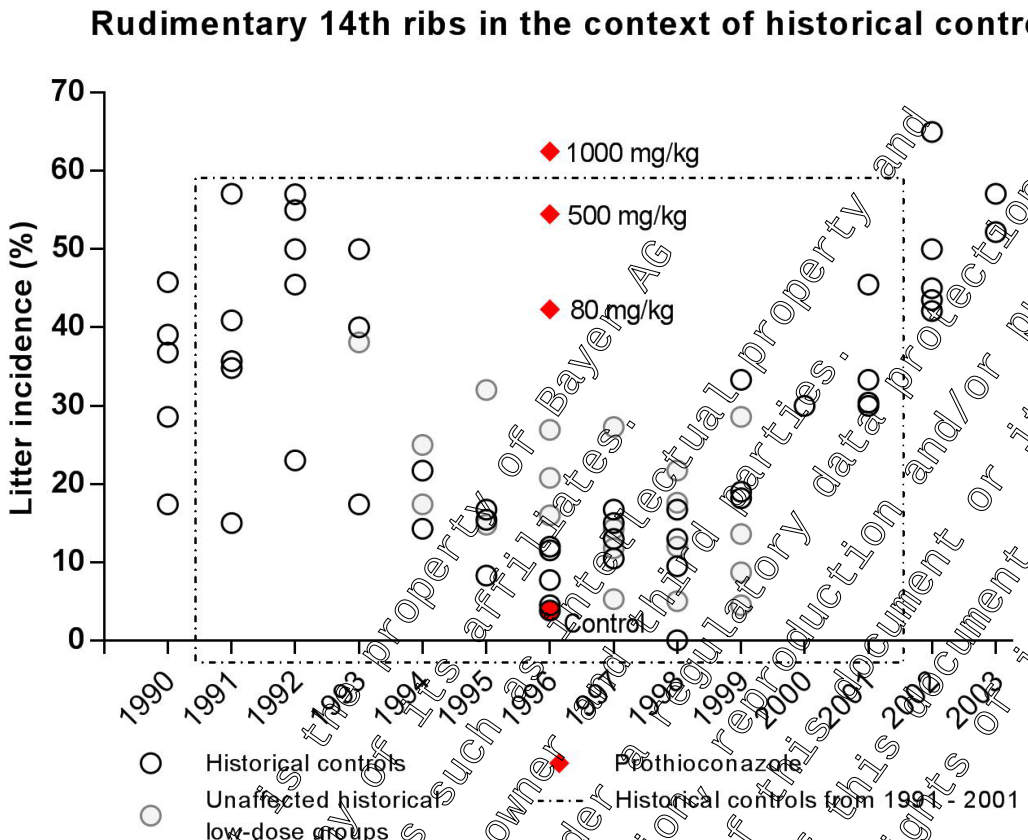


Figure 5.6.2/01- 2 Rudimentary 14th ribs in the context of historical control data (rat strain Hsd Cpb:WU) Historical control data of 53 studies from 1990 - 2003 (Sources: original report, M-129256-02-1, M-576707-01-1, M-577830-01-1). Low dose groups were considered unaffected if there was no effect on the incidence of supernumerary 14th ribs up to and including the highest dose tested.

Table 5.6.2/01- 19: Historical control data – rudimentary supernumerary 14th ribs (comma shaped and punctiform) (Species: Rat, Strain: Hsd Cpb:WU)

Year	Study	No. of Fetuses investigated	Fetuses with Supernumerary 14th ribs		No. of Litters investigated	Litters with Supernumerary 14th ribs	
			No.	%		No.	%
1990	T6034739+	222	13	5.98	23	4	17.4
	T9037072	258	14	10.22	23	9	39.1
	T3037265	62	4	12.12	7	2	28.6
	T7037368+	170	16	17.98	19	7	36.8
	T6039518	248	16	12.31	24	11	45.8
1991	T3038066	236	14	11.3	22	9	40.9
	T4039958	137	21	15.3	14	8	57.1
	T6040039	220	4	3.5	20	3	15.0
	T4040307	262	12	8.8	23	8	34.8
	T3040711	134	9	13.0	14	5	35.7

Year	Study	No. of Fetuses investigated	Fetuses with Supernumerary 14th ribs		No. of Litters investigated	Litters with Supernumerary 14th ribs	
			No.	%		No.	%
1992	T9040474	113	19	16.8	22	11	50
	T3041008/A+++	126	32	24.4	21	12	57
	T3041008/V+++	138	26	19.8	22	12	55
	T4040848	120	14	11.5	12	10	45.5
	T9044173	77	7	9.1	13	3	23
apr 1993	T4050072	108	9	8.3	20	8	40.0
	T4050072 LD	n.a.	n.a.	11.3	n.a.	n.a.	38.1
aug 1993	T7050318	131	6	4.6	23	4	17.4
nov 1993	T1050105	123	15	12.2	24	12	50.0
1994	T7055548	141	5	3.5	23	5	21.7
	T7055548 LD	n.a.	n.a.	6.9	n.a.	n.a.	25.0
	T2058027	164	5	3.0	28	4	14.3
	T2058027 LD	n.a.	n.a.	3.3	n.a.	n.a.	17.4
1995	T8058014	147	4	2.7	24	4	16.7
	T2055246	143	2	1.4	24	2	8.3
	T2055246 LD	n.a.	n.a.	4.7	n.a.	n.a.	14.8
	T1055245	n.a.	n.a.	3.2	n.a.	n.a.	15.4
	T1055245 LD	n.a.	n.a.	7.0	n.a.	n.a.	32.0
feb 1996	**T2060240 ***	**152**	**1**	**0.7**	**26**	**1**	**3.8**
1996	T4060260	142	3	2.1	25	3	12.0
	T1054291	152	3	2.0	26	2	7.7
	T1054291 LD	n.a.	n.a.	3.3	n.a.	n.a.	20.8
	T3055247	163	3	1.8	26	3	11.5
	T3055247 LD	n.a.	n.a.	3.8	n.a.	n.a.	16.0
	T8054289	135	2	1.5	22	1	4.5
	T8054289 LD	n.a.	n.a.	4.5	n.a.	n.a.	26.9
1997	T2055255	147	4	2.7	23	3	13.0
	T2055255 LD	n.a.	n.a.	0.8	n.a.	n.a.	5.3
	T0060860	116	4	3.4	20	3	15.0
	T0060860 LD	n.a.	n.a.	5.1	n.a.	n.a.	27.3
	T3060250	113	3	2.7	19	2	10.5
	T3060250 LD	n.a.	n.a.	2.5	n.a.	n.a.	11.8
	T8060255	117	6	5.1	18	3	16.7
	T8060255 LD	n.a.	n.a.	2.4	n.a.	n.a.	14.3
1998	T2061366	144	8	5.6	24	4	16.7
	T2061366 LD	n.a.	n.a.	1.9	n.a.	n.a.	12.0
	T7061370	128	2	1.6	21	2	9.5
	T7061370 LD	n.a.	n.a.	6.1	n.a.	n.a.	21.7
	T8061380	136	4	2.9	23	3	13.0
	T8061380 LD	n.a.	n.a.	0.9	n.a.	n.a.	5.0
	T2061375	120	0	0.0	21	0	0.0
	T2061375 LD	n.a.	n.a.	4.6	n.a.	n.a.	17.6
	T9061390	125	2	1.6	21	2	9.5

Year	Study	No. of Fetuses investigated	Fetuses with Supernumerary 14th ribs		No. of Litters investigated	Litters with Supernumerary 14th ribs	
			No.	%		No.	%
1999	T9067880	128	12	9.4	21	7	33.3
	T9067880 LD	n.a.	n.a.	0.8	n.a.	n.a.	4.5
	T2061311	126	6	4.8	22	4	18.2
	T2061311 LD	n.a.	n.a.	1.5	n.a.	n.a.	8.7
	T9061318	133	8	6	21	4	19.0
	T9061318 LD	n.a.	n.a.	2.2	n.a.	n.a.	13.6
	T0061319 [b]	-	-	-	-	-	-
	T0061319 LD	n.a.	n.a.	7.8	n.a.	n.a.	28.6
2000	T5068551	123	16	13	20	6	30
2001	T5067750	n.a.	n.a.	6.6	n.a.	n.a.	30
	T1067765	n.a.	n.a.	10	n.a.	n.a.	45.5
	T8068563	n.a.	n.a.	8	n.a.	n.a.	33.3
	T6062800	n.a.	n.a.	5	n.a.	n.a.	30.4
2002	T3068568	n.a.	n.a.	12.8	n.a.	n.a.	50
	T7062784	n.a.	n.a.	9.2	n.a.	n.a.	43.5
	T6071558	n.a.	n.a.	11.1	n.a.	n.a.	45
	T9062786	n.a.	n.a.	15	n.a.	n.a.	50
	T3063590	n.a.	n.a.	11.6	n.a.	n.a.	42.1
	T5063600	n.a.	n.a.	16.3	n.a.	n.a.	65
2003	T7063008	n.a.	n.a.	13.6	n.a.	n.a.	52.2
	T7063955	n.a.	n.a.	12.4	n.a.	n.a.	57.1

LD unaffected low dose group of studies from 1993-1999 (M-576707-01-1). Low dose groups were considered unaffected if there was no effect on the incidence of supernumerary 14th ribs up to and including the highest dose tested.

[a] present study

[b] same control group as study T9061318

n.a. not available

Control data of 53 studies from 1990 – 2003; Sources: original report, M-129256-02-1, M-576707-01-1, M-577830-01-1

Rudimentary 14th supernumerary ribs also showed a high historical inter-group variability within studies conducted in the same rat strain and laboratory as the present study. An example is given in Table 5.6.2/01- 20. By exchanging the incidences of control and low-dose group of study T9067880 one could create a distribution pattern similar to the control and low dose in the present study. The assessment of the high variability of supernumerary 14th ribs in the present study in the context of the historical control and variability data is therefore considered crucial.

Table 5.6.2/01- 20: Examples for inter-group variability for rudimentary supernumerary 14th ribs

	% Fetal (litter) incidences for group:			
	Control	Low	Mid	High
Study number T9067880 (1999)	9.4 (33.3)	0.8 (4.5)	3.9 (13.6)	5.0 (18.2)
Present study	0.7 (3.8)	7.1* (42.3)**	10.6* (54.5)**	25.2** (62.5)**

Source of data: M-576707-01-1

In the special rat developmental toxicity study ([redacted] 2004, see summary further down below), 750 mg/kg bw/d caused a comparably marked maternal toxicity as 1000 mg/kg bw/d in the present study. In the special study 750 mg/kg bw/d caused only marginal effects on the incidence of 14th rudimentary ribs. Therefore it is plausible to conclude that the treatment-related increase of 14th rudimentary ribs in the present study at 1000 mg/kg bw/d (which was only slightly outside the historical control data range) is also only a marginal effect (secondary to maternal toxicity) and, thus, the mid dose of 500 mg/kg bw/d (incidences were within historical controls) is a respective NOAEL.

The relevance of rudimentary supernumerary ribs as a marker of developmental toxicity has also been discussed in public literature and it is concluded that the toxicological significance of an increased rudimentary 14th rib incidence is somewhat confounded by its spontaneous occurrence in control and treated groups and by its disappearance during normal postnatal maturation[5,6,7]. Interpretation of this skeletal variation as an endpoint of concern is further confounded by its occurrence resultant to maternal stress. Due to their high variability, spontaneous incidence, and normal disappearance, rudimentary 14th ribs should not be considered biologically significant in the absence of more profound signs of developmental toxicity (i.e. malformations, embryolethality, and/or fetal weight reduction). There is no scientific evidence that an increase in supernumerary ribs in a developmental toxicity study is a reliable predictor on increased risk to human development. Examination of historical control data and determination of whether the concurrent control falls within the range of historical control values is needed to support any toxicological interpretation of rudimentary 14th rib incidence.

Furthermore, as in the present study rudimentary 14th ribs occur along with retarded fetal development (lower fetal weights, incomplete ossification), they are assessed as a non-specific enhancement of this common variation secondary to the strong maternal toxicity (as discussed in a paper prepared by the UK Advisory Committee on Pesticides Medical and Toxicological Panel (ACP, 1998 "The significance of supernumerary ribs in teratology studies", M-091112-01-1) and not as a specific developmental toxic effect.

According to EFSA Scientific Committee[8], the benchmark dose (BMD) approach is an adequate method for deriving a reference point when the NOAEL is uncertain and dose-response data are available to further inform selection of a reference dose. A BMD approach provides quantitative support for selection of a NOAEL or reference dose via consideration of the shape of the dose-response curve using mathematical models, resulting in calculation of a BMD and its 95 % lower-bound confidence limit (i.e. the BMDL). This methodology enables determination of a dose corresponding to a specified effect in a manner that is not limited to experimental doses and less dependent on dose spacing than the traditional NOAEL/LOAEL approach. In the present rat developmental toxicity study, dose-dependent increases in rudimentary 14th rib incidence were observed after prothioconazole exposure; however, as discussed above, the incidence seen in control animals was unusually low in this study. Calculation of the $BMDL_{10}$ was conducted according to EFSA (2016)[8].

When the BMD confidence interval was derived by model averaging using all models available for quantal data in MADr-BMD, it resulted in a $BMDL_{10}$ of 347.1 mg/kg bw/d. When only the models

[5] [redacted]. (1988). The post-natal fate of supernumerary ribs in rat teratogenicity studies. *J. Appl. Toxicol.* **8**:91-94. [M-236121-01-1]

[6] [redacted]. (1991). Significance of supernumerary ribs in rodent developmental toxicity studies: postnatal persistence in rats and mice. *Fundam. Appl. Toxicol.* **17**(3):448-453. [M-184999-01-1]

[7] [redacted] (2013). Data interpretation: Using historical control data to understand supernumerary ribs, a common skeletal variation. In: Teratogenicity testing, methods and protocols, Barrow PC (editor), ISSN 1064-3745, ISBN 978-1-62703-130-1, Humana Press, Springer New York, Heidelberg, Dordrecht, London, 290-294. [M-577059-01-1]

[8] European Food Safety Authority (EFSA) Scientific Commitee 2016. Use of the benchmark dose approach in risk assessment. Draft Guidance.

which gave an acceptable fit in PROAST were used, model averaging with MADr-BMD resulted in a $BMDL_{10}$ of 317.75 mg/kg bw/d (M-579365-01-1). Those results confirm the outcome of a former benchmark dose analysis of rudimentary 14th ribs observed in the present study conducted in 2015 (M-531958-01-1) using US-EPA software, which resulted in a $BMDL_{10}$ of 384.44 mg/kg bw/d.

In the special rat developmental study ([redacted], 2004) with dose levels of 0, 20, 80 and 750 mg/kg bw/d the fetal incidence of rudimentary 14th ribs (comma-shaped) was only marginally (but statistically significantly) increased at the highest dose tested (750 mg/kg bw/d). Based on these results, in the special study the NOAEL for increased incidence of rudimentary 14th rib in fetal rats was established at 80 mg/kg bw/d. Considering the fact that the increase in incidences at 750 mg/kg bw/d was only slight and there were no effects at 20 and 80 mg/kg bw/d this NOAEL setting is very conservative. Also in the EFSA-DAR[9] this NOAEL was stated to be *"probably conservative"*. The recent results of the BMD approach further support the assumption that the true NOAEL for 14th rudimentary ribs is probably considerably higher than 80 mg/kg bw/d.

The maternal toxicity observed in the present study at 1000 mg/kg bw/d and in the special study at 750 mg/kg bw/d is considered comparable, leading to the conclusion that the effects on 14th ribs at 1000 mg/kg bw/d should also be considered comparable and therefore only slight and making 500 mg/kg bw/d the NOAEL in this study. Since at 500 mg/kg bw/d the fetal and litter incidences were well within the historical control data, the study director of the present study also concluded the NOAEL for developmental toxicity to be 500 mg/kg bw/d. A NOAEL for rudimentary 14th ribs of 500 mg/kg bw/d would also be supported by the aforementioned published conclusion that rudimentary 14th ribs should not be considered biologically significant in the absence of more profound signs of developmental toxicity – which are clearly absent at 500 mg/kg bw/d.

However, as a very conservative approach, in the present study a NOAEL for rudimentary 14th ribs is set at 80 mg/kg bw/d, as supported by historical control data, both BMD approaches and the results of the special supplementary study.

There were no notable increases in any other findings and the overall incidences of abnormal fetuses and litters in all treated groups were not significantly different from the control group.

III. CONCLUSION

The NOAEL for dams of 80 mg/kg bw/d is based on reduced bodyweight gains, strongly increased water consumption (up to 131 % of control), increased urination and slight effects on AST, cholesterol and T4 at 500 mg/kg bw/d. At the highest tested dose (1000 mg/kg bw/d) transient body weight loss and drastically increased water consumption (up to 175 % of control) evidenced the very strong (sublethal) maternal toxicity related to kidney dysfunction and resulting dehydration. Slight increases of ALT and ALP were also observed at 1000 mg/kg bw/d.

A plausible NOAEL for developmental toxicity could be set at 500 mg/kg bw/d, based on a slight increase (compared to historical controls) of the incidence of the in the present rat strain common spontaneous variation rudimentary 14th ribs and other findings (see below) observed at 1000 mg/kg bw/d.

However, as a very conservative approach, the NOAEL for developmental toxicity is set at 80 mg/kg bw/d, based on incidences of rudimentary 14th ribs which were increased compared to concurrent controls at 500 mg/kg bw/d, but are still within the historical control range. Respective benchmark dose calculations confirm $BMDL_{10}$ values of >300 mg/kg bw/d. At 1000 mg/kg bw/d an increased incidence

[9] EFSA-DAR (2004), Prothioconazole – Volume 3, Annex B.6.: Toxicology - Prothioconazole

of engorged placentas, retarded fetal development (reduced fetal weights, delayed ossification and renal pelvis dilatation) and increased incidences of the in the present rat strain common spontaneous malformation microphthalmia were observed.

All developmental effects, including the in that rat strain common spontaneous variation rudimentary 14th ribs and common spontaneous malformation microphthalmia, are considered as unspecific enhancements secondary to strong (at 500 mg/kg bw/d) or very strong (sublethal) (at 1000 mg/kg bw/d) maternal toxicity. Therefore, these findings are no indications for a specific or direct developmental toxic or teratogenic potential of prothioconazole. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, such a constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. Specifically, the Guidance states: *"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species.*

Report:	KCA 5.6.2/10 [redacted] C; [redacted]; [redacted]; [redacted]; 1996; M-041671-02-1
Title:	FCR 1272 - Explantory report on results and mechanistic studies for embryortoxicity effects in rats after inhalation
Report No.:	23219
Document No.:	M-041671-02-1
Guideline(s):	--
Guideline deviation(s):	--
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.6.2/10. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.6.2/02** (also in numbering of tables/figures).

Executive summary:

In this report results of an embryotoxicity study with inhalation of cyfluthrin are evaluated in consideration of supplementary mechanistic information.

The document contains data from 4 studies conducted with cyfluthrin:

1) Inhalation Study for Embryotoxic Effects in Rats, B. [redacted] (1993), Report No. 22581 (Further details on the embryotoxicity study can be found in a brief study summary below.)
2) Determination of the FCR 1272 Concentration in the Plasma of Rats Following Inhalative Exposure, U. [redacted] (1993), Report No. 22726
3) Pilot Study for Acid-base Status Following Inhalation Exposure to the Rat, J. [redacted] (1992), Report No. 21865
4) Study for Acute Oral Toxicity in Rats, W. [redacted] (1991), Report No. 19852

In the inhalation study for embryotoxic effects 6 groups of 25 females were nose-only exposed to cyfluthrin by inhalation (6 h daily, 7 days/week) at analytical concentrations of 0 (air), 0 (vehicle), 0.46, 2.55, 11.9 and 12.8 mg/m³ air (equivalent to 0, 0, 0.2, 1.0, 3.0 and 3.0 mg/kg bw/d). The inhaled air of the last dose group was supplemented with an enriched oxygen concentration (39% instead of approx.

21%). Satellite groups comprised of five females each were used to investigate specific maternal toxicity parameters.

Clinical findings were apparent in the dams at levels of ≥ 2.55 mg/m³ air (bloody snout, ungroomed fur and piloerection); respiratory disturbances and hypoactivity were noted at the 11.9 mg/m³ and 12.8 mg/m³ + O_2 levels, and a high-stepping gait and salivation at 11.9 mg/m³ only. Whereas only isolated animals were affected in the 2.55 mg/m³ group, most of the animals in the two high-dose groups exhibited clinical findings. The feed intakes and body weight gains were depressed at levels of 0.46 mg/m³ air and above (only marginally at 0.46 mg/m³). With respect to intrauterine development, the gestation rate, the resorption rate and, accordingly, the number of fetuses, and the fetal sex were unaffected at all dose levels. At levels of 2.55 mg/m³ air and above the placental and fetal weights were reduced, and the fetuses exhibited retarded ossification as well as an increased incidence of the (in that rat strain) common spontaneous malformation microphthalmia. Oxygen supply resulted in reduction of maternal as well as developmental effects, particularly of the incidences of fetuses with microphthalmia.

Thus, the no-observed-adverse-effect concentration for maternal toxicity was below 0.46 mg/m³ air, while 0.46 mg/m³ air was the no-observed-adverse-effect concentration for developmental toxicity.

The results from the inhalation embryotoxicity study show that all embryotoxic findings correlated with maternal toxicity. There were no specific malformations caused by cyfluthrin. The unspecific nature of the embryotoxicity (retarded development, increase in the common malformation microphthalmia) indicates that these findings are due to unspecific maternal effects. The fact that oxygen supplementation minimises both maternal and developmental effects at the high concentration further substantiates this assumption.

The satellite animals revealed bradypnea (hypoventilation) and hypothermia at the lowest dose level and above. Bradypnea and concomitant hypothermia after cyfluthrin inhalation is reflexively induced by sensory irritation of the respiratory tract, as shown on a subacute inhalation study with cyfluthrin ([redacted], 1989). Investigations on blood-gases and acid-base-status in the animals with arterial catheters show that, besides bradypnea and hypothermia, inhalation of cyfluthrin induces a decrease in the arterial CO_2 pressure along with an increase in the arterial pH during exposure reflecting respiratory alkalosis. Alkalemia (respiratory alkalosis) secondary to hypothermia is a known and generally valid physiological reaction amongst mammals[10].

Thus, the effects on the thermoregulatory system (hypothermia) and blood-gases as well as acid-base-status (respiratory alkalosis) after inhalation of cyfluthrin are physiological compensation mechanisms following reflex bradypnea after sensory irritation.

Hypothermia after exposure to cyfluthrin is specific to inhalation. Acute oral administration up to 500 mg FCR 1272/ kg body weight did not induce hypothermia ([redacted], 1991). This matches well with the results from a developmental toxicity studies with oral administration of cyfluthrin (up to an approx. 10-fold higher dose of 30 mg/kg bw/d), in which no embryotoxicity was observed. Therefore, a primary embryotoxic or teratogenic potential of cyfluthrin can be excluded.

The correlation of embryotoxicity and maternal toxicity, the unspecific type of embryotoxicity and the reduction of embryotoxic effects by oxygen supplement together with the reflectory induced effects of cyfluthrin on the respiratory and thermoregulatory system of the dams lead to the conclusion that reflectory induced hypoxia of the dams with its physiological compensation mechanisms (hypothermia with respiratory alkalosis) is the cause for embryotoxicity after inhalation of cyfluthrin.

The embryotoxic effects (including increased incidence of the common spontaneous malformation microphthalmia) observed in the inhalation study with cyfluthrin are induced by disturbed maternal health.

[10] [redacted] (1987). Gas exchange and acid-base disturbances. Handbook of physiology - The respiratory system IV, Chapter 20, 421-438

**Short summary of "Inhalation Study for Embryotoxic Effects in Rats";
[redacted], 1993; Report No. 22581**

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Cyfluthrin
Synonym:	FCR 1272
Description:	Yellow brown, solidified mass, clear yellow-brown oil above 50 °C
Batch No.:	238005176
Purity:	96.2 %
CAS No.:	68359-37-5
Stability of the test compound:	The stability was guaranteed for the duration of the study.
2. Vehicle:	Blend of polyethylene glycol 400 and ethanol
3. Test animals:	
Species:	Rat
Strain:	Wistar Bor:WISW (synonym to Wistar (Hsd Cpb:WU))
Sex:	females
Age:	12-23 weeks
Weight:	At mating: females: 186 – 244 g
Source:	[redacted], Germany
Acclimation period:	At least 7 days before mating
Diet:	Standard rat diet Altromin® 1324 (Altromin company in Lage, Germany), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Individually in Type II Makrolon® cages on low-dust wood shavings supplied by Ssniff GmbH in Soest, Germany. The males were kept individually in Type III Makrolon® cages
Environmental conditions:	
Temperature:	22.5 ± 0.5°C
Humidity:	50 ± 10 %
Air changes:	At least 10 times per hour
Photo period:	Artificial illumination, 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: May 11, 1992 – June 05, 1992

2. Animal assignment and treatment

6 groups of 25 females were nose-only exposed to the test substance by inhalation under dynamic exposure conditions for six hours daily (seven days per week) from day 6 to 15 p.c.. In addition, satellite groups comprised of five females each (treatment from day 6 - 13 p.c.) were used to investigate specific maternal toxicity parameters (reflexes, rectal temperature and respiration parameters), and to determine the cyfluthrin plasma levels.

Table 5.6.2/02- 1: Study design and inhalation chamber parameters

	Control (air)	Control (vehicle)	Group 1	Group 2	Group 3	Group 4
Number of dams*	25 + 5	25 + 5	25 + 5	26 + 5	25 + 5	25 + 5

	Control (air)	Control (vehicle)	Group 1	Group 2	Group 3	Group 4
Nominal Concentration (mg/m³)	0	0	3.3	11.0	60.0	60.0
Mean analytical concentration (mg/m³ air)	0	0	0.46	2.55	11.9	12.8 + 39.5 O_2
MMAD (µm)	1.1					
GSD	1.5					
Mass < 3 µm (%)	> 98					
Chamber temperature (°C)	22.5	22.4	22.8	22.8	22.9	23.5
Chamber humidity (%)	3.9	4.1	11.6	5.0	6.9	9.7
Oxygen concentration (%)	20.5	20.5	20.4	20.5	20.5	39.2

* main groups + satellite groups

Observations:

Clinical signs (daily before and after exposure), feed and water consumption, excretory products, mortality, body weight development, gross pathology, caesarian section (on GD 20) with assessment of: corpora lutea, no of implantations, uterus weight, weight and appearance of placentas, resorptions, no and sex of live fetuses, fetal weights, external, visceral (approx. half of the fetuses were evaluated according to the mod. WILSON technique) and skeletal observation (DAWSON technique)

Dams in satellite groups were used to determine cyfluthrin plasma levels and to investigate specific maternal toxicity parameters (reflexes, rectal temperature (1st an 7th day of exposure) and respiration parameters (1st day of exposure)) which were not determined in the dams of the main groups due to the potential invasive effects of these measurements

II. RESULTS

Maternal toxicity:

Clinical signs: ≥ 0.46 mg/m³: concentration dependent hypothermia and bradypnea (satellite groups)

≥ 2.55 mg/m³: bloody snout, ungroomed fur and piloerection (at 2.55 only isolated animals affected, at high dose groups most animals exhibited clinical findings)

11.9 mg/m³ and 12.8 mg/m³ + O_2: high-stepping gait and salivation (without O_2 supplementation only), respiratory disturbances and hypoactivity

Feed intakes and body weight gains were depressed at 0.46 mg/m³ air and above (Table 5.6.2/02- 2).

Table 5.6.2/02- 2: Maternal toxicity (main groups)

Conc. (mg/m³)	Control (air)	Control (vehicle)	Group 1 0.46	Group 2 2.55	Group 3 11.9	Group 4 12.8 with O_2 supplementation
Mean Feed intake (g/animal/d)						
Day 0-6 p.c.	19.6	19.9	19.5	20.1	19.8	19.7
Day 6-11 p.c.	17.5	17.4	**16.4****	**14.5*****	**13.0*****	**12.6*****
Day 11-16 p.c.	20.2	19.9	19.3	**18.1*****	**16.3*****	**16.3*****

Conc. (mg/m³)	Control (air)	Control (vehicle)	Group 1 0.46	Group 2 2.55	Group 3 11.9	Group 4 12.8 with O_2 supplementation
Day 16-20 p.c.	22.8	23.5	**21.9****	22.8	**22.2****	**21.6*****
Day 0-20 p.c.	19.9	20.0	**19.1****	**18.7*****	**17.7*****	**17.4****
Body weight gain (g)						
Day 6-15	12.0	13.3	**9.8***	**2.6*****	**-4.3*****	**-2.6*****
Day 0-20	83.6	88.8	**76.8***	**74.7*****	**58.7*****	**62.3*****
Day 0-20 (corrected)	20.0	23.0	19.8	**19.3***	**13.6*****	**12.5*****

* Statistically significant difference to vehicle control $p < 0.05$
** Statistically significant difference to vehicle control $p < 0.01$
*** Statistically significant difference to vehicle control $p < 0.001$
Findings considered related to treatment with cyfluthrin are written in **bold letters**

In the satellite groups increase in partial pressure of oxygen in the inhalation chamber apparently produced an attenuation of the maternal toxic effects (feed intake, body weight gain, hypothermia) (Table 5.6.2/02- 3).

The rectal temperature determinations showed concentration-dependent hypothermia, coupled with bradypnea (hypoventilation), after the first exposure to levels of 0.46 mg/m³ air and above. After the seventh exposure, this hypothermia could only still be determined in the 11.9 and 12.8 mg/m³ air groups, that in the (12.8 mg/m³ air) group of rats receiving oxygen supplementation being less severe than in the 11.9 mg/m³ air group.

Inhaled air concentrations can be transformed into equivalent dose levels in mg/kg bw/d by multiplication with the measured respiratory volume and the daily exposure time of 6 h. The inhaled air concentrations of 0.46, 2.55, 11.9 and 12.8 mg/m³ are equivalent to 0.2, 1.01, 3.03 and 3.0 mg/kg bw/d respectively.

There was no difference in plasma levels with and without oxygen supplementation.

Table 5.6.2/02- 3: Maternal toxicity (satellite groups)

Conc. (mg/m³)	Control (air)	Control (vehicle)	Group 1 0.46	Group 2 2.55	Group 3 11.9	Group 4 12.8 with O_2 supplementation
Mean Feed intake (g/animal/d)						
Day 6-11	82.6	83.4	75.2	70.2	**62.2****	70.4
Body weight gain (g)						
Day 6-11	-5.6	-2.2	-6.4	-11.6	**-15.8**	-9.8
Difference in rectal temperatures before and after exposure (°C)						
1st exposure	+0.48	+0.70	**-2.02***	**-3.40**	**-4.76****	**-5.16****
7th exposure	+0.14	+0.92	+0.54	-0.36	**-2.64**	-1.14

Conc. (mg/m³)	Control (air)	Control (vehicle)	Group 1 0.46	Group 2 2.55	Group 3 11.9	Group 4 12.8 with O_2 supplementation
Respiratory function measurements						
Mean rate (breath/min)	143	148	**115**	**107**	**111**	**89**
Mean resp. minute vol. (ml/min)	382	411	**298**	**258**	**164**	**155**
Mean resp. minute vol. (ml/min/kg)	1524	1682	**1202**	**1099**	**706**	**650**
Mean substance intake in mg/kg bw/d						
	--	--	0.20	1.01	3.09	3.00
Plasma levels following 7-day inhalation (pmol/mL)						
	--	--	--	--	19.0±13.3	14.7±4.4

* Statistically significant difference to vehicle control $p < 0.05$
** Statistically significant difference to vehicle control $p < 0.01$
*** Statistically significant difference to vehicle control $p < 0.001$
Findings considered related to treatment with cyfluthrin are written in **bold letters**

Developmental toxicity:

There was no effect on gestation rate, resorption rate, the number of fetuses, and the fetal sex at any dose level.

At 2.55 mg/m³ and above placental and fetal weights were reduced, and the fetuses exhibited retarded ossification as well as an increased incidence of the (in that rat strain) common spontaneous malformation microphthalmia (Table 5.6.2/02- 4).

Table 5.6.2/02- 4: Selected parameters of intrauterine development in developmental toxicity study with cyfluthrin (inhalation)

Conc. (mg/m³)		Control (air)	Control (vehicle)	Group 1 0.46	Group 2 2.55	Group 3 11.9	Group 4 12.8 with O_2 suppl.
Fetuses	n	243	263	245	251	239	240
Litters	n	21	22	23	23	23	23
Fetal weight (g)		3.41	3.50	3.48	**3.13*****	**2.48*****	**2.83*****
Microphthalmia [a]	n	1 (1)	2 (2)	1 (1)	3 (2)	**13**** **(8*****)**	**7 (5)**
	%	0.41 (4.76)	0.76 (9.09)	0.41 (4.35)	1.20 (8.70)	**5.44 (34.78)**	**2.91 (21.74)**

[a] fetal (litter) incidences
* Statistically significant difference to control $p < 0.05$
** Statistically significant difference to control $p < 0.01$
*** Statistically significant difference to control $p < 0.001$
Findings considered related to treatment with cyfluthrin are written in **bold letters**

Oxygen supply resulted in reduction of the number of fetuses with microphthalmia.

For comparison, in an embryotoxicity study with oral application of cyfluthrin at doses up to 30 mg/kg bw/d (10-fold of the maximum dose in the inhalation study), no increased incidence of fetuses with microphthalmia was observed (Table 5.6.2/02- 5). In this study the dams showed clinical signs (high stepping gait from the second week of application after the application; individual animals in the top

dosage group were occasionally ataxic or exhibited decreased motility) but there was no effect on body weight gain.

Table 5.6.2/02- 5: Intrauterine development in developmental toxicity study with cyfluthrin (application orally by gavage)

Dose (mg/kg)		Control	Group 1 3 mg/kg bw/d	Group 2 10 mg/kg bw/d	Group 3 30 mg/kg bw/d
Fetuses	n	277	261	257	251
Litter	n	25	23	25	22
Fetal weight (g)		4.09	4.26	4.39**	4.29*
Microphthalmia	n	1[##] (1)	0	0	0

[##] fetus with multiple cranial malformations (microphthalmia, anophthalmia, hydrocephalus)
* Statistically significant difference to control $p < 0.05$
** Statistically significant difference to control $p < 0.01$
[redacted] 1982, Report No. 10562, [M-037361-01-1], Rat strain BAY:FV30, dosing: gestation days 6 – 15

III. CONCLUSION

A clear no-observed-adverse-effect level for developmental toxicity was established at the concentration of 0.46 mg/m³ air. All embryotoxic findings correlated with maternal toxicity. There were no specific malformations caused by cyfluthrin.

The comparison of the maternal and embryotoxicity data in both high concentration groups of the embryotoxicity phase (12.8 mg/m³ air with oxygen adjustment and 11.9 mg/m³ air without oxygen adjustment) showed that maternal toxicity and developmental toxicity at the high concentration with oxygen supplement were less pronounced than without oxygen supplement. The unspecific nature of the embryotoxicity (retarded development, increase in common spontaneous malformations including microphthalmia) which was correlated with a decrease in placental weight indicates that these findings are due to unspecific maternal effects. This conjecture is supported by the fact that reduced maternal toxicity in the high concentration group with adjustment of the oxygen level is accompanied by a comparable decrease in embryotoxicity.

Supplementary mechanistic investigations after inhalation exposure of cyfluthrin in rats revealed that the embryotoxicity of cyfluthrin is induced by physiologically maternal compensation mechanisms (hypothermia with respiratory alkalosis) following reflex bradypnea after sensory irritation.

Report:	KCA 5.6.2/04[redacted],; 2004; M-067839-01-1
Title:	Technical grade JAU 6476: A supplementary prenatal developmental toxicity study in the Wistar Hanover (Crl:WI(HAN) rat to investigate ocular abnormalities and supernumerary ribs
Report No.:	201037
Document No.:	M-067839-01-1
Guideline(s):	OPPTS 870.3700; OECD 414 (2001); Health Canada PMRA DACO 4.5.2; JMAFF 12-Nousan no. 8147; Guideline 88/302/EEC
Guideline deviation(s):	none
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.6.2/04. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.6.2/03** (also in numbering of tables/figures).

Deviations: Deviations from the current OECD guideline (2001):
No visceral investigations were conducted (heads of all fetuses were used for special ocular investigations; the torso of all fetuses was used for a complete skeletal investigation with special emphasis on rib alterations).
These modifications enable an increased power of the study to investigate the effects identified previously as of primary concern and do not affect the overall acceptability of the study.

Executive summary:

In order to investigate the specificity of microphthalmia formation, in January 2004 a new (second) developmental toxicity study with prothioconazole was required by the UK Advisory Committee on Pesticides (ACP). Further requirements were the use of a "low-background incidence" rat strain for microphthalmia and "objective measurements" for microphthalmia. Therefore the OECD-guideline study design was adapted with the assistance of an external expert for developmental toxicity (Mildred S. Christian, Ph.D. (developmental anatomy (teratology)), at that time director of the Academy of Toxicological Sciences, President and CEO of Argus International, Inc. with more than 35 years of experience in industrial toxicology, personally involved in over 1200 developmental, reproductive and general toxicology evaluations) and was approved by the ACP.

The second study was conducted in a different Wistar rat substrain for which the available historical control database reveals a virtually-zero background incidence of microphthalmia; thus, a non-specific enhancement of microphthalmia secondary to maternal toxicity could not be elicited in this strain. Since the strain was nevertheless sensitive to a direct, specific oculo-teratogenic effect (15 mg/kg bw/d of the positive control all trans retinoic acid cause increased litter incidences of anophthalmia (41.7 %) and microphthalmia (16.7 %)) it is well suited to decisively investigate the specificity of microphthalmia formation caused by prothioconazole.

Prothioconazole (batch no. 6253/0031, purity: 98.7 %) was administered to the Wistar Hannover (Crl:WI(HAN)) rat. Twenty-five inseminated animals/dose/sex were orally gavaged on days 6-19 *post coitum* at nominal concentrations of 0 (aqueous 0.5 % CMC), 20, 80, or 750 mg/kg bw/d. The fetuses were delivered by caesarean section on day 20 of gestation. Doses were selected to reflect the doses of the first study (extending the range at the lower end) but the high dose had to be adapted from 1000 mg/kg to 750 mg/kg based on the higher sensitivity of the rat strain (as observed in a pilot maternal toxicity dose range finding study in which a 25 % mortality due to dehydration was the most prominent finding at 1000 mg/kg bw/d).

According to the study objectives, some aspects of the study design deviated from the standard guideline. Maternal evaluations included organ weight determination, clinical chemistry and histopathology to investigate possible effects on kidneys and liver, in addition to the minimum requirements. Fetal visceral examinations were not conducted since all fetuses were decapitated and the torso was evaluated for general skeletal and cartilage development with emphasis on the occurrence of supernumerary rudimentary (punctiform and comma-shaped) and extra ribs. All fetal heads were skinned; fetal brains and eyes (distinguishing right from left) were extracted and weighed. In order to

establish an objective measure for the ocular size, rather than the more subjective standard guideline observation according to the Wilson freehand slicing technique, the eyes were photographed so that both the horizontal and vertical diameters of the entire eye and of the cornea (including the area of the cornea) as well as, longitudinal length of the distance from the optic nerve remnant to the very front of the cornea could be measured.

750 mg/kg bw/d resulted in decreased corrected body weight gain (excluding gravid uterus) during gestation and a marked reduction in overall body weight gain during the first half of gestation, drastically increased water consumption, decreased feed consumption and clinical chemical indicators of functional impairments of kidneys (increased blood urea nitrogen) and liver (increased blood cholesterol and alkaline phosphatase activity, decreased aspartate aminotransferase activity). There were no treatment-related findings in the low- and mid-dose groups. In the context of the 25% mortality observed in the pilot maternal toxicity range finding study at 1000 mg/kg, 750 mg/kg, the highest dose tested in this study, is considered to be a maximum tolerated dose (MTD). There were no treatment-related reproductive effects nor were there any significant differences in the litter size, the median percent male fetuses, or fetal or placental weights in any group tested. No treatment-related effects were observed on external or skeletal malformations or on external variations. The external examinations before and after skinning did not reveal any single fetus exhibiting microphthalmia in any dose group tested up to and including the 750 mg/kg dose group. No compound-related effects were observed on the individual or mean eye weights, eye to fetal weight ratios, or on eye measurements. There was no evidence that prothioconazole caused microphthalmia in any dose group.

Skeletal evaluation revealed a possible treatment related increase in the fetal incidence of supernumerary rudimentary (comma-shaped) ribs at 750 mg/kg while litter incidence was not significantly affected. A treatment-related effect on punctiform or fully-formed supernumerary ribs was not discernible. The fetal incidence of the comma shaped rudimentary ribs was only marginally outside the historical control range for the same laboratory and rat strain, and that for punctiform ribs was well within the historical control range. There were no alterations in supernumerary ribs at 20 and 80 mg/kg bw/d. The marginal increase of rudimentary (comma-shaped) supernumerary ribs at 750 mg/kg bw/d is considered as an enhancement of this common variation secondary to the marked maternal toxicity at this dose.

The NOAEL for maternal toxicity was established at 80 mg/kg bw/d, based on decreased net body weight gain, markedly increased water consumption, decreased feed consumption and clinical chemical indications for functional impairments of kidneys and liver at 750 mg/kg bw/d.

A NOAEL for developmental toxicity was also established at 80 mg/kg bw/d, based on a marginal increase in the incidence of fetal supernumerary rudimentary (comma-shaped) ribs at 750 mg/kg bw/d.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: Prothioconazole

Synonym:	JAU 6476
Description:	Beige powder
Batch No.:	6233/0031
Purity:	98.7 %
CAS No.:	178928-70-6
Stability of the test compound:	Confirmed at least for the time of study duration

2. Vehicle: 0.5 % carboxymethylcellulose (CMC) in deionized water

3. Test animals:

Species:	Rat
Strain:	Wistar Hannover, Crl:WI(HAN)

Sex:	Males and females (nulliparous and not pregnant) (Males were not treated with prothioconazole and were only used for mating)
Age:	20–22 weeks (females)
Weight at dosing:	239–313 g (females)
Source:	[redacted], USA
Acclimation period:	At least 6 days before mating
Diet:	Purina Mills Certified Rodent Diet 5002, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Upon arrival animals were individually housed in suspended stainless steel wire-mesh cages. Females found sperm positive were individually housed in polycarbonate cages with corn-cob bedding.
Environmental conditions:	
Temperature:	18–26 °C
Humidity:	30–70 %
Air changes:	At least 10 times per hour
Photo period:	Artificial illumination, 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: February 24, 2004 – March 23, 2004

2. Animal assignment and treatment

Mating and start of gestation

The estrous cycle was determined for females (by examining vaginal smears) just prior to cohousing and a selection from those animals exhibiting either a proestrus or estrus stage was put with a male in order to limit the number of caesarean sections performed on a given day. Vaginal smears were taken each morning and examined for the presence of sperm and/or internal vaginal plug. Females found to be inseminated were placed in a polycarbonate nesting cage. The day on which insemination was observed in the vaginal smear was designated Day 0 of gestation for that female.

Dose levels, experimental group and rationale for choice of dose levels

The male animals were used for mating only and were not treated. After insemination was ascertained, 25 females each were allocated to four experimental groups according to a computer-generated randomization plan.

The animals were treated daily from day 6 to 19 *post coitum* (p.c.). Doses were administered by oral gavage, the suggested route of administration for studies of this type, in the vehicle at a dosage volume of 10 ml/kg (adjusted daily based on dam body weight during the dosing period). The animals of the control group received vehicle only (0.5 % carboxymethylcellulose, CMC) at the same volume.

The following doses (related to the test compound) were administered:

Table 5.6.2/03- 1: Study design

	Control	Group 1	Group 2	Group 3
Number of dams	25	25	25	25
Concentration in mg/L	0	2	8	75
mg/kg body weight	0	20	80	750

Doses were selected to reflect the doses of the first study (extending the range at the lower end) but the high dose had to be adapted from 1000 mg/kg to 750 mg/kg based on the higher sensitivity of the rat

strain (as observed in a pilot maternal toxicity dose range finding study: at 1000 mg/kg bw/d dams showed decreased body weights and feed consumption as well as clearly increased water consumption. Dehydration even resulted in 25 % mortality at this dose group. Already at 500 mg/kg bw/d, this pilot study showed dehydration (but no mortality) and clearly increased water consumption (presented in study report p. 596 f.)).

3. Test substance preparation and analysis

Prothioconazole was suspended in a mixture of 0.5 % CMC in deionized water. Dose vehicle was prepared in advance of the dose suspension preparation and stored under the hood (room temperature) since doses were prepared the following day. Following preparation, the concentration of the test compound at each dose level was determined prior to dosing, as well as at the end of the in-life portion of the study. The stock suspensions were refrigerated. Dosing suspensions and aliquots were continuously stirred on a stir plate (highest possible speed) until dispensing to the animals. On the days of dosing, an aliquot of each stock suspension was taken, from which the appropriate animals were dosed.

The homogeneity and 35-day stability of the dosing suspensions were previously confirmed ([redacted], 1999 (see 5.6.1/01), and [redacted], 2001 ([M-053225-01-1] **study summary not included yet**)) for concentrations which bracketed the range of dosages used in this study, 1.0 and 103.0 mg/ml. The liquid chromatographic methodologies used to perform the concentration verifications required for this study have been described previously ([redacted] and [redacted], 1998 [M-091268-01-1]).

The analytically confirmed mean concentrations of prothioconazole in the nominal 0, 20, 80, and 750 mg prothioconazole/kg body weight dose suspensions were 0, 1.9, 8.3, and 76.1 mg/ml, respectively. These concentrations correspond to doses of 0, 19.0, 83.0, and 761.0 mg/kg based on a dosage volume of 10 ml/kg. However, in the interest of clarity and consistency between the text, tables, and appendices, the dose levels are presented throughout the report as the nominal 0, 20, 80, and 750 mg/kg doses.

4. Statistics

The data (with the litter as the primary experimental unit) was analyzed using applications provided by DATATOX (Instem Computer Systems), SAS (SAS Institute, Inc.), or TASC (Toxicology Analysis Systems Customized, 1993).

Differences between the control and test compound-treated groups were considered statistically significant when $p \leq 0.05$ or $p \leq 0.01$. Statistical significance was tested using the following methods:

Statistical test	Parameter
Analysis of Variance (ANOVA) (and in case of significant results Dunnett's t-Test) as posthoc test for:	Parametric data (including dam body weights and feed consumption)
CHI^2 test; in case of significant differences Fisher's exact test with Bonferroni correction for:	Nonparametric dichotomous data (e.g. number normal/abnormal)
Kruskall-Wallis test (and in case of significant differences Dunn's test) for:	Nonparametric data (e.g. litter size and number of corpora lutea)

Both the fetal and litter incidence of the absolute difference between the left and right eye were analyzed by Dunnett's test to compare each treatment group with the control group. Dunnett's test was conducted without regard to the outcome of the analysis of variance F-test.

C. METHODS

1. Observations

From day 0 to 20 p.c. all animals were inspected twice daily - only once daily on weekends and holidays - and all findings were recorded. Mortality checks were performed 2 ± 1 times/d.

2. Feed and water consumption

Feed and water consumption was measured on gestation Days 0 to 1 and 5 to 6 and then daily on gestation Days 6 through 20.

3. Body weight development

The body weights of the animals were determined on day 0 p.c. and daily from day 6 to 20 p.c..

4. Clinical Pathology

Blood was taken via retro-orbital sinus from each female dose group on their Day 20 of gestation prior to caesarean section. The blood samples were collected in two serum microtainers and one EDTA tube. Hematology and clinical chemistries were evaluated from only those females found to be pregnant at caesarean section.

Clinical Chemistry		
Enzymes	*Electrolytes*	*Metabolites and Proteins*
Alanine aminotransferase (ALT)	Calcium (Ca)	Cholesterol (CHOL)
Aspartate aminotransferase (AST)	Chloride (Cl)	Triglycerides (TRIGL)
Alkaline phosphatase (ALP)	Phosphate (Phos)	Bilirubin, total (T-Bili)
Gamma-glutamyltranspeptidase (GGT)	Potassium (K)	Blood urea nitrogen (BUN)
Lactic dehydrogenase (LDH)	Sodium (Na)	Creatinine (Creat)
Creatine phosphokinase (CK)		Protein, total (T-Prot)
		Uric Acid (Uric-A)
		Glucose (Gluc)
		Albumin (Alb)

Hematology	
Blood Cell morphologies	Mean corpuscular volume (MCV)
Erythrocytes (RBC)	Mean corpuscular hemoglobin (MCH)
Hematocrit (Hct)	Mean corpuscular hemoglobin concentration
Hemoglobin (Hgb)	Platelets (PLTS)
Leucocytes (WBC) (total & differential)	Reticulocytes

5. Investigations at Cesarean Section

On Day 20 of gestation, dams were terminated by CO_2 asphyxiation and a gross external exam and internal necropsy was performed. The following parameters were determined and assessed at cesarean section:

- Number of corpora lutea
- Number of implantations
- Uterus weight
- Liver, kidney: weight and histopathological examination
- Individual weight and appearance of the placentas

- Number of (early/late) resorptions
- Number of live fetuses
- Sex of live fetuses
- Individual weights of fetuses
- External examination of fetuses (with special emphasis on the size of the eye bulges prior to and after skinning)
- Skeletal and cartilage development examination (with emphasis on the occurrence of supernumerary rudimentary (punctiform and comma-shaped) and extra ribs). Fetuses were eviscerated immediately after cesarean section, skinned and placed in ethanol for at least three days. The fetal skeleton was then double-stained with Alizarin Red S and Alcian Blue (according to [redacted] et al., 2000[11]).
- Examination of fetal heads: all fetal heads were skinned, fetal brains and eyes (distinguishing right from left) were extracted and weighed. The eyes were photographed so that both the horizontal and vertical diameters of the entire eye and of the cornea (including the area of the cornea), as well as, longitudinal length of the distance from the optic nerve remnant to the very front of the cornea could be measured (see Figure 5.6.2/03- 1 for an example).
  Fetal head dimensions were measured (anterior to posterior and right to left sides) using a digital caliper. After extraction of the eyes, the right and left orbit of each skull was photographed. The skull was retained in Bouin's solution (minimum of one week) and changed to 70% alcohol, after decalcification. Fetal head dimensions and photographs of the orbits were collected in the case that microphthalmia would be observed. Since microphthalmia did not occur in this study, the fetal head dimensions and photographs of the orbits were not reported.

Fetuses were sacrificed prior to skinning and decapitation.
Visceral examination was not performed.

[11] [redacted]AD, [redacted]DE, [redacted] AB (2000). Large-scale double-staining of rat fetal skeletons using Alizarin Red S and alcian blue. *Teratology*, **61**(4):273-6.

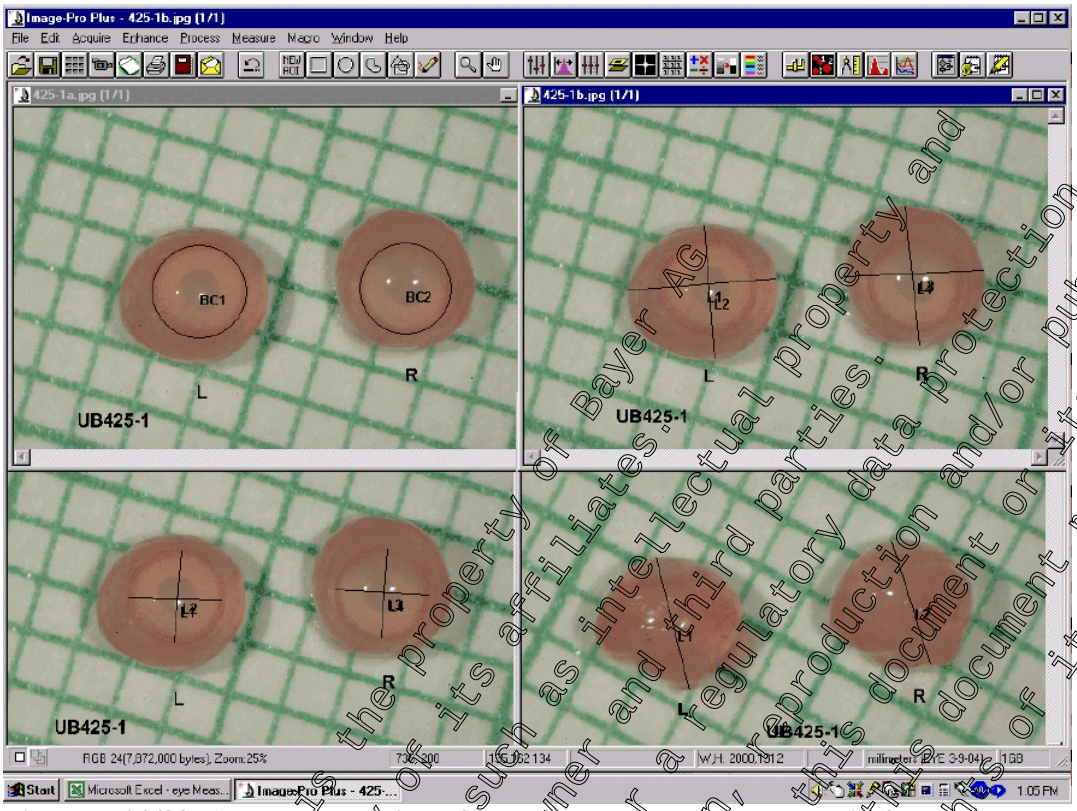


Figure 5.6.2/03- 1: Illustration of morphometric measurements in freshly extracted fetal eyes
(scale: one square = 1 mm²)

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSIS

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

No mortality occurred up to and including 750 mg/kg bw/d.

2. Clinical signs of toxicity

There were no compound-related clinical findings observed in any dose level up to and including 750 mg/kg.

C. FEED AND WATER CONSUMPTION, BODY WEIGHT AND BODY WEIGHT GAIN

1. Feed and water consumption

In the 750 mg/kg dose group significant declines in feed consumption from Days 6-12 (up to -27 % compared to control) and markedly increased water consumption from Days 11-20 (up to +74 %

compared to control) were observed (Figure 5.6.2/03- 2, Table 5.6.2/03- 2). There were no compound-related effects observed in any other group tested.

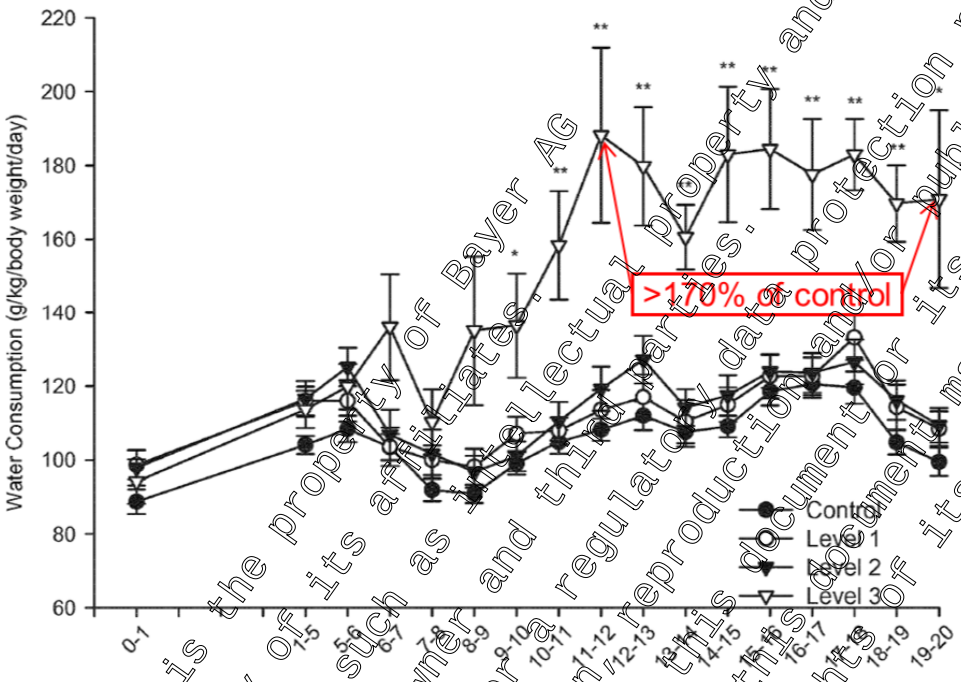


Figure 5.6.2/03- 2: Mean gestational water consumption (* $p \leq 0.05$, ** $p \leq 0.01$)

Table 5.6.2/03- 2: Feed and water consumption during gestation

	Prothioconazole (mg/kg bw/d)					
	0	**20**		**80**		**750**	
Mean Feed Consumption (g/kg bw/d) *(% difference to control)*							
Day 5-6	70.2	72.0	*(+3)*	74.0	*(+5)*	75.0	*(+7)*
Day 6-7	70.7	72.5	*(+3)*	69.9	*(-1)*	**55.4****	***(-22)***
Day 7-8	64.8	70.1	*(+8)*	66.9	*(+3)*	**52.3****	***(-19)***
Day 8-9	68.5	68.3	*(-0)*	67.7	*(-1)*	**49.8****	***(-27)***
Day 9-10	68.6	70.7	*(+3)*	69.6	*(+1)*	**55.4****	***(-19)***
Day 10-11	65.7	66.9	*(+2)*	63.4	*(-4)*	**58.1****	***(-12)***
Day 11-12	67.4	69.5	*(+3)*	67.5	*(±0)*	**60.2****	***(-11)***
Day 12-13	71.6	67.6	*(-6)*	71.6	*(±0)*	70.7	*(-1)*
Day 13-14	64.0	69.4*	*(+8)*	62.7	*(-2)*	66.1	*(+3)*
Day 14-15	62.3	67.9	*(+9)*	64.6	*(+4)*	66.1	*(+6)*
Day 15-16	66.4	64.6	*(-3)*	67.8	*(+2)*	64.2	*(-3)*
Day 16-17	66.4	69.5	*(+5)*	66.8	*(+1)*	66.6	*(±0)*
Day 17-18	64.1	68.1	*(+6)*	66.4	*(+4)*	67.8	*(+6)*
Day 18-19	59.5	64.3	*(+8)*	63.4	*(+7)*	64.4	*(+8)*
Day 19-20	56.2	58.9	*(+5)*	55.8	*(-1)*	61.2	*(+9)*

	Prothioconazole (mg/kg bw/d)							
	0	20		80		750		
Mean Water Consumption (g/kg bw/d) *(% difference to control)*								
Day 0-1	88.7	98.7	*(+11)*	97.9	*(+10)*	94.2	*(+6)*	
Day 1-5	104.1	116.0	*(+11)*	116.6	*(+12)*	113.7	*(+9)*	
Day 5-6	108.4	116.0	*(+7)*	125.2	*(+15)*	119.9	*(+11)*	
Day 6-7	103.5	103.6	*(±0)*	106.9	*(+3)*	136.1	*(+31)*	
Day 7-8	91.9	100.0	*(+9)*	101.7	*(+11)*	110.3	*(+20)*	
Day 8-9	90.8	98.2	*(+8)*	96.8	*(+7)*	135.1	*(+49)*	
Day 9-10	99.1	107.1	*(+8)*	101.0	*(+2)*	166.1	*(+68)*	
Day 10-11	104.9	107.9	*(+3)*	110.5	*(+5)*	198.4	*(+89)*	
Day 11-12	108.2	113.6	*(+5)*	119.3	*(+10)*	**188.2****	***(+74)***	
Day 12-13	112.0	117.0	*(+4)*	127.2	*(+14)*	**179.8****	***(+61)***	
Day 13-14	107.6	110.4	*(+3)*	114.2	*(+6)*	**160.6****	***(+49)***	
Day 14-15	109.1	115.0	*(+5)*	117.5	*(+8)*	**183.0****	***(+68)***	
Day 15-16	118.6	122.7	*(+3)*	124.0	*(+5)*	**184.5****	***(+56)***	
Day 16-17	120.6	122.8	*(+2)*	123.6	*(+2)*	**177.6****	***(+47)***	
Day 17-18	119.6	133.2	*(+11)*	126.6	*(+6)*	**183.0****	***(+53)***	
Day 18-19	104.8	114.3	*(+9)*	116.2	*(+11)*	**169.7****	***(+62)***	
Day 19-20	99.5	108.5	*(+9)*	109.4	*(+10)*	**170.9***	***(+72)***	

* significantly different from control, $p \leq 0.05$
** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

2. Body weight and body weight gain

750 mg/kg resulted in a reduction of -46 % in overall body weight gain during days 6–12 of gestation (Table 5.6.2/03- 3). In the corrected body weight gains (minus gravid uterus) a biologically significant decline of -13 % was observed when compared to controls. There were no compound-related effects observed in any other group tested.

Table 5.6.2/03- 3: Maternal body weight and body weight gain

Day post coitum	Prothioconazole (mg/kg bw/d)							
	0	20		80		750		
Final body weight (g) *(% difference to control)*								
Day 20	347.6	340.9	*(-2)*	344.6	*(-1)*	343.7	*(-1)*	
Body weight gain (g)								
Days 0 – 6	17.6	18.7		16.7		19.1		
Days 6 – 7	3	3.2		5.2		1.6		
Days 7 – 8	2.1	0.4		1.3		-1.4		
Days 8 – 9	2.2	2.6		2.5		0.4		
Days 9 – 10	3.8	4.4		3.6		2.5		
Days 10 – 11	4.7	3.7		3.8		4.2		
Days 11 – 12	2.6	4		2.3		2.6		

Day post coitum	Prothioconazole (mg/kg bw/d)							
	0	20		80		750	
Gravid uterus weight (g)							
Day 20	59.7	49.0		50.3		58.0	
Cumulative body weight gain (g) *(% difference to control)*							
Days 6 – 12	18.4	18.3	*(-1)*	18.7	*(+2)*	**9.9**	***(-46)***
Days 6 – 19	66.4	58.4	*(-12)*	62.5	*(-6)*	60.3	*(-9)*
Days 0 – 20	97.7	87.8	*(-10)*	89.9	*(-8)*	91.3	*(-7)*
Days 0 – 20 (corrected [a])	38.1	38.9	*(+2)*	39.7	*(+4)*	**33.2**	***(-13)***

[a] for uterus weight
Findings considered related to treatment with prothioconazole are written in **bold letters**

D. CLINICAL CHEMISTRY AND HEMATOLOGY

The clinical chemistry data revealed evidence of maternal toxicity at the 750 mg/kg dose group and included: statistically significant increases in urea nitrogen (BUN) and cholesterol (CHOL), and statistically decreased aspartate aminotransferase (AST) activity, as well as a non-statistical increase in alkaline phosphatase (ALP) activity. The urea nitrogen increase correlates with the increased water consumption seen at this level. There were no findings attributed to the test compound in the 20 or 80 mg/kg dose groups. Hematological examination did not reveal treatment-related effects in any dose group.

Table 5.6.2/03- 4: Clinical chemistry results (Day 20)

Parameter	Prothioconazole (mg/kg bw/d)			
	0	20	80	750
Enzymes				
ALT (U/L)	47	53	48	53
AST (U/L)	67	73	62	**56***
ALP (U/l)	65	77	73	**104**
GGT (U/L)	1	1	0	0
LDH (U/L)	164	216	155	109
CK (U/L)	222	229	238	195
Electrolytes				
Calc (mg/dL)	10.2	10.2	10.3	10.3
Cl (mmol/L)	101	101	101	101
Phos (mg/dL)	4.4	4.6	4.5	4.7
K (mmol/L)	5.9	5.8	5.9	5.9
Na (mmol/L)	141	142	141	141
Metabolites and Proteins				
CHOL (mg/dL)	83	76	74	**98***
TRIGL (mg/dL)	807	762	723	995
T-Bili (mg/dL)	0.1	0.1	0.1	0.1
BUN (mg/dL)	16	17	17	**19***

Parameter	Prothioconazole (mg/kg bw/d)			
	0	**20**	**80**	**750**
Creat (mg/dL)	0.9	0.8	0.9	0.9
T-Prot (g/dL)	6.1	6.2	6.2	5.8
Uric-A (mg/dL)	0.6	0.6	0.5	0.5
Gluc (mg/dL)	83	85	91*	86
Alb (g/dL)	3.7	3.8	3.8	3.5

* significantly different from control, $p \leq 0.05$
Findings considered related to treatment with prothioconazole are written in **bold letters**

E. NECROPSY OBSERVATIONS

There were no necropsy observations attributed to the test compound in any dose group. No compound-related effects were observed on the final body weight or liver or kidney weights, or on the micropathology in any group tested.

Table 5.6.2/03- 5: Organ weights

Parameter	Prothioconazole (mg/kg bw/d)			
	0	**20**	**80**	**750**
Final body weight (g)	348	341	345	344
Liver weight (g)	12.3	12.1	13.0	12.7
Liver weight/ body weight ratio	3.6	3.5	3.8*	3.7
Kidney weight (g)	1.8	1.8	1.9	1.8
Kidney weight/ body weight ratio	0.5	0.5	0.6	0.5

* significantly different from control, $p \leq 0.05$

Maternal toxicity:

The signs of maternal toxicity observed in the present study (reduction in body weight gain, markedly increased water consumption, decreased feed consumption and clinical chemical indicators of functional impairment of the kidneys) are consistent with the findings in the subacute ([redacted] and [redacted], 1997 [M-012338-01-1], *(*study summary not included yet*))* and subchronic ([redacted] and [redacted], 1999 [M-011757-01-1], *(*study summary not included yet*)*) rat toxicity studies with prothioconazole. Furthermore, increased water intake had also been observed in the first rat developmental toxicity study ([redacted], 1997) and in the pilot maternal toxicity dose range finding study for the present study ([redacted], 2004 [M-067839-01-1]).

The results of the present study correlate well with the established toxicological profile of prothioconazole in the rat (disturbed kidney function and resulting impaired systemic water homeostasis), although the study duration was obviously too short to render the functional renal impairment into morphological changes as seen in the subacute to long-term rat studies.

There were no treatment-related findings in the low- and mid-dose groups. In the context of the 25 % mortality observed in the pilot maternal toxicity range finding study at 1000 mg/kg (obviously due to overt dehydration), 750 mg/kg, the highest dose tested in this study, is considered to be a maximum tolerated dose (MTD).

E. CAESAREAN SECTION DATA

There were no treatment-related reproductive effects nor were there any significant differences in the litter size, the median percent male fetuses, or fetal or placental weights in any group tested.

Table 5.6.2/03- 6: Summary of reproductive data

Parameter	Prothioconazole (mg/kg bw/d)			
	0	20	80	750
No. pregnant / no. mated				
No. dams with resorptions only	0	2	1	0
No. dams with live fetuses	21	18	18	23
Mean no. corpora lutea / dam	13.5	12.9	13.7	13.4
Mean no. implantation sites / dam	11.8	10.4	10.5	11.9
Pre-implantation loss (mean no. / dam)	1.7	2.5	3.6	1.5
Pre-implantation loss (% of corpora lutea)	12.6	19.5	25.9	12.1
Mean no. resorptions/dam	1.3	1.5	1.3	1.4
early resorptions	1.3	1.5	1.2	1.3
late resorptions	0.0	0.1	0.1	0.1
No. dead fetuses	0	0	0	1
Post-implantation loss (mean no. / dam)	1.3	1.5	1.2	1.4
Post-implantation loss (% of implantations)	11.7	27.4	15.1	13.3
Mean no. live fetuses/dam	10.5	8.8	8.9	10.5
Sex ratio (% males)	56.1	49.4	52.9	51.5
Mean fetal weight, both sexes (g)	3.7	3.7	3.8	3.6
Mean fetal weight of males (g)	3.8	3.8	3.9	3.7
Mean fetal weight of females (g)	3.6	3.6	3.6	3.5
Placental weight (g)	0.51	0.48	0.52	0.52

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

No treatment-related effects were observed on external or skeletal malformations or on external variations. In the pilot maternal toxicity dose range finding study for the present study no external findings attributed to the test compound at any dose group tested (up to and including 1000 mg/kg bw/d).

Ocular Investigations

The external examinations before and after skinning did not reveal any single fetus exhibiting microphthalmia in any dose group tested up to and including the 750 mg/kg dose group. No compound-related effects were observed on the individual or mean eye weights, eye to fetal weight ratios, or on eye measurements (Table 5.6.2/03- 9 and Table 5.6.2/03- 8). There was no evidence that prothioconazole caused microphthalmia in any dose group.

Table 5.6.2/03- 7: Summary of brain/eye weight

Parameter	Prothioconazole (mg/kg bw/d)							
	0		20		80		750	
	Left	Right	Left	Right	Left	Right	Left	Right
Mean eye weights, absolute (mg ± SD)	7.79 ± 0.59	7.83 ± 0.55	7.78 ± 0.96	7.85 ± 0.91	7.81 ± 0.92	7.86 ± 0.75	7.57 ± 0.63	7.61 ± 0.59
Mean eye weights, relative to fetal weight (mg/g ± SD)	2.12 ± 0.12	2.13 ± 0.14	2.11 ± 0.12	2.13 ± 0.14	2.08 ± 0.15	2.10 ± 0.14	2.13 ± 0.25	2.11 ± 0.16
Mean brain weight (g ± SD)	0.16 ± 0.01		0.16 ± 0.02		0.16 ± 0.02		0.16 ± 0.01	

Table 5.6.2/03- 8: Summary of morphometry of fetal eyes

Parameter	Prothioconazole (mg/kg bw/d)							
	0		20		80		750	
	Left	Right	Left	Right	Left	Right	Left	Right
Mean cornea diameter (mm ± SD)	1.78 ± 0.04	1.79 ± 0.05	1.78 ± 0.07	1.80 ± 0.07	1.78 ± 0.06	1.80 ± 0.06	1.77 ± 0.04	1.79 ± 0.04
Mean cornea area (mm ± SD)	2.40 ± 0.12	2.45 ± 0.13	2.43 ± 0.21	2.46 ± 0.21	2.43 ± 0.16	2.49 ± 0.17	2.40 ± 0.15	2.42 ± 0.11
Mean globe diameter (mm ± SD)	2.58 ± 0.06	2.60 ± 0.07	2.57 ± 0.11	2.60 ± 0.13	2.59 ± 0.09	2.62 ± 0.09	2.58 ± 0.07	2.60 ± 0.08
Mean globe length (lateral) (mm ± SD)	2.44 ± 0.07	2.44 ± 0.07	2.44 ± 0.09	2.45 ± 0.11	2.42 ± 0.16	2.44 ± 0.08	2.43 ± 0.09	2.43 ± 0.10

The large sample size collected in this study (control: 442 eyes; 750 mg/kg group: 482 eyes) allows for a graphical presentation of the data as a normal distribution curve. The figure below (Figure 5.6.2/03-3) demonstrates the normal distribution pattern of eye weight in the control and high dose fetuses. At the left (and right) end of the curve there is no indication for any individual values being outside the normal range of control animals. Also the frequency distribution between the two groups is remarkably similar. This illustrates the complete lack of any fetus with reduced eye size in the high dose group of this study when compared to concurrent control animals.

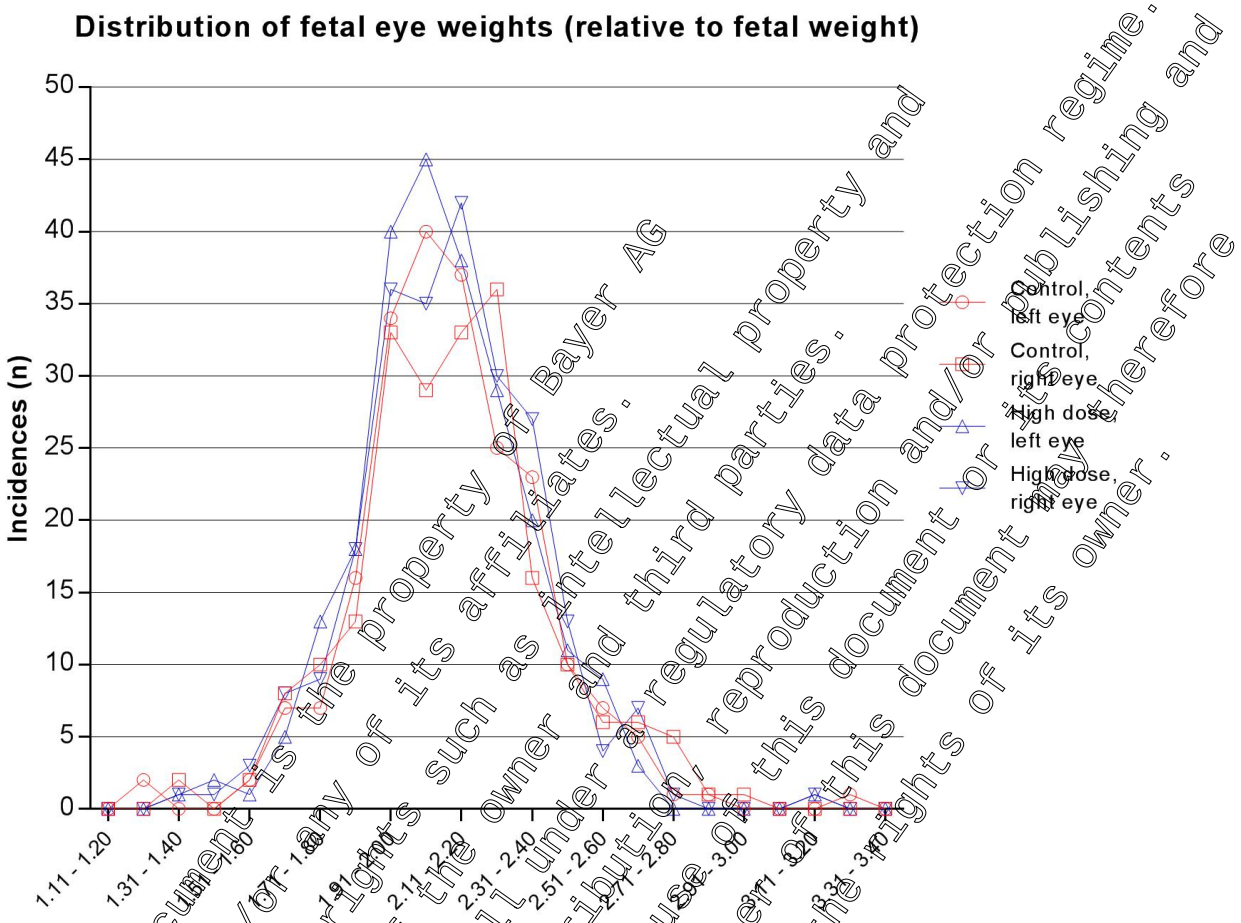


Figure 5.6.2/03- 3: Distribution of fetal eye weights (relative to body weight)

Table 5.6.2/03- 9: Incidences (n) of the distribution of fetal eye weights (relative to body weight) in the control and high dose group

Eye/pup weight intervals (mg/g)	Control, left eye	Control, right eye	High dose, left eye	High dose, right eye
1.11 - 1.20	0	0	0	0
1.21 - 1.30	2	0	0	0
1.31 - 1.40	0	2	1	1
1.41 - 1.50	0	0	2	1
1.51 - 1.60	3	2	1	3
1.61 - 1.70	7	8	5	8
1.71 - 1.80	7	10	13	9
1.81 - 1.90	16	13	18	18
1.91 - 2.00	34	33	40	36
2.01 - 2.10	40	29	45	35
2.11 - 2.20	37	33	38	42
2.21 - 2.30	25	36	29	30
2.31 - 2.40	23	16	20	27
2.41 - 2.50	10	10	11	13
2.51 - 2.60	7	6	9	4
2.61 - 2.70	5	6	3	7

Eye/pup weight intervals (mg/g)	Control, left eye	Control, right eye	High dose, left eye	High dose, right eye
2.71 - 2.80	1	5	0	1
2.81 - 2.90	1	1	0	0
2.91 - 3.00	0	1	0	0
3.01 - 3.10	0	0	0	0
3.11 - 3.20	0	0	1	1
3.21 - 3.30	1	0	0	0

For Wistar Hannover rats (Crl:WI(HAN)), the available historical control database reveals a virtually zero background incidence of microphthalmia ([M-576725-01-1]). That this substrain is nevertheless sensitive to a direct, specific oculo-teratogenic effect was demonstrated in a study with the positive control substance All Trans-Retinoic Acid (study number 01-T82-DA1, [M-517045-01-1]) (Table 5.6.2/03- 10). Therefore Wistar Hannover rats are well suited to decisively investigate the specificity of microphthalmia formation, while excluding a non-specific enhancement of microphthalmia secondary to maternal toxicity.

Table 5.6.2/03- 10: Specific oculo-teratogenic effect induced by All Trans-Retinoic Acid in Wistar Hannover rats (results from study number 01-T82-DA1, [M-517045-01-1])

	Control	All Trans-Retinoic Acid 15 mg/kg
External findings		
Fetuses evaluated (n)	186	83
Litters evaluated (n)	16	14
Eyes – no eye bulge (fetal (litter) incicende in %)	0.0 (0.0)	71.1** (100.0**)
Visceral findings		
Fetuses evaluated (n)	99	36
Litters evaluated (n)	16	12
Microphthalmia (fetal (litter) incicende in %)	0.0 (0.0)	5.6 (16.7)
Anophthalmia (fetal (litter) incicende in %)	0.0 (0.0)	22.2** (41.7**)

* $p \leq 0.05$; ** $p \leq 0.01$

The fact that in this rat strain prothioconazole did not cause a single case of microphthalmia (even at the maximum tolerated dose of 750 mg/kg) confirms that the increased incidence of microphthalmia which had been observed in the fetuses of the first developmental study ([redacted], 1997) at 1000 mg/kg (using a "high background incidence" rat strain for microphthalmia) was not caused by a specific or direct teratogenic effect of prothioconazole but was an enhancement of a common spontaneous malformation in that rat strain secondary to strong maternal toxicity at this dose level. The present study thus provides a mechanistic clarification for the pathomechanism of microphthalmia formation; it does not just show a simple "difference in the sensibility of the strains used".

Skeletal investigations

Skeletal evaluation revealed a possible treatment related increase in the fetal incidence of supernumerary rudimentary (comma-shaped) ribs at 750 mg/kg. The litter incidence was not significantly affected

(Table 5.6.2/03- 11). A treatment-related effect on punctiform or fully-formed supernumerary ribs was not discernible. The fetal incidence of the comma shaped rudimentary ribs (21.2%) was only marginally outside the historical control range for the same laboratory and rat strain (max. 18 %), and that for punctiform ribs (33.6) was well within the historical control range (max. 82 %). There were no alterations in supernumerary ribs at 20 and 80 mg/kg bw/d. The marginal increase of rudimentary (comma-shaped) supernumerary ribs at 750 mg/kg bw/d is considered as an enhancement of this common variation secondary to the marked maternal toxicity at this dose.

Table 5.6.2/03- 11: Summary of effects on supernumerary ribs (Fetal (litter) incidence in %)

	Prothioconazole (mg/kg bw/d)				
	0	**20**	**80**	**750**	**HCD range [a]**
Rudimentary (punctiform)	23.5 (95.2)	18.2 (77.8)	27.6 (88.9)	33.6 (95.7)	19 – 82 (57 – 91)
Rudimentary (comma-shaped)	11.8 (52.4)	7.4 (66.7)	12.4 (38.9)	**21.2*** (69.6)	4 – 18 (9 – 58)
Extra (full-size)	6.3 (33.3)	3.4 (16.7)	1.2* (11.1)	8.3 (39.1)	0 3 (0 – 14)

* $p \leq 0.05$; ** $p \leq 0.01$

[a] Historical control data from 4 studies (522 fetuses, 97 litters) [M-576725-01-1]

Findings considered related to treatment with prothioconazole are written in **bold letters**

There were no compound-related skeletal malformations observed in any group up to and including 750 mg/kg. Malformations considered incidental to treatment were extra thoracic arches and centra (2 fetuses of 2 litters) in the control group and (2 fetuses of 1 litter) in the 20 mg/kg dose group (Table 5.6.2/03- 12).

The only other statistically significant finding for fetal morphology was an increase in the fetal (but not in the litter) incidence of delayed ossification of the second sternebra at 750 mg/kg. This minimal reversible retardation in sternal ossification was considered a threshold effect at most because the litter incidence was unaffected, and the value (33.6 %) was well within this laboratory's historical control range (10.3 % to 49.2 %). Therefore, a treatment-related effect of prothioconazole is not assumed for the delayed ossification of the second sternebra.

Table 5.6.2/03- 12: Summary of further skeletal findings

	Prothioconazole (mg/kg bw/d)				
	0	**20**	**80**	**750**	**HCD range [b]**
Thoracic arches and centra - extra	0.2 (9.5)	1.1 (5.6)	0 0.0	0 0.0	0.0 – 0.9 (0.0 – 4.5)
Second sternebra – incompletely ossified	21.7 (76.2)	23.3 (77.8)	26.5 (66.7)	33.6* (78.3)	10.3 – 49.2 (38.5 – 80.8)

* $p \leq 0.05$

[b] Historical control data from 4 studies (522 fetuses, 97 litters) included in original report (p.588 f.)

III. CONCLUSION

At 750 mg/kg bw/d dams showed decreased body weight gain, drastically increased water consumption and clinical chemistry indications of functional impairments of kidneys and liver. In a pilot maternal toxicity dose range finding study also caused decreased body weights and increased water consumption

at doses ≥ 500 mg/kg bw/d. A dose of 1000 mg/kg bw/d even resulted in 25 % mortality due to dehydration. Based on this strong, even sublethal maternal toxicity at 750 mg/kg bw/d, the **maternal NOAEL is 80 mg/kg bw/d**.

Based on the results from the external examinations before and after skinning, the individual or mean eye weights, the eye measurements, or the eye to fetal weight ratios, there was no evidence to support that prothioconazole caused microphthalmia in any dose group tested up to and including the 750 mg/kg dose group. At 750 mg/kg bw/d there was a marginal increase of fetal supernumerary rudimentary (comma-shaped) ribs (variation), which is assessed as a secondary non-specific consequence of the disrupted maternal water homeostasis at the same dose. The **developmental NOAEL is 80 mg/kg bw/d**. Given the marginality of the increase above the historical control range; the absence of other developmental effects (microphthalmia, punctiform or fully formed supernumerary 14th ribs); the actual impact of comma-shaped supernumerary 14th ribs on the well-being on the animal and the large dose spacing, this NOAEL is conservative.

Report:	KCA 5.6.2/03 [redacted]; 2001; M-035764-01-1
Title:	A dermal developmental toxicity study with (JAU 6476, technical material and products) in the Wistar rat
Report No.:	737673
Document No.:	M-035764-01-1
Guideline(s):	US EPA, OPPTS 870.3700 (1998) EU, 91/414/EEC OECD, Section 4, Guideline 414 (1981) Japan MAFF, 59 NohSan No. 4200 (1985) Health Canada PMRA DACO No. 4.5.2
Guideline deviation(s):	yes (for supplemental study only), see report.
GLP/GEP:	**yes**

In the original dossier this study received the reference number KCA 5.6.2/03. This numbering is fixed and cannot be changed retroactively. However, for logical reasons, in the present document the study is named **5.6.2/04** (also in numbering of tables/figures).

Deviations:	Deviations from the current OECD guideline (2001): The number of pregnant animals was below 20 in one group (diluted EC250 group) but overall the performance of the study is considered acceptable.

Executive summary:

In a 1998 GLP study, groups of 20-30 mated female Wistar rats (actual numbers pregnant were 17-23) received daily topical applications of test material (6 hours/d, non-occlusive dressing) from days 6 to 19 post coitum. The test material was one of the following:
- technical prothioconazole as a dry powder (batch no. 6233/0031, purity 98.1-98.8%) moistened with water at a dose level of 1000 mg/kg bw/d,
- an EC formulation (EC250) containing 25 % prothioconazole at a dose level of 1000 mg/kg bw/d (equivalent to 250 mg prothioconazole/kg bw d),
- a 1:3 aqueous dilution of the above EC formulation (EC250) at a dose level of 1000 mg/kg bw/d (equivalent to 62.5 mg prothioconazole/kg bw d),
- deionised water (vehicle control).

The study was compliant with or exceeded the requirements of the contemporary OECD Guideline 414 (1981). Dosing was extended from day 15 to day 19 to comply with the appropriate US-EPA Guideline

(which brings the dosing pattern in this study in line with the 2001 OECD Guideline 414). The dose level of 1000 mg/kg bw/d is a limit dose for this type of study.

There were no deaths. There were signs of skin irritation noted in animals treated with the undiluted EC250 formulation, consisting of erythema and edema in one animal, eschar formation in 6 animals and scaling and sloughing of the application site in 20 animals. Red vaginal discharge was observed sporadically in all treated groups except the control group, in both pregnant and non-pregnant animals, from days 14-17. A supplementary study was performed to investigate the relationship of this finding to treatment with prothioconazole. From the results of the supplementary study it can be concluded that this red vaginal discharge was not related to treatment with prothioconazole. There were no effects of treatment on bodyweights, feed consumption, gross necropsy findings and reproductive parameters in any treatment group. The overall litter and fetal incidences of abnormalities were comparable to controls in all treated groups and the nature of the abnormalities recorded did not indicate any particular effect of treatment. The pattern of malformations, visceral abnormalities and variations and skeletal abnormalities between the control and treated groups did not indicate any effect of treatment.

The NOAEL for maternal and developmental toxicity via dermal application was 1000 mg/kg bw/d for prothioconazole technical material, 1000 mg/kg bw/d for the EC250 formulation (equivalent to 250 mg/kg bw/d prothioconazole) and 1000 mg/kg bw/d for the diluted EC 250 formulation (equivalent to 62.5 mg/kg bw/d prothioconazole).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole (technical)
Synonym:	JAU 6476 technical
Description:	Beige powder
Batch No.:	6233/0031
Purity:	98.1-98.8 %
CAS No.:	178928-70-6
Stability of the test compound:	Guaranteed at least for the time of study duration
1. Further Test Material:	Prothioconazole EC250 (formulation of prothioconazole)
Synonym:	JAU 6476 EC250
Description:	Clear brown liquid
Batch No.:	0202 (based on # 06025/0003)
Purity:	25 % active ingredient
CAS No.:	178928-70-6
Stability of the test compound:	Guaranteed at least for the time of study duration
2. Vehicle/solvent:	Dry material was only moistened with water for application EC250: deionized water
3. Test animals:	
Species:	Rat
Strain:	Wistar Hannover, Crl:WI(HAN)
Sex:	Males and females (nulliparous and not pregnant) (Males were not treated with prothioconazole technical or EC250 and were only used for mating)
Age:	12–15 weeks (females) 15–17 weeks (males)
Weight at dosing:	175–248 g (females)

Source:	[redacted], USA
Acclimation period:	At least 6 days before mating
Diet:	Purina Mills Rodent Lab Chow 5001-4, *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	Upon arrival animals were individually housed in suspended stainless steel cages. During co-housing phase a maximum of two females were housed with one male. Females found sperm positive were individually housed in plastic cages with corn cob bedding.
Environmental conditions:	
Temperature:	18–26 °C
Humidity:	30–70 %
Photo period:	Artificial illumination, 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: main study: September 7, 1998 – February 17, 1999
suppl. study: June 14, 1999 – July 7, 1999

2. Animal assignment and treatment

Mating and start of gestation

Rats were co-housed with a maximum of two females per male at one time. Following cohabitation, morning vaginal smears were taken and examined for the presence of sperm. Females found to be sperm-positive were randomized into groups as described below. The day on which sperm was observed in the vaginal smear was designated day 0 of gestation for that female.

Dose levels, experimental group and rationale for choice of dose levels

The male animals were used for mating only and were not treated. After insemination was ascertained, females each were allocated to four experimental groups according to a computer-generated randomization plan.

Based on the anticipated absence of any effects during the conduct of a developmental toxicity study, the limit dose for this study type, 1000 mg/kg/d, was selected. The proposed dose groups were 0 and 1000 mg/kg prothioconazole (technical material), 1000 mg/kg of the EC250 formulation (equivalent to 250 mg of the active ingredient/kg), and a 1:3 aqueous dilution of the EC250 formulation (equivalent to 62.5 mg of the active ingredient/kg) (Table 5.6.2/04-d). The latter dose group was included to investigate the potential effects of an aqueous dilution of the test material. All doses were administered dermally on non-occlusive dressings. The treatment sites were wiped with water after removal of the dressings.

Table 5.6.2/04- 1: Study design of the main study

	Control	Group I technical	Group II diluted EC250 (1:3)	Group III EC250
Number of dams:	30	29	29	30
mg/kg body weight	0	1000	1000	1000
Concentration of active ingredient (mg/kg)	0	1000	62.5	250

The females in the prothioconazole technical (dry material) treatment group received 1000 mg/kg body weight. Based on an approximate density of 1 g/ml, both EC 250 formulation treatment groups were administered 1 ml/kg. The dry technical grade material, the undiluted formulation, and the diluted

formulation were administered dermally, daily on days 6 through 19 of gestation. Control animals were similarly administered 1 ml deionized water/kg body weight. Dosing weight/volume was adjusted daily, based on dam body weight during the dosing period.

Dermal administration of the test substance

Prior to application of the first dose, and as necessary depending on hair growth and body weight gain, an area representing approximately 10 % of the total surface area of each female was shaved. The area to be clipped was determined based on the following table:

Table 5.6.2/04- 2: Determination of the area to be shaved prior to application of the test substance

Body weight (g)	Surface area (cm^2)	10 % of surface area	Square (cm)
100-199	300	30	5.5
200-299	394	39	6.3
300-399	477	48	6.9
400-499	554	55	7.4
500-599	625	63	7.9
600-699	693	69	8.3

On each day of dosing the animals were weighed, the test material amount was determined based on the body weights and it was then applied to the shaved dose area. Specifically, the dry active ingredient was applied to gauze, moistened, and the gauze was then placed (compound-side toward the skin) on the area shaved as described above. The gauze was moistened with deionized water using an aerosol atomizer both prior to, and following application of the dry test material. The undiluted liquid EC250 formulation and the diluted EC250 formulation was also applied to gauze and the gauze was then placed on the shaved dose site. For the control group, deionized water was applied to the gauze and placed on the shaved dose site. For all animals, the gauze was held in place with a minimal amount of tape to keep the gauze on the dose site while still maintaining non-occlusive conditions. Approximately six hours (± 30 minutes) following dose application the gauze was removed, and the dose site was wiped of any residual test material using clean gauze moistened with deionized water.

Supplementary study

This study was conducted to investigate the potential relationship between prothioconazole and a maternal clinical finding, red vaginal discharge, observed during the conduct of the main dermal developmental toxicity study. The supplementary study featured groups of 5 or 10 mated female Wistar rats treated as follows:

Table 5.6.2/04- 3: Study design of the supplementary study

	Group A	Group B	Group C	Group D
Number of dams	10	10	10	5
Treatment	Prothioconazole technical	Deionized water	Prothioconazole technical	Colored dye (green)*
Dose	1000 mg/kg	--	33 mg (13.5-58.1 mg)	1000 mg/kg
Application route	Dermal (replicating main study)	Directly to vaginal area (on the surface of the vaginal opening)		Dermal (as group I)
Duration of treatment	Gestation days 6-19			Gestation days 6-8

* Rit tint and dye, Kelly green #32

As the main study was conducted via the dermal route of exposure, it was thought that during normal grooming the animals may have transferred some of the test compound from the dose site to the vaginal area, potentially causing irritation, resulting in the red vaginal discharge observed during the study. The supplementary study sought to investigate this possibility by applying the test compound directly to the vaginal area. The supplementary study also attempted to reproduce the previous findings by replicating the study procedures and utilizing the highest dose of the main study. A fourth group (group D) with dermal application of a green dye was included in order to establish whether a dry material, applied as described in the main study, could migrate from the dosage site. Only the maternal findings, and in particular the gestational clinical signs, were considered in the supplementary study.

In all other respects, the study replicated the conditions of the main study (i.e. animals wore gauze patches (dry gauze in the case of vaginally treated animals), collars etc.). From five animals from the water control group (direct vaginal application, group B) and the 1000 mg/kg bw/d dermal application group (group A), serum and vaginal wipe samples were taken and analysed for the presence of prothioconazole. A gross necropsy and histopathological examination of the uterus, cervix and vagina was also performed.

3. Test substance preparation and analysis (main study)

The technical material and EC250 formulation were used undiluted; therefore, dose analysis was not performed on these samples. Homogeneity, stability, and analysis of concentration were performed on the aqueous dilution (1:3) of the EC250. The solution was stable for 46 days and the mean actual concentration in the dosing solution used for the study was 66.7 mg/ml.

A liquid chromatographic method was used to measure the actual concentration of the diluted EC250 formulation utilized in this study and the homogeneity and stability of the diluted EC250 formulation [M-091268-01-1]. The analytically determined concentration and the percent nominal value for the study were 64.7 and 104 %, respectively. The homogeneity of this preparation is presented in Table 5.6.2/04- 4.

Table 5.6.2/04- 4: Homogeneity of the diluted EC250 formulation used for the study

Sample	Target concentration (mg/mL) 1
1	66.81
2	66.96
3	66.45
Mean	**66.74 mg/mL**
SD	**0.2621**
CV	**0.3927**
% Theoretical	**106.7840**

SD: standard deviation
CV: coefficient of variation

Based on a coefficient of variation of 0.3927, the diluted EC250 formulation was considered homogeneous. The results from the analysis of the room temperature stability of the diluted formulation are presented in Table 5.6.2/04- 5.

Table 5.6.2/04- 5: Stability of diluted EC250 formulation stored at room temperature

Time	Test concentration	
	62.5 mg/mL	% initial
Day 0 (=start)	66.7	100
Day 7	64.6	96.8
Day 14	63.3	94.8
Day 21	61.2	91.7
Day 28	61.5	91.8
Day 33	64.3	96.3
Day 39	63.1	94.5
Day 46	63.8	95.5
Mean	**63.52**	
SD	**1.81**	
CV	**2.85**	

SD: standard deviation
CV: coefficient of variation

The diluted EC250 formulation was considered stable at room temperature for 7 weeks with less than a 5 % decline in concentration.

4. Statistics (both studies)

The data was analyzed with the litter as the primary experimental unit using applications provided by TASC.

Differences between the control and test compound-treated groups were considered statistically significant when $p \leq 0.05$. Statistical significance was tested using the following methods:

Statistical test	Parameter
Analysis of Variance (ANOVA) (and in case of significant results Dunnett's t-Test) as posthoc test for:	Parametric data (including dam body weights and feed consumption) (Fetal and placental weights were specifically analyzed via the Healy's Test if significance was observed in the ANOVA)
CHI² test; in case of significant differences Fisher's exact test with Bonferroni correction for:	Nonparametric dichotomous data (e.g. number normal/abnormal)
Kruskall-Wallis test (and in case of significant differences Dunn's test) for:	Nonparametric data (e.g. litter size and number of corpora lutea)

C. METHODS

1. Observations

From day 0 to 20 p.c. all animals were inspected once daily. Mortality checks were performed twice daily during the workweek and once daily on weekends and holidays.

2. Feed and water consumption

Feed and water consumption was measured on gestation days 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20.

3. Body weight development

The body weights of the animals were determined daily from day 0 to 20 p.c..

4. Investigations at Cesarean Section (main study)

On day 20 of gestation, the dams were terminated by carbon dioxide asphyxiation and a gross external examination was performed. The abdomen and thoracic cavities were opened and a gross internal necropsy performed. The ovaries were excised, corpora lutea counted, and pregnancy determined. A sample of skin from the dose site of all gravid dams was collected and put in formalin but evaluation of these skin samples was not performed. The intact uterus was removed and weighed. The uterus was then opened and resorptions, if any, characterized.

Fetuses were removed from the uterine wall and each implant noted. The placentas were trimmed of extraneous tissue, blotted, and weighed. Fetuses were sacrificed and all fetuses were individually identified, sexed, weighed, and externally examined. Approximately half of all fetuses from each litter were fixed in 70-95 % alcohol (processing to include skinning, evisceration, and staining) and evaluated for general skeletal (including cartilage) development. The remaining fetuses were subjected to a gross visceral examination and placed in Bouin's solution. Prior to fetal cranial examination, the fetuses were transferred to 70 % alcohol. Sectioning of the head was performed according to the method of Wilson.

Dams sacrificed on gestation day 20 and suspected to be not pregnant underwent a gross examination. The abdomen was opened and the uterus was flushed with saline or water to verify the patency of the cervical/uterine os. If the openings were patent the uterus was excised and examined for implantation sites to confirm pregnancy. If the dam was not pregnant no further examinations were performed. Dams found dead, moribund, or delivering prematurely while on study, were sacrificed and a gross necropsy was performed.

Evaluated parameters

Reproductive and dam assessment included:

- Fertility, gestation and mating indices
- Body weight gain (corrected for gravid uterine weight)
- Feed consumption
- Clinical signs
- Necropsy findings
- Organ weights: gravid uterus
- Number of corpora lutea
- Total number if implantations

Litter assessment included:

- Total number of fetuses
- Number of viable fetuses
- Number of non-viable fetuses
- Fetal sex distribution
- Number type of resorptions
- Number of affected (i.e. nonviable and malformed) implants
- Number of malformed males or females
- Pre-implantation loss: # corpora lutea - # implants / # corpora lutea x 100
- Post-implantation loss: # implantations - # viable progeny / # implantations x 100
- Number of affected litters

Fetal assessment included:

- Placental weight
- Fetal weight
- Type and incidence of:
  - external malformations and variations
  - visceral malformations and variations
  - skeletal malformations and variations

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSIS

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

No mortality occurred in any group (neither in the main nor in the supplementary study).

2. Clinical signs of toxicity

Results of the main study

There were remarkable clinical observations noted in all test compound-treated groups.

Clinical signs observed only in the EC250 formulation group (group III) included:

- 1 animal exhibiting erythema and edema at the dose site
- 6 animals exhibiting eschar at the dose site and
- 20 animals exhibiting scaling sloughing at the dose site.

Also noted in the EC250 formulation group were 5 animals self-vocalizing just after administration of test material (Table 5.6.2/04- 6).

Also possibly attributed to the test compound, and observed in all groups except the concurrent control, and regardless of pregnancy status, was red vaginal discharge. This finding was noted sporadically during days 14-17 of gestation when removing the gauze from the dose site just after the six hour exposure. Specifically, four out of twenty-nine females in the diluted EC250 formulation group, six out of thirty females in the EC250 formulation group, and twenty-three out of twenty-nine females in the technical material group exhibited this observation. The potential relationship between the test compound and red vaginal discharge was specifically evaluated in a supplemental developmental toxicity study. The results of the supplemental study did not demonstrate any relationship between the test compound and red vaginal discharge.

Table 5.6.2/04- 6: Summary of clinical observations during gestation (day 6-20) – main study

Parameter	Dose group			
	Control 0 mg/kg	**Group I 1000 mg/kg technical**	**Group II 1000 mg/kg diluted EC250**	**Group III 1000 mg/kg EC250**
No remarkable clinical observations	23	21	17	21

Parameter	Dose group			
	Control 0 mg/kg	**Group I 1000 mg/kg technical**	**Group II 1000 mg/kg diluted EC250**	**Group III 1000 mg/kg EC250**
Self-vocalization	0	0	0	**5**
Lacrimation	1	0	1	2
Nasal stain	9	6	7	8
Urine stain	1	0	0	1
Scaling/sloughing	0	0	0	**20**
Eschar	0	0	0	**6**
Erythema	0	0	0	**1**
Edema	0	0	0	**1**
Red vaginal discharge	0	10	4	6

Females may exhibit more than one sign.
Table includes only females found pregnant at termination (day 20).
Findings considered related to treatment with test substance are written in **bold letters**

Results of the supplementary study

Given the purpose of this study, the clinical sign of interest was red vaginal discharge. This clinical sign was observed in groups A, B, and C. As noted in the methods section, the fourth group of animals were used to determine the potential for a dry material to migrate from the dose site. Migration of the applied material, observed as dye outside the dose site, was observed within gestation days 6-8. Following this observation, these animals were sacrificed. Red vaginal discharge was not observed in group 4 as these animals were sacrificed prior to the onset of this clinical sign.

Table 5.6.2/04- 7: Summary of clinical observations during gestation (day 6-20) – supplementary study

	Group A 1000 mg/kg technical dermal application	**Group B deionized water vaginal application**	**Group C 1000 mg/kg technical vaginal application**
Number of dams examined	10	10	10
Red vaginal discharge	8	6	8

In group A (1000 mg prothioconazole/kg administered as described in the main study), red vaginal discharge was observed in 8 of 10 dams, initially observed on gestation day 13 and resolved in all affected females by gestation day 18 (Table 5.6.2/04- 7). Group B (direct application of water to the vaginal area, no exposure to the test compound), exhibited red vaginal discharge in 6 of 10 dams, with onset on gestation day 13 and resolution in all affected females by gestation day 18. In group C (direct vaginal application of 1000 mg prothioconazole/kg), red vaginal discharge was observed in 8 of 10 dams, initially observed on gestation day 12 and resolved in all affected females by gestation day 18. Hence, both the incidence and duration were similarly observed in all three affected groups.

The fact that red vaginal discharge was recorded in control animals which had not been exposed to prothioconazole indicates that this clinical sign is not related to treatment with prothioconazole.

C. FEED CONSUMPTION, BODY WEIGHT AND BODY WEIGHT GAIN

In the main study, there were no statistically significant findings for feed consumption or body weight for any of the groups (Table 5.6.2/04- 8) nor any statistically significant effects on uterine weight or corrected body weight change.

Table 5.6.2/04- 8: Maternal feed consumption, body weights during gestation and gravid uterus weights

	Dose group							
	Control 0 mg/kg	**Group I 1000 mg/kg technical**		**Group II 1000 mg/kg diluted EC250**		**Group III 1000 mg/kg EC250**	
Mean Feed Consumption (g/kg bw/d) *(% difference to control)*							
Day 6-8	72.3	76.6	*(+6)*	76.1	*(+5)*	71.7	*(-1)*
Day 8-10	78.6	80	*(+2)*	78.5	*(±0)*	76.6	*(-3)*
Day 10-12	82.5	80.6	*(-2)*	77.8	*(-4)*	82.3	*(±0)*
Day 12-14	88.3	87.2	*(-1)*	86.2	*(-2)*	88.4	*(±0)*
Day 14-16	85	82	*(-4)*	83.1	*(-2)*	85.2	*(±0)*
Day 16-18	83.5	84.3	*(+1)*	84.6	*(+1)*	83.4	*(±0)*
Day 18-20	78.1	78.3	*(±0)*	79.4	*(+2)*	78.5	*(+1)*
Body weight (g) *(% difference to control)*							
Day 0	211.5	212.6	*(+1)*	207.2	*(-2)*	209.4	*(-1)*
Day 6	226.0	232.1	*(+3)*	225.3	*(±0)*	227.9	*(+1)*
Day 9	230.6	236.2	*(+2)*	228.9	*(-1)*	230.2	*(±0)*
Day 12	241.2	244.3	*(+1)*	236.7	*(-2)*	236.9	*(-2)*
Day 15	254.1	254.7	*(±0)*	250.3	*(-1)*	251.9	*(-1)*
Day 18	277.1	278.7	*(+1)*	273.6	*(-1)*	275.9	*(±0)*
Day 20	296.1	297.5	*(±0)*	293.9	*(-1)*	294.1	*(-1)*
Gravid uterus weight (g)							
Day 20	52.2	53.7		53.2		52.5	
Cumulative body weight gain (g) *(% difference to control)*							
Days 0 – 20	84.6	84.9	*(±0)*	86.7	*(+2)*	84.8	*(±0)*

[a] for uterus weight
* significantly different from control, $p \leq 0.05$
** significantly different from control, $p \leq 0.01$
Findings considered related to treatment with test substance are written in **bold letters**

The no-observed-effect-level (NOEL) for effects on maternal body weight and feed consumption was 1000 mg (technical AI and EC250 formulations)/kg bw/d.

In the supplementary study, there were no effects on body weight or feed consumption.

D. MATERNAL ANALYTICAL RESULTS

In the main study no serum concentrations of prothioconazole were determined.

In the supplementary study the serum concentration of prothioconazole was determined in five dams from the 1000 mg/kg bw/d dermal application group (group A) and five dams from the water control

group (direct vaginal application, group B). No prothioconazole was detected in the serum of animals not administered the test compound. In contrast, prothioconazole was detected in the serum of the dams of the 1000 mg/kg dermal application group. This evaluation was conducted to verify that there was no cross contamination of the test compound from one study group to another. Also determined on this study, utilizing the same females noted above, was the presence of the test compound in the wipe of the vaginal area. No prothioconazole was detected in the vaginal wipe of the dams administered water. As might be expected, prothioconazole was detected in the vaginal wipe of the dams administered 1000 mg/kg dermally. The presence of the test compound in the vaginal area may have resulted from transfer of the material from the dermal dose site during normal grooming, or via urinary excretion of the test compound. Therefore, based on the similarity of clinical findings observed in these two groups (described above), the presence or absence of the test compound on the vaginal area does not appear to have predisposed the dam to any particular clinical finding, including the red vaginal discharge.

D. NECROPSY OBSERVATIONS

In the main study, there were no statistically significant findings noted at necropsy.

Table 5.6.2/04- 9: Summary of maternal necropsy findings

Parameter	Dose group			
	Control 0 mg/kg	**Group I 1000 mg/kg technical**	**Group II 1000 mg/kg diluted EC250**	**Group III 1000 mg/kg EC250**
No of dams examined	23	21	17	21
Ovaries – cystic (n)	1	0	1	0
Ovaries – enlarged (n)	0	0	0	1
Uterus – cystic	0	1	0	0

Findings considered related to treatment with test substance are written in **bold letters**

In the supplementary study there were no remarkable findings noted either at necropsy or following histopathological examination of the uterus, cervix, or vagina.

E. CAESAREAN SECTION DATA

In the main study, all gravid dams terminated on gestation day 20, from all groups, had viable fetuses. One dam from the EC250 formulation group had one non-viable fetus. No statistically significant differences were seen in the number of corpora lutea or the number of implantation sites. There were no statistically significant effects on resorptions, early or late; or pre or post-implantation loss (Table 5.6.2/04- 10).

Table 5.6.2/04- 10: Summary of reproduction data

Parameter	Dose group			
	Control 0 mg/kg	**Group I 1000 mg/kg technical**	**Group II 1000 mg/kg diluted EC250**	**Group III 1000 mg/kg EC250**
Fertility Index	76.7	72.4	58.6	70.0
Gestation Index	100	100	100	100
Mating Index	100	100	100	100

Parameter	Dose group			
	Control 0 mg/kg	**Group I 1000 mg/kg technical**	**Group II 1000 mg/kg diluted EC250**	**Group III 1000 mg/kg EC250**
No. dams with resorptions only	0	0	0	0
No. dams with live fetuses	23	21	17	21
Mean no. corpora lutea / dam	11.5	12.1	11.5	12.2
Mean no. implantation sites / dam	10.4	11.0	10.2	10.7
Pre-implantation loss (mean no. / dam)	1.1	1.1	1.3	1.5
Pre-implantation loss (% of corpora lutea)	9.4	10.4	11.1	12.3
Mean no. resorptions/dam	0.6	0.9	0.8	0.6
early resorptions	0.5	0.8	0.8	0.4
late resorptions	0.0	0.1	0.0	0.1
No. non-viable fetuses	0	0	0	1
Post-implantation loss (mean no. / dam)	0.6	0.9	0.8	0.6
Post-implantation loss (% of implantations)	5.9	9.9	7.4	6.2
Mean no. live fetuses/dam	9.8	10.1	9.4	10.0
Sex ratio (% males)	49.3	42.9	42.6	48.7
Mean fetal weight, both sexes (g)	3.4	3.5	3.8	3.3
Mean fetal weight of males (g)	3.5	3.6	3.9*	3.4
Mean fetal weight of females (g)	3.3	3.4	3.7	3.2
Placental weight (g)	0.49	0.48	0.52	0.46

* significantly different from control, $p \leq 0.05$
Fertility Index: # pregnant (with implants) / # sperm positive x 100
Gestation Index: # with viable fetuses / # pregnant (with implants) x 100
Mating Index: # sperm-positive / # co-housed x 100
Findings considered related to treatment with test substance are written in **bold letters**

No statistically significant differences in the litter size, the number and proportion of live fetuses/litter, or the percent of male fetuses were observed between the control and the test substance-treated groups. A statistically significant increase in the male mean fetal body weight was noted in the diluted EC250 formulation group. This is not considered test-compound related since similar findings were not observed in the undiluted formulation group. No effect on fetal weight was observed in any other group. No effects on placental weights were observed in any group.

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

In the main study, the overall litter and fetal incidences of abnormalities were comparable to controls in all treated groups and the nature of the abnormalities recorded did not indicate any particular effect of treatment. The pattern of malformations, visceral abnormalities and variations and skeletal abnormalities between the control and treated groups did not indicate any effect of treatment (Table 5.6.2/04- 11).

Table 5.6.2/04- 11: Summary of malformations and variations (fetal (litter) incidence in %)

	Dose group			
	Control 0 mg/kg	**Group I 1000 mg/kg technical**	**Group II 1000 mg/kg diluted EC250**	**Group III 1000 mg/kg EC250**
External examination				
Number of fetuses (litters) evaluated	226 (23)	212 (21)	160 (17)	211 (21)
External malformations	0.0 (0.0)	0.9 [a] (9.5)	0.0 (0.0)	0.0 (0.0)
External variations	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Visceral examination				
Number of fetuses (litters) evaluated	106 (23)	103 (21)	75 (17)	101 (21)
Visceral malformations	4.7 [b] (21.7)	2.9 [b] (14.3)	0.0 (0.0)	4.0 [b] (14.3)
Visceral variations	9.4 [c] (34.8)	9.7 [c] (33.3)	12.0 [c] (47.1)	4.0 [c] (19.0)
Skeletal examination				
Number of fetuses (litters) evaluated	120 (23)	109 (20)	85 (17)	110 (21)
Skeletal malformations	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Skeletal variations	99.2 [d] (100.0)	100.0 [d] (100.0)	98.8 [d] (100.0)	100.0 [d] (100.0)

* $p \leq 0.05$; ** $p \leq 0.01$

[a] one fetus with exencephaly, one fetus with domed head and subcutaneous edema (torso).

[b] malformations included: fetuses with heart reduced in size (among all dose groups except group II), one fetus with dilated brain ventricles (control), one fetus with brain malformed (group I)

[c] variations included: fetuses with left sided umbilical artery, one fetus with hydroureter (group I)

[d] Skeletal variations, of one form or another, were observed in all fetuses examined. Three incidental, albeit statistically significantly decreased findings, as compared to the control group, were observed: Incompletely ossified sternebra 4 in group I, enlarged posterior fontanelles in group III, and unossified metacarpals in both group I and group II.

Findings considered related to treatment with test substance are written in **bold letters**

III. CONCLUSION

Dermal application of the undiluted EC250 formulation was associated with skin irritation. There were no systemic toxic effects identified in any group, and no effects on developing offspring. The red vaginal discharge observed sporadically in all treated group is considered not related to treatment with prothioconazole, as confirmed by results of a supplementary study. The NOAEL for maternal and developmental toxicity via dermal application was 1000 mg/kg bw/d for prothioconazole technical material, 1000 mg/kg bw/d for the EC250 formulation (equivalent to 250 mg/kg bw/d prothioconazole) and 1000 mg/kg bw/d for diluted EC250 (equivalent to 62.5 mg/kg bw/d prothioconazole).

Report:	KCA 5.6.2/05 [redacted],; [redacted]; 1997; M-012332-01-1
Title:	Dose toleration study to a developmental toxicity study with JAU 6476 in the rabbit
Report No.:	R7003
Document No.:	M-012332-01-1
Guideline(s):	not specified
Guideline deviation(s):	not specified
GLP/GEP:	**no**

Deviations: As a range finding study, compliance with OECD Guidelines was not required

Executive summary:

In 1997 a limited (non GLP) range finding study was performed to determine suitable dose levels for a developmental toxicity study in rabbits. Groups of 3 or 5 mated female Chinchilla rabbits were treated by gavage from days 6 to 27 *post coitum* with prothioconazole (batch no. NLL 6096-12, purity 99.7 %). Dose levels were 480, 100, 300 and 80 mg/kg bw/d (sequentially in that order) and the vehicle was 0.5 % aqueous carboxymethylcellulose. Clinical signs, bodyweights and feed consumption were recorded in dams. A gross necropsy was performed on day 28, uterine and implantation site parameters were assessed and fetuses received a limited examination for abnormalities (including degree of ossification of the cranium, a single cross section through the brain, and abnormalities of the major blood vessels, heart and kidneys by dissection). As a range finding study, compliance with OECD Guidelines was not required.

Since the present range finding study included no concurrent control group and the number of investigated pregnant females in the dose groups was only 2-3, the study is of very limited value to establish a toxicological profile including a dose-response relationship and the setting of NOAELs.

It is plausible that the following observations are related to treatment with prothioconazole: Maternal mortality, reduced feed consumption and body weight effects at 480 and 300 mg/kg bw/d, total post implantation loss and reduced fetal weight (due to the number of runts) at 480 mg/kg bw/d. Thus, possible NOAELs have been established at 100 mg/kg bw/d (maternal toxicity) and at 300 mg/kg bw/d (developmental toxicity).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-12
Purity:	99.7 %
CAS No.:	Not reported
Stability of the test compound:	Confirmed at least for the time of study duration
2. Vehicle:	0.5 % carboxymethylcellulose sodium salt (CMC) in bi-distilled water
3. Test animals:	
Species:	Rabbit
Strain:	Chinchilla (CHbb:CH, Hybrids)

Sex:	Males and females (nulliparous and not pregnant) (Males were not treated with prothioconazole and were only used for mating)
Age:	At delivery: 13–18 weeks
Weight at dosing:	3000 g (± 500 g)
Source:	[redacted], Germany
Acclimation period:	At least 7 days prior to pairing
Diet:	Pelleted standard Kliba 341 rabbit maintenance diet ([redacted] CH-[redacted] / Switzerland), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	The animals were individually housed in suspended stainless steel cages equipped with an automatic cleaning system.
Environmental conditions:	
Temperature:	20 ± 3 °C
Humidity:	40–70 %
Air changes:	10–15 per hour
Photo period:	Artificial illumination, 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: February 11, 1997 – May 16, 1997

2. Animal assignment and treatment

Mating and start of gestation

After acclimatization, the females were placed in cages with sexually mature males (1:1) until copulation had been observed. After mating, the females were removed and caged individually. The day of mating was designated day 0 *post coitum* (p.c.). The male rabbits used for mating were in the possession of RCC. The fertility of these males is known and was continuously controlled.

Dose levels, experimental group and rationale for choice of dose levels

The test article was administered orally, by gavage, once daily in the morning from day 6 through to day 27 p.c., inclusive. The females received a dose volume of 4 ml/kg bw, with a daily adjustment of the individual volume to the actual body weight. A control group was not assigned to this study.

Table 5.6.2/05-1: Dose groups

	Group 1	Group 2	Group 3	Group 4
Number of dams	5	3	3	3
mg/kg body weight	480[#]	100[##]	300[##]	80[###]

[#] Dose level was proposed by the Sponsor
[##] Dose levels were selected based on the results obtained from group 1.
[###] Dose level was selected based on the results obtained from groups 2 and 3.

3. Test substance preparation and analysis

Prothioconazole was suspended in a mixture of 0.5 % CMC in bi-distilled water. The mixtures of the test article and vehicle were prepared daily before administration.

Concentration, homogeneity and stability of the test article/ vehicle mixtures were determined on one occasion during the dosing period. Samples were taken immediately after preparation and again 4 hours later. Analyses were performed by the RCC Analytical Chemistry Laboratory, using a method supplied by the Sponsor.

Table 5.6.2/05- 2: Concentration, homogeneity and stability of test article in vehicle

Dose group	Nominal Concentration (mg/ml)	TMB	Time of Storage at Room Temperature (h)	Concentration Found (µg/ml)	% of Nominal Concentration	Mean % of Nominal Concentration
1	0	--	0	0.000	--	--
2	120	T	0	111.0	92.5	95.5
		M	0	116.0	96.7	
		B	0	116.6	97.2	
			4	108.3	90.2	90.2

T/M/B: **T**op/**M**iddle/**B**ottom (segment of mixing container)

4. Statistics

The following methods were used to analyze body weights, feed consumption and reproduction data:

- Means and standard deviation of various data were calculated and included in the report.

C. METHODS

1. Observations

The animals were checked at least twice daily for any mortality.

Any female sacrificed or found dead during the study was subjected to macroscopic examination with emphasis on the uterus and its contents. Specimens of abnormal tissue were fixed in neutral phosphate buffered 4 % formaldehyde solution. The animals were observed at least twice daily for signs of reaction to treatment and/or symptoms of ill health.

2. Feed consumption and body weight

Feed consumption was recorded for the following periods: days 0-6, 6-11, 11-15, 15-19, 19-24 and 24-28 *post coitum*.

Body weights were recorded daily from day 0 until day 28 *post coitum*.

3. Investigations at Cesarean Section

On day 28 *post coitum*, the females were killed by an intravenous injection of 1 ml/kg body weight sodium pentobarbital (Narcoren) and the fetuses were removed by cesarean section. The following parameters were determined and assessed at cesarean section:

- Gross macroscopic examination of all internal organs (only tissues or organs with abnormal findings were retained)
- Number of corpora lutea
- Uterus weight
- Number of implantations
- Individual weight and appearance of the placentas
- Number of (early/late) resorptions
- Number of live fetuses
- Sex of live fetuses
- Individual weights of fetuses
- Examination of fetuses:

1) The skin was removed from the head and the eyes were examined, removed and discarded. The cranium was examined for the degree of ossification and the brain for the internal structure by one cross section throughout both cerebral hemispheres, respectively.
2) The thorax and abdomen were opened and the fetuses examined by a microdissection technique. This included detailed examination of the major blood vessels and sectioning of the heart and kidneys. Any abnormalities were recorded and, if appropriate, retained in neutral phosphate 4 % formaldehyde solution. The sex was recorded and then the fetuses discarded.

Fetuses with external or visceral abnormalities were photographed and preserved in 70 % alcohol.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSIS

See Section B.3 above.

B. OBSERVATIONS

1. Mortality

Two females from group 4 (480 mg/kg bw/d) died on day 23 and 26 p.c. (day 17 and 20 of treatment). One female from group 3 (300 mg/kg bw/d) died on day 24 p.c. (day 18 of treatment). One female from group 2 (100 mg/kg bw/d) died on day 26 p.c. (day 20 of treatment). At 80 mg/kg one female died on day 27 p.c. (day 21 of treatment). Based on the fact that in the main developmental study in rabbits (see below) only the highest tested dose of 350 mg/kg bw/d caused the death of only one dam (and no further maternal mortality was observed up to and including 80 mg/kg bw/d), the maternal mortality at 80 and 100 mg/kg bw/d in the present range finding study are not considered to be related to the treatment with prothioconazole. It is plausible that the maternal mortality at 480 and at 300 mg/kg bw/d was caused by the treatment with prothioconazole.

C. FEED CONSUMPTION AND BODY WEIGHT

1. Feed consumption

Since no concurrent control group was included in the present range finding study, the number of investigated females in the dose groups was only 2-3, and the day 0-6 feed consumption was highly variable between dose groups, the feed consumption data during the treatment period (days 6-27 p.c.) are difficult to assess. Prothioconazole seems to have caused a reduced feed consumption at 480 and 300 mg/kg bw/d. But since in the main developmental study in rabbits (see below) only the highest tested dose of 350 mg/kg bw/d caused reduced feed consumption and no effects on feed consumption were observed up to and including 80 mg/kg bw/d, the feed consumption data at 80 and 100 mg/kg bw/d in the present range finding study are not considered to indicate a treatment-related effect of prothioconazole.

Table 5.6.2/05- 3: Feed consumption

	Prothioconazole (mg/kg bw/d)			
	Group 1 (480)	**Group 2 (100)**	**Group 3 (300)**	**Group 4 (80)**
Mean Feed Consumption (g/animal/d)				
Day 0-6	268	206	155	176
Day 6-11	**103**	133	**67**	118
Day 11-15	**88**	106	**87**	96
Day 15-19	**81**	66	--	122
Day 19-24	**110**	92	--	135
Day 24-28	**90**	70	**98**	113
Mean of means	123	113	102	127

Only values of pregnant dams were used for calculation
-- Spillage of feed
Findings considered related or possibly related to treatment with prothioconazole are written in **bold letters**

2. Body weight, body weight gain and uterus weight

Since no concurrent control group was included in the present range finding study, the number of investigated females in the dose groups was only 2-3, and the day 0-6 body weight gain was highly variable between dose groups, the body weight data during the treatment period (days 6-27 p.c.) are difficult to assess. Prothioconazole seems to have caused intial body weight loss, followed by decreased body weight gain at 480 and 300 mg/kg bw/d. But since in the main developmental study in rabbits (see below) only the highest tested dose of 350 mg/kg bw/d caused effects on body weights and no effects on body weights were observed up to and including 80 mg/kg bw/d, the maternal body weight data at 80 and 100 mg/kg bw/d in the present range finding study are not considered to indicate a treatment-related effect of prothioconazole.

Table 5.6.2/05- 4: Maternal body weight, body weight gain and gravid uterus weight

	Prothioconazole (mg/kg bw/d)			
	Group 1 (480)	**Group 2 (100)**	**Group 3 (300)**	**Group 4 (80)**
Body weight [a] (g)				
Day 0	3872	4281	4143	3871
Day 1	4022	4349	4273	3919
Day 2	4048	4357	4310	3905
Day 3	4100	4221	4297	3876
Day 4	4120	4305	4305	3896
Day 5	4154	4363	4289	3934
Day 6	4175	4331	4287	3948
Day 7	4113	4295	4214	3909
Day 8	4098	4272	4152	3937
Day 9	4075	4278	4071	3908
Day 10	4058	4283	4108	3816
Day 11	3989	4280	4088	3783
Day 12	3970	4266	4112	3786
Day 13	3885	4261	4063	3807

	Prothioconazole (mg/kg bw/d)			
	Group 1 (480)	**Group 2 (100)**	**Group 3 (300)**	**Group 4 (80)**
Day 14	3849	4264	4101	3798
Day 15	3839	4264	4103	3771
Day 16	3819	4257	4137	3813
Day 17	3786	4249	4136	3843
Day 18	3764	4211	4124	3826
Day 19	3739	4215	4107	3829
Day 20	3696	4232	4168	3847
Day 21	3669	4242	4246	3841
Day 22	3632	4250	4262	3850
Day 23	3367	4151	4230	3869
Day 24	3514	4273	4266	3871
Day 25	3493	4275	4255	3886
Day 26	3445	4328	4252	3886
Day 27	3444	4309	4198	3892
Day 28	3396	4298	4170	3902
Gravid uterus weight (g)				
Day 20	263	346	284	268
Body weight gain [a] (g)				
Days 0–6	303	50	144	75
Days 6–11	**-186**	-51	**-199**	-165
Days 11–15	**-150**	-16	15	-12
Days 15–19	**-100**	-49	94	58
Days 19–24	**-225**	58	69	42
Days 24–28	**-118**	25	96	31
Days 6–28	**-779**	-33	**-117**	-46
Days 0 – 20 (corrected for gravid uterus weight)	**-761**	-580	**-401**	-313

[a] only values of pregnant dams were used for calculation
Findings considered related or possibly related to treatment with prothioconazole are written in **bold letters**

D. NECROPSY OBSERVATIONS

During scheduled necropsy on day 28 *post coitum*, one female of group 1 (480 mg/kg) showed following necropsy findings: Colon, contents whitish mass; gallbladder, bile finely granulated, mucosa, several gray white foci, diameter = 1 mm. This finding was considered to be unrelated to the treatment with the test article.

No abnormal findings were evident in any other female of any other group during scheduled necropsy.

The following abnormal test article-unrelated findings were seen during post mortem examination of the females which died before scheduled necropsy.

At 480 mg/kg bw/d one female showed a reddish discoloration of the lungs. The caecum was filled with liquid contents. The other female showed no abnormal findings.

At 100 mg/kg bw/d and 300 mg/kg bw/d the females showed dark red discoloration of the lungs.

At 80 mg/kg bw/d the female showed dark red discoloration of the lungs and a claycolored liver.

E. CAESAREAN SECTION DATA

Since no concurrent control group was included in the present range finding study and the number of investigated females in the dose groups was only 2-3, the Caesarean section data are difficult to assess. One of three surviving pregnant group 1 dams (480 mg/kg) showed a total post implantation loss and was not used for calculation of the reproduction data. A test article relation is plausible (Table 5.6.2/05-5).

The mean reproduction data of dams (implantation sites, post implantation loss and number of fetuses) did not indicate test article related effects.

The external examination of the placentas did not reveal any test article related findings.

All differences noted were within the normal range of biological variation and did not indicate a treatment dependency.

No differences in sex ratios were noted which were considered to be test article related. The mean weight of live fetuses was reduced in group 1 (480 mg/kg) when compared to the other groups and to the historical range of control data (stated to be 31.5-36.8 g ("mean over years") on p. 19 of original report). The reduction was considered to be due to the number of runts (9 of 16 fetuses).

All other body weights were close to those noted in the historical control data (and not dose-related for the 100 mg/kg bw/d group) and do therefore not indicate a treatment dependency.

Table 5.6.2/05-5: Summary of reproductive data

Parameter	Prothioconazole (mg/kg bw/d)			
	Group 1 (480)	**Group 2 (100)**	**Group 3 (300)**	**Group 4 (80)**
No. pregnant / no. mated	3/5	2/3	2/3	2/3
No of dams found dead prior to scheduled necropsy	**2**	1	1	1
No. dams with implantations only	**1**	0	0	0
No. dams with live fetuses	2	2	2	2
Mean no. corpora lutea / dam	12.0	12.5	10.5	10.0
Mean no. implantation sites / dam	8.0	12.0	5.5	8.0
Pre-implantation loss (mean no. / dam)	4.0	0.5	5.0	2.0
Pre-implantation loss (% of corpora lutea)	33.3	4.0	47.6	20.0
Mean no. resorptions/dam	0	0.5	0.5	2.5
early resorptions	0	0	0.5	1.5
late resorptions	0	0.5	0	1.0
No. dead fetuses	0	0	0	0
Post-implantation loss (mean no. / dam)	0	0.5	0.5	2.5
Post-implantation loss (% of implantations)	0	4.2	9.1	31.3

Parameter	Prothioconazole (mg/kg bw/d)			
	Group 1 (480)	**Group 2 (100)**	**Group 3 (300)**	**Group 4 (80)**
Mean no. live fetuses/dam	8.0	11.5	5.0	9.5
Sex ratio (% males)	43.8	60.9	50.0	54.5
Mean fetal weight, both sexes (g)	**25.0**	30.9	36.4	39.4
Mean fetal weight of males (g)	**24.5**	30.7	34.3	30.3
Mean fetal weight of females (g)	**25.2**	31.7	37.5	28.5

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

In group 1 (480 mg/kg) 9 out of 16 fetuses were runts (small fetuses, body weights between 9.8 and 17.7 g). A treatment relation of this finding is plausible. In groups 2 (100 mg/kg) and 3 (300 mg/kg) no abnormal findings were noted.

In group 4 (80 mg/kg), one of 11 fetuses showed an encephalocele in the region of the large fontanelle. This finding was considered not to be related to test article treatment.

Table 5.6.2/05- 6: External and fresh visceral examination of fetuses (Fetal (litter) incidence in %)

Parameter	Prothioconazole (mg/kg bw/d)			
	Group 1 (480)	**Group 2 (100)**	**Group 3 (300)**	**Group 4 (80)**
Fetuses evaluated (n)	16	23	10	11
Litters evaluated (n)	2	2	2	2
Runt	**56.3 (50.0)**	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Encephalocele	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.1 (50.0)

Runt: small fetus < 19 g, not assessed as external malformation

III. CONCLUSION

Since the present range finding study included no concurrent control group and the number of investigated pregnant females in the dose groups was only 2-3, the study is of very limited value to establish a toxicological profile including a dose-response relationship and the setting of NOAELs.

It is plausible that the following observations are related to treatment with prothioconazole: Maternal mortality, reduced food consumption and body weight effects at 480 and 300 mg/kg bw/d, total post implantation loss and reduced fetal weight (due to the number of runts) at 480 mg/kg bw/d. Thus, possible NOAELs have been established at 100 mg/kg bw/d (maternal toxicity) and at 300 mg/kg bw/d (developmental toxicity).

Report:	KCA 5.6.2/06[redacted],;[redacted]; 1998; M-012237-01-1
Title:	Developmental toxicity study with JAU 6476 in the rabbit
Report No.:	R7235
Document No.:	M-012237-01-1
Guideline(s):	OECD 414 (1981), Direcivte 67/548/EEC (1987), US-EPA OPPTS870.3700 (1997)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Deviations: Deviations from the current OECD guideline (2001):
Feed consumption was recorded in five-day intervals instead of three-day intervals.
However this does not affect the overall acceptability of the study.

Executive summary:

In a 1998 GLP study, groups of 24 mated female Chinchilla rabbits received daily gavage doses of 0, 10, 30 and 80 mg/kg bw/d prothioconazole (batch no. NLL 6096-12, purity 99.5 - 99.7%) from days 6 to 27 *post coitum*. The vehicle was 0.5 % aqueous carboxymethylcellulose. An additional group of 24 rabbits was added to the study at a dose level of 350 mg/kg bw/d due to the absence of clear maternal toxicity at 80 mg/kg bw/d. A further 6 and 7 mated females were treated with 10 and 80 mg/kg bw/d, respectively, due to low pregnancy incidences. In life observations in dams were clinical signs, bodyweights and feed consumption. Fetuses were delivered by caesarean section on day 28 of gestation, a gross necropsy was performed on dams, and liver and adrenal glands were weighed. The study was compliant with or exceeded the requirements of the contemporary OECD Guideline 414 (1981) with the dosing pattern and fetal examinations being in line with the 2001 OECD Guideline 414.

At 350 mg/kg bw/d very strong maternal toxicity was observed, including mortality, bodyweight losses/reduced gains and reduced feed consumption. Secondarily to the slightly decreased terminal body weight, absolute liver weight was also minimally decreased. Relative liver weights as well as absolute and relative adrenal weights were not affected in any dose group. None of the gross necropsy findings were considered treatment-related.

At 350 mg/kg bw/d there were 3 females with abortions and 3 females with total litter resorption, resulting in decreased overall litter size in this group, and post-implantation losses were correspondingly higher. Gravid uterus weights and fetal weights were reduced at 350 mg/kg bw/d and mean placental weight was slightly decreased. Pre-implantation loss, the incidence of dead fetuses and fetal sex ratio were unaffected at 350 mg/kg bw/d. All reproductive parameters and fetal weights were unaffected at dose levels up to and including 80 mg/kg bw/d. In fetal examination the nature and incidences of external, visceral and skeletal abnormalities did not indicate an effect of treatment at any dose level

Based on the very strong maternal toxicity evident at 350 mg/kg bw/d, the NOAEL for maternal toxicity was 80 mg/kg bw/d.

Treatment at 350 mg/kg bw/d was also associated with abortions, total litter losses and reduced fetal weights, which are considered secondary to the very strong maternal toxicity. The NOAEL for developmental effects was 80 mg/kg bw/d. There was no evidence of a teratogenic effect up to 350 mg/kg bw/d.

All observed developmental effects are considered as unspecific and secondary to the very strong (partially lethal) maternal toxicity. Therefore, these findings are no indications for a specific or direct developmental toxic potential of prothioconazole. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, such a constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. Specifically, the Guidance states: *"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species."*

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	Prothioconazole
Synonym:	JAU 6476
Description:	White powder
Batch No.:	NLL 6096-12
Purity:	99.5 – 99.7 %
CAS No.:	not reported
Stability of the test compound:	Guaranteed at least for study duration
2. Vehicle:	0.5 % carboxymethylcellulose (CMC) in bi-distilled water
3. Test animals:	
Species:	Rabbit
Strain:	Chinchilla (CHbb:CH, Hybrids)
Sex:	Males and females (nulliparous and not pregnant) (Males were not treated with prothioconazole and were only used for mating)
Age:	At pairing: 4.5–8.5 months
Weight:	2733–4683 g (females, day 0 *post coitum*)
Source:	[redacted] Germany
Acclimation period:	At least 7 days prior to pairing
Diet:	Pelleted standard Kliba 341 rabbit maintenance diet (KLIBA Mühlen AG, Switzerland), *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	The animals were housed individually in stainless steel cages equipped with an automatic cleaning system.
Environmental conditions:	
Temperature:	20±3 °C
Humidity:	40–70 %
Air changes:	At least 10 times per hour
Photo period:	Artificial illumination, 12 hour light/dark cycle

B. STUDY DESIGN

1. Dates of work: June 10, 1997 – December 08, 1997

2. Animal assignment and treatment

Mating and start of gestation

After acclimatization, the females were placed in cages with sexually mature males (1:1) until copulation had been observed. After mating, the females were removed and caged individually. The day of mating was designated day 0 *post coitum*.

The male rabbits used for mating were in the possession of RCC. The fertility of these males is known and was continuously controlled.

Dose levels, experimental group and rationale for choice of dose levels

The male animals were used for mating only and were not treated with prothioconazole. After copulation had been observed, 24 females each were manually assigned to four experimental groups. After termination of the initial study phase (June– August 1997) it became apparent that the number of

pregnant females in groups 2 and 4 was not sufficient. Therefore, in a second study phase starting in October 1997, 6 mated females were added to group 2 and 7 mated females to group 4. Further, because no clear effects of toxicity were noted up to and including 80 mg/kg bw/d, an additional dose group was included (350 mg/kg bw/d).

The test article was administered orally, by gavage once daily in the morning from day 6 through day 27 *post coitum*, inclusive. The females received a dose volume of 4 ml/kg bw with a daily adjustment of the individual volume to the actual body weight.

The following doses of prothioconazole were administered:

Table 5.6.2/06- 1: Dose groups

	Group 1	Group 2	Group 3	Group 4	Group 5
Number of dams	24	24 + 6	24	24 + 7	24
mg/kg bw/d	0	10	30	80	350

3. Test substance preparation and analysis

Prothioconazole was suspended in a mixture of 0.5 % CMC in bi-distilled water.

The mixtures of the test article and vehicle were prepared daily before administration.

The test article was weighed into a glass beaker on a suitable tared precision balance and the vehicle added (w/v). The mixtures were prepared with a homogenizer. During the daily administration period, homogeneity of suspensions was maintained using a magnetic stirrer.

Analytical verifications of concentration, homogeneity and stability of prothioconazole in vehicle (see Table 5.6.2/06- 2) were performed by the RCC Analytical Chemistry Laboratory, using a method supplied by the Sponsor.

Table 5.6.2/06- 2: Concentration, homogeneity and stability of prothioconazole in vehicle

Dose group	Nominal Concentration (mg/ml)	Time of Storage at Room Temperature (h)	Date of Analysis (1997)	Concentration Found Mean % of Nominal Concentration
Date of preparation: June 19, 1997				
1	0	0	24-JUN	--
2	2.5	0 5		87.5 88.1
3	7.5	0 5		84.5 80.6
4	20	0 5		72.3 70.3
Date of preparation: June 25, 1997				
1	0	0	26-JUN	--
2	2.5	0 4		92.7 90.8
3	7.5	0 4		91.4 91.6
4	20	0 4		90.5 100.1

Dose group	Nominal Concentration (mg/ml)	Time of Storage at RoomTemperature (h)	Date of Analysis (1997)	Concentration Found Mean % of Nominal Concentration
Date of preparation: August 13, 1997				
1	0	0	13-AUG	--
2	2.5	0 4		98.4 100.5
3	7.5	0 4		98.1 78.5
4	20	0 4		93.7 91.6
Date of preparation: November 10, 1997				
1	0	0	28-NOV	--
2	2.5	0 2		106.0 105.0
4	20	0 2		106.9 104.4
5	87.5	0 2		111.0 110.6
Date of preparation: November 24, 1997				
1	0	0	28-NOV	--
2	2.5	0 2		104.8 103.4
4	20	0 2		99.6 100.4
5	87.5	0 2		110.7 105.4

4. Statistics

The following statistical methods were used to analyze body weights, feed consumption, reproduction and skeletal examination data:

Means and standard deviation of various data were calculated and included in the report.

Statistical test	Parameter
Dunnett many-one t-test, based on a pooled variance estimate	For intergroup comparisons (i.e. single treatment groups against the control group)
Steel test (many-one rank test)	When the data cannot be assumed to follow a normal distribution
Fisher's Exact test for 2x2 tables	If the variables can be dichotomized without loss of information.

C. METHODS

1. Observations

The animals were checked at least twice daily for any mortalities, morbidity or signs of abortion. Any female found dead during the course of this study wase subjected to macroscopic examination with emphasis on the uterus and its contents.

2. Feed consumption

Feed consumption was recorded for the following periods: days 0-6, 6-11, 11-15, 15-19, 19-24 and 24-28 *post coitum*.

3. Body weight development

Body weights were recorded daily from day 0 until day 28 *post coitum*.

4. Investigations at Cesarean Section

On day 28 *post coitum*, the females were killed by an intravenous injection of 1 ml/kg body weight sodium pentobarbital and the fetuses were removed by Caesarean section.

The following parameters were determined and assessed at cesarean section:

- Gross macroscopic examination of all internal organs (with emphasis on the uterus, uterine contents, position of fetuses in the uterus and number of corpora lutea in each ovary)
- Liver and adrenals weight
- Gravid uterus and placenta weight
- Individual weight and appearance of the placentas
- Number and distribution of implantations in uterine horns
- Embryonic/fetal resorptions
- Number of dead/live fetuses
- Sex of live fetuses
- Individual weights of live fetuses

Preparation for fetal examination:

1) With the exception of over the paws, the skin and the dorsal cervical fat pads were removed and discarded.
2) The eyes were examined, removed and discarded. The skin was removed and the cranium was examined for the degree of ossification. From one half of the fetuses, the brain was examined for internal structure by one cross section throughout both cerebral hemispheres, respectively. From the other half of the fetuses, the heads were separated from the trunks and fixed in a solution of trichloroacetic acid and formaldehyde. They were serially sectioned. Descriptions of any abnormalities were recorded. After examination, the sections were preserved in a solution of ethyl alcohol and glycerin (one head per container).
3) The thorax and abdomen was opened and the fetuses examined by a microdissection technique. This included detailed examination of the major blood vessels and kidneys. The hearts of the fetuses were examined for septum defects or other malformation of the heart after fixation in neutral phosphate-buffered 4% formaldehyde solution for minimum of 2 days. Any abnormalities were recorded and, if appropriate, retained in neutral phosphate-buffered 4% formaldehyde solution. The sex was recorded and the viscera removed and discarded.
4) The carcasses were then processed through solutions of ethanol, glacial acetic with Alcian blue (for cartilage staining), potassium hydroxide with Alizarin red S (for clearing and staining ossified bone) and aqueous glycerin for preservation and storage. The skeletons were examined and all abnormal findings and variations were recorded. The specimens were preserved individually.

   Fetuses with external or visceral abnormalities were photographed

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSIS

See Section B.3 above.

B. OBSERVATIONS

1. Mortality and clinical signs of toxicity

At 350 mg/kg bw/d one female was found dead in the morning of day 25 *post coitum* after showing reduced feed consumption from the first day of treatment on, resulting in bodyweight loss throughout treatment until death. The death is therefore considered treatment-related.

At 350 mg/kg bw/d one female showed tachypnea and red discharge was found on the feces tray of another female of the same group. There was no effect on reproductive parameters in those two females. Therefore these findings were considered to be incidental.

Up to and including 80 mg/kg bw/d, no death occurred and no signs of reaction to treatment with prothioconazole were observed.

C. FEED CONSUMPTION AND BODY WEIGHT DEVELOPMENT

1. Feed consumption

At 350 mg/kg bw/d mean feed consumption was reduced during the treatment period (max. -50 % on days 6-11, overall -31 % on days 6-28). This reduction attained statistical significance between days 6 and 19 *post coitum* and correlated with the reduction in body weight gain. Up to and including 80 mg/kg bw/d no test article related differences in feed consumption were noted.

Table 5.6.2/06- 3: Feed consumption

	Prothioconazole (mg/kg bw/d)								
	0	10		30		80		350	
Mean Feed Consumption (g/animal/d) *(% difference to control)*									
Day 0-6	206	213	*(+3)*	212	*(+3)*	198	*(-4)*	197	*(-4)*
Day 6-11	219	203	*(-7)*	211	*(-4)*	185	*(-16)*	**110****	***(-50)***
Day 11-15	185	183	*(-1)*	189	*(+2)*	175	*(-5)*	**105****	***(-43)***
Day 15-19	196	205	*(+5)*	172	*(-12)*	169	*(-14)*	**143****	***(-27)***
Day 19-24	174	180	*(+3)*	170	*(-2)*	168	*(-3)*	174	*(±0)*
Day 24-28	118	120	*(+2)*	107	*(-9)*	110	*(-7)*	**87**	***(-26)***
Day 6-28	180	179	*(-1)*	172	*(-4)*	163	*(-9)*	**125**	***(-31)***

* significantly different from control, $p \le 0.05$
** significantly different from control, $p \le 0.01$
Findings considered related to treatment with prothioconazole are written in **bold letters**

2. Body weight, body weight gain, gravid uterus weight

Correlating with the reduced feed consumption, at 350 mg/kg bw/d the dams showed initial body weight loss after start of treatment; this was only partially compensated during the rest of the treatment period. This is reflected also in the reduced non-corrected (days 6-28) and corrected (days 0-28) body weight gain values. Gravid uterus weight was also decreased at 350 mg/kg bw/d. Up to and including 80 mg/kg bw/d no test article related differences in body weights, body weight gain and gravid uterus weight were observed.

Table 5.6.2/06- 4: Maternal body weight, gravid uterus weight and body weight gain

	Prothioconazole (mg/kg bw/d)									
	0	**10**		**30**		**80**		**350**		
Mean Body Weight [a] (g) *(% difference to control)*										
Day 0	3154	3266	*(+4)*	3239	*(+3)*	3296	*(+5)*	3337	*(+6)*	
Day 1	3274	3365	*(+3)*	3333	*(+2)*	3387	*(+3)*	3417	*(+4)*	
Day 2	3319	3420	*(+3)*	3383	*(+2)*	3439	*(+4)*	3465	*(+4)*	
Day 3	3339	3442	*(+3)*	3394	*(+2)*	3462	*(+4)*	3471	*(+4)*	
Day 4	3372	3480	*(+3)*	3443	*(+2)*	3497	*(+4)*	3504	*(+4)*	
Day 5	3404	3511	*(+3)*	3470	*(+2)*	3512	*(+3)*	3544	*(+4)*	
Day 6	3444	3545	*(+3)*	3497	*(+2)*	3526	*(+2)*	3561	*(+3)*	
Day 7	3464	3579	*(+3)*	3521	*(+2)*	3534	*(+2)*	**3516**	*(+2)*	
Day 8	3496	3594	*(+3)*	3543	*(+1)*	3553	*(+2)*	**3491**	*(-0)*	
Day 9	3536	3610	*(+2)*	3569	*(+1)*	3572	*(+1)*	**3471**	*(-2)*	
Day 10	3555	3614	*(+2)*	3595	*(+1)*	3579	*(+1)*	**3473**	*(-2)*	
Day 11	3589	3618	*(+1)*	3622	*(+1)*	3602	*(±0)*	**3472**	*(-3)*	
Day 12	3587	3657	*(+2)*	3649	*(+2)*	3620	*(+1)*	**3466**	*(-3)*	
Day 13	3593	3675	*(+2)*	3653	*(+2)*	3645	*(+1)*	**3479**	*(-3)*	
Day 14	3621	3704	*(+2)*	3681	*(+2)*	3669	*(+1)*	**3474**	*(-4)*	
Day 15	3669	3735	*(+2)*	3706	*(+1)*	3711	*(+1)*	**3496**	*(-5)*	
Day 16	3701	3775	*(+2)*	3740	*(+1)*	3718	*(±0)*	3532	*(-5)*	
Day 17	3728	3802	*(+2)*	3769	*(+1)*	3731	*(±0)*	3564	*(-4)*	
Day 18	3775	3820	*(+1)*	3760	*(+1)*	3749	*(+1)*	3598	*(-3)*	
Day 19	3753	3840	*(+2)*	3770	*(+1)*	3766	*(±0)*	3641	*(-3)*	
Day 20	3773	3865	*(+2)*	3798	*(+1)*	3799	*(+1)*	3686	*(-2)*	
Day 21	3789	3895	*(+3)*	3819	*(+1)*	3831	*(+1)*	3722	*(-2)*	
Day 22	3815	3927	*(+3)*	3856	*(+1)*	3846	*(+1)*	3764	*(-1)*	
Day 23	3837	3939	*(+3)*	3892	*(+2)*	3871	*(+1)*	3777	*(-1)*	
Day 24	3840	3959	*(+3)*	3913	*(+2)*	3896	*(+1)*	3791	*(-1)*	
Day 25	3831	3974	*(+4)*	3920	*(+2)*	3904	*(+2)*	**3763**	*(-2)*	
Day 26	3838	3974	*(+4)*	3921	*(+2)*	3913	*(+2)*	**3753**	*(-2)*	
Day 27	3846	3977	*(+3)*	3924	*(+2)*	3913	*(+2)*	**3739**	*(-3)*	
Day 28	3850	3996	*(+4)*	3916	*(+2)*	3935	*(+2)*	**3752**	*(-3)*	
Mean Gravid uterus weight (g) *(% difference to control)*										
Day 28	408.1	441.7	*(+8)*	488.8	*(+20)*	438.7	*(+7)*	**316.2**	*(-23)*	
Mean Body weight gain [a] (g) *(% difference to control)*										
Days 0–6	290	279		258		230		224		
Days 6–11	145	73		125		76		**-89**		
Days 11–15	80	117		84		109		**24**		
Days 15–19	84	105		64		55		145		

	Prothioconazole (mg/kg bw/d)				
	0	**10**	**30**	**80**	**350**
Days 19–24	87	119	143	130	150
Days 24–28	10	37	3	39	**-39**
Days 6–28	406	451 *(+11)*	419 *(+3)*	409 *(+1)*	**191** ***(-53)***
Days 6–28 (corrected for gravid uterus weight) [a, b]	-38.5	-8.3 *(+78)*	-70.1 *(-82)*	-47.3 *(-23)*	**-175.6** ***(-356)***

[a] Only values of pregnant dams were used for calculations

[b] (Weight on day of section) - (weight on day 6 p.c.) - (gravid uterus weight), calculated individually before deriving mean value

Findings considered related to treatment with prothioconazole are written in **bold letters**

D. NECROPSY OBSERVATIONS

Terminal body and organ weights:

Secondarily to the slightly decreased terminal body weight, absolute liver weight was also minimally decreased. Relative liver weights as well as absolute and relative adrenal weights were not affected in any dose group.

Table 5.6.2/06- 5: Mean terminal body and organ weights

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**10**	**30**	**80**	**350**
Terminal body weight (g)	3826	3991	3880	3937	**3641**
Liver weight (g)	110.4	109.5	106.9	110.7	**99.6**
Liver / body weight ratio (%)	2.9	2.7	2.8	2.8	2.7
Adrenals weight (g)	0.228	0.231	0.225	0.240	0.230
Adrenal / body weight ratio (%)	0.006	0.006	0.006	0.006	0.007

Findings considered related to treatment with prothioconazole are written in **bold letters**

Necropsy findings:

The following abnormal findings were noted during post mortem examination of the females during scheduled necropsy:

In the control group one female showed enlarged liver and red foci on the right adrenal gland. At 10 mg/kg bw/d one female showed enlarged liver and another female showed agenesia of the left uterine horn, the left kidney and ureter. A further female of this dose group showed watery cysts in both kidneys. At 30 mg/kg bw/d there was one female with red discoloration of the right adrenal gland, one with reduced liver size and one female showed inflamed crateriform retractions in the pyloric part of the stomach. At 80 mg/kg bw/d there was one female with red foci on the left adrenal gland, one with a reddish discolored thickened uterus, and one female with inflamed crateriform retractions of the stomach wall. At 350 mg/kg bw/d one female showed several watery cysts in both kidneys.

All these gross necropsy findings are considered incidental and not related to treatment with prothioconazole.

D. CAESAREAN SECTION DATA

At 350 mg/kg bw/d, there were 3 females with abortions (between day 22 and 28 *post coitum*) and 3 females with total litter resorption, resulting in decreased mean number of live fetuses per dam in this group, but not in the subset of dams which maintained live litters to day 28. Post-implantation losses were correspondingly higher at 350 mg/kg bw/d. Mean fetal weights were significantly reduced at 350 mg/kg bw/d (10-13 % lower than controls) and mean placental weight was slightly (5.8 %) lower (Table 5.6.2/06- 6). All these parameters were not affected at dose levels up to and including 80 mg/kg bw/d.

All further reproductive parameters, including pre-implantation loss, the incidence of dead fetuses and fetal sex ratio, were unaffected by treatment up to and including 350 mg/kg bw/d (differences noted were within the normal range of biological variation and considered to be incidental).

Table 5.6.2/06- 6: Summary of reproductive data

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**10**	**30**	**80**	**350**
No. mated	24	30	24	31	24
No. pregnant (used for calculations)	22	25	22	24	24
No. dying	0	0	0	0	**1**
No. aborting	0	0	1	0	**3**
No. with implantation sites only	2	1	0	1	**3**
No. pregnant dams surviving to D28	22	25	21	24	**20**
No. dams with live fetuses on D28	20	24	21	23	**17**
Mean no. corpora lutea/dam	10.6	10.4	11.9	10.6	9.5
Implantation sites - % corpora lutea	84.1	84.6	83.5	86.5	99.1**
Implantation sites - mean/dam	8.9	8.8	9.9	9.0	9.4*
Pre-implantation loss - % of corp. lutea	15.9	15.4	16.5	13.5	0.9**
Pre-implantation loss - mean/dam	1.7	1.6	2.0	1.4	0.1*
Post-implantation loss - % impl.	10.2	4.5*	12.8	5.5	**29.6****
Post-implantation loss - mean/dam	0.9	0.4	1.3	0.5	**2.8**
Empty implantation sites - % impl.	3.6	1.4	3.2	0.9	**23.1****
Empty implantation sites - mean/dam	0.3	0.1	0.3	0.1	2.2
Embryonic resorptions - % impl.	3.6	0.5*	6.0	1.4	3.2
Embryonic resorptions - mean/dam	0.3	0.0	0.6	0.1	0.3
Fetal resorptions - % impl.	3.1	2.7	3.7	3.2	3.2
Fetal resorptions - mean/dam	0.3	0.2	0.4	0.3	0.3
No. live fetuses - % impl.	89.8	95.5*	87.2	94.5	**70.4***
No. live fetuses - mean/dam	8.0	8.4	8.6	8.5	**6.6**
No. of live fetuses - mean/dam (dams with live fetuses on D28 only)	8.8	8.8	9.0	8.9	8.9
No. dead fetuses	0	0	0	0	0
Sex ratio (% males)	45.5	50.0	50.0	48.3	48.7

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**10**	**30**	**80**	**350**
Mean fetal weight (g) - combined	34.5	35.3 *(+2%)*	35.6 *(+3%)*	34.5 *(±0%)*	**30.5 *(-12%)***
Mean fetal weight (g) - males	35.2	35.4 *(+1%)*	36.1 *(+3%)*	34.8 *(-1%)*	**30.6* *(-13%)***
Mean fetal weight (g) - females	34.0	35.0 *(+3%)*	35.3 *(+4%)*	34.2 *(+1%)*	**30.5 *(-10%)***
Placental weight (g)	5.2	5.4 *(+3.8%)*	5.5 *(+5.8%)*	5.2 *(±0%)*	**4.9 *(-5.8%)***

* p<0.05, ** p<0.01 (Fishers's Exact Test, Steels Test or Dunnett's Test)
Findings considered related to treatment with prothioconazole are written in **bold letters**

E. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

The nature and incidences of external, visceral and skeletal abnormalities did not indicate an effect of treatment at any dose level (Table 5.6.2/06-7). The percentage incidence of fetuses with any abnormality was similar across all treated groups with no dose-response relationship, which does not suggest a treatment-related effect. The incidence of fetuses with abnormal skeletal findings was well within the normal range for controls from this strain of rabbits (range 0.0-3.6 % in studies from 1991-1995, see attachment V of report). Although the incidence of fetuses with rib fusion/bifurcation and/or thoracic vertebral defects at 350 mg/kg bw/d was slightly raised relative to concurrent controls (3 cases vs. 1 control case), it is not considered as a treatment related effect since incidences of up to 4 cases in comparable numbers of fetuses have occurred in the laboratory historical control data (from studies performed 1991-1995, see attachment V of report).

Table 5.6.2/06-7: Group mean incidences of fetal findings (fetal /litter) incidences)

		Prothioconazole (mg/kg bw/d)				
		0	**10**	**30**	**80**	**350**
External / fresh visceral examination						
No. litters evaluated		20	24	21	23	17
No. fetuses evaluated (external)		176	210	190	205	152
Any external / fresh visceral abnormal finding	n	0 (0)	4 (3)	0 (0)	4 (3)	1 (1)
	%	0.0 (0.0)	1.9 (12.5)	0.0 (0.0)	2.0 (13.0)	0.7 (5.9)
Visceral examination						
No. fetuses evaluated (heart + vessels / head)		176 / 87	210 / 103	190 / 98	205 / 103	152 / 76
Any visceral abnormal finding	n	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	%	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Skeletal examination						
No. fetuses evaluated (skeletal)		176	210	190	205	152
Any skeletal abnormal finding	n	2 (2)	2 (2)	0 (0)	3 (3)	4 (4)
	%	1.1 (10.0)	1.0 (8.3)	0.0 (0.0)	1.5 (13.0)	2.6 (23.5)

		Prothioconazole (mg/kg bw/d)				
		0	10	30	80	350
Total						
Any abnormal finding	F	2[a] (2)	5[b] (4)	0 (0)	6[c] (5)	4[d] (4)
	L	1.1 (10.0)	2.4 (16.7)	0.0 (0.0)	2.9 (21.7)	2.6 (23.5)

F: Fetus, L: Litter
[a] one fetus with thoracic vertebral defect, one fetus with fused and abnormally ossified sternebrae;
[b] one runt (small fetus, <19 g) with missing rib and vertebral body, 2 fetuses with right kidney/ureter agenesis, one fetus with cheilognathopalatoschisis, encephalocele, micro/anophthalmia, prolaps linguae, one fetus with fused and abnormally ossified sternebrae; [c] 2 runts, one with fused and abnormally ossified sternebrae, one fetus with fused ribs, one fetus with abnormally ossified sternebrae, 2 fetuses with agenesis of left kidney/ureter; [d] one fetus with enlarged fontanelle, shortened and bent ulna/radius, humerus/tibia/fibula divided and bent, wavy ribs, 3 fetuses with fused ribs (2 with some vertebral bodies absent and one with bifurcated ribs
Findings considered related to treatment with prothioconazole are written in **bold letters**

Incidences of skeletal variants are presented in Table 5.6.2/06- 8. At 350 mg/kg bw/d there were notable differences from the controls in the fetal incidences of incomplete and absent ossification of one or more sternebrae and phalanges of the digits, and of unossified 13[th] rib. However, in some cases the incidence was lower than controls and in others the incidence was higher than controls, indicating either advanced or retarded ossification in different structures. This does not suggest a clear effect of treatment, though an increased incidence of retarded ossification would not be unexpected given the lower fetal bodyweights recorded in this group.

Table 5.6.2/06- 8: Summary of notable skeletal variants (Fetal (litter) incidence in %)

Parameter	Prothioconazole (mg/kg bw/d)				
	0	10	30	80	350
No. fetuses evaluated	176	210	190	205	152
No. litters examined	20	24	21	23	17
Sternebra 5 - unossified	13 (50)	12 (54)	16 (62)	18 (65)	20* (76)
13[th] rib - unossified	49 (100)	63** (100)	65** (100)	54 (100)	37* (82)
Left forelimb					
Metacarpal 1 - incomplete ossification	34 (95)	31 (67*)	28 (95)	27 (61**)	0** (0**)
Digit 5 phalanx - incomplete ossification	56 (100)	58 (100)	64 (100)	48 (96)	28** (76*)
Digit 5 phalanx - unossified	43 (95)	41 (100)	34 (90)	52* (91)	72** (100)
Right forelimb:					
Metacarpal 1 - incomplete ossification	34 (95)	31 (67*)	28 (90)	28 (61**)	0** (0*)
Digit 5 phalanx - incomplete ossification	53 (95)	57 (100)	61 (95)	44* (96)	27** (82)
Digit 5 phalanx - unossified	46 (95)	42 (96)	38 (90)	55* (96)	72** (100)
Left hindlimb					
Digit 4 phalanx - incomplete ossification	59 (100)	52 (96)	52 (95)	51 (96)	26** (76*)
Digit 4 phalanx - unossified	14 (65)	17 (63)	12 (52)	22* (65)	26** (71)

Parameter	Prothioconazole (mg/kg bw/d)				
	0	**10**	**30**	**80**	**350**
Right hindlimb :					
Digit 4 phalanx - incomplete ossification	59 (100)	50 (96)	51 (95)	50 (96)	26** (76**)
Digit 4 phalanx - unossified	14 (60)	19 (63)	13 (48)	23* (70)	26** (71)

* p<0.05, ** p<0.01
Findings considered related to treatment with prothioconazole are written in **bold letters**

III. CONCLUSION

Very strong maternal toxicity was evident at 350 mg/kg bw/d, including mortality, reduced feed consumption, bodyweight losses/reduced gains, and minimally decreased absolute liver weights (secondarily to the slightly decreased terminal body weight). The NOAEL for maternal toxicity was 80 mg/kg bw/d.

Treatment at 350 mg/kg bw/d was also associated with abortions, total litter losses and reduced gravid uterus and fetal weights, which are considered secondary to maternal toxicity. The NOAEL for developmental effects was 80 mg/kg bw/d. There was no evidence of a teratogenic effect up to 350 mg/kg bw/d.

All observed developmental effects are considered as unspecific and secondary to the very strong (partially lethal) maternal toxicity. Therefore, these findings are no indications for a specific or direct developmental toxic potential of prothioconazole. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, such a constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. Specifically, the Guidance states: *"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and certain malformations in some strains of certain species."*

CA 5.7 Neurotoxicity studies

CA 5.7.1 Neurotoxicity studies in rodents

Table 5.7-1 Summary of neurotoxicity studies in rodents

Study	Sex	NOAEL (mg/kg bw/d)	LOAEL	Findings at LOAEL	Reference
Acute oral neurotoxicity rat 0, 200, 750 and 2000 mg/kg/d	M/F	200	750	Partially formed stools and perianal stain, decreases in motor and locomotor activity	[redacted] 2000 M-023861-01-1
13 week neurotoxicity rat (gavage) 0, 100, 500 and 1000 mg/kg/d	M/F	100	500	Reduced bodyweight gain, urine stain, ventrum wet/stain	[redacted] 2001 M-053225-01-1

Acute and subchronic neurotoxicity studies in rats did not identify specific neurobehavioural effects in the absence of general systemic toxicity and there were no neurohistopathological changes in nerve tissue or muscle (mild reductions in activity in animals showing general toxicity are not considered to be a specific neurotoxic effect). It was concluded that prothioconazole is not selectively toxic to the nervous system.

CA 5.7.2 Delayed polyneuropathy studies

No data submitted. Since prothioconazole is not a member of a chemical class associated with delayed neurotoxicity and since there is no evidence of changes in nervous tissues, testing for delayed neurotoxicity is not required.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies of metabolites

Some toxicological studies have been conducted on the following wheat metabolites:

Prothioconazole-desthio (M04, JAU 6476-desthio, SXX 0665) accounts for the majority of the residue found in wheat grain. It is only a minor systemic metabolite found in the rat, dog and goat.

JAU 6476-sulfonic acid (M02, JAU 6476-SA) is a major metabolite found in the rotational wheat straw or hay. However, it was not identified in the rat metabolism studies.

JAU 6476-alpha-hydroxy-desthio (M18), JAU 6476-alpha-acetoxy-desthio (M19), JAU 6476-benzylpropyldiol (M49) were also found in wheat but not in rat metabolism studies or in any other animal tested, and their toxicity is also considered here. Trace quantities of JAU 6476-triazolinone (M03) occurred in the rat, but it was a substantial metabolite in fodder wheat.

JAU 6476-desthio (M04, prothioconazole-desthio, SXX 0665)

In plants (and only to a minor extend also in mammals) prothioconazole is converted to its desthio-metabolite, which appears to be more potent toxicologically than prothioconazole (JAU 6476). Therefore, reference values are also been proposed for prothioconazole-desthio for use in the consumer and operator risk assessments.

Acute toxicity

Table 5.8.1-2: Summary of the acute toxicity of JAU 6476-desthio

Species	Sex	Route/Study	Comments	Classification (EU Directive 93/21/EEC)	Reference
Rat	M F	Oral	LD_{50} 2806 mg/kg 2506 mg/kg	Not classified	[redacted] (1991a) M-008355-01-1
Mouse	M F	Oral	LD_{50} 2235 mg/kg 3455 mg/kg	Not classified	[redacted] (1991d) M-008521-01-1
Rat	M/F	Dermal	LD_{50} >2000 mg/kg	Not classified	[redacted] (1991b) M-008359-01-1
Rat	M/F	Inhalation	LC_{50} >5077 mg/m³ (dust)	Not classified	[redacted] (1991) M-008361-01-1
Rat	M F	Intraperitoneal	LD_{50} 430-500 mg/kg 632 mg/kg	Not classified	[redacted] (1991) M-008353-01-1
Rabbit	M/F	Skin irritation	Not irritant	Not classified	[redacted] (1991) M-031139-01-1
Rabbit	M/F	Eye irritation	Slightly irritating	Not classified	[redacted] (1991) M-031139-01-1
Guinea pig	M/F	Skin sensitisation Buehler method	Not sensitising	Not classified	[redacted] (1991) M-008358-01-1

SXX 0665 was not classifiable for any of the acute effects tested for according to current EC classification criteria.

In the inhalation study the acute LC_{50} of SXX 0665 dust in the rat is >5077mg/m³. Despite the MMAD being 39.9 µm for this value there were no suitable practical methods available to reduce the particle size of the dust at high concentrations. The 4-hour LC_{50} attained for the dust is hence considered valid for human risk assessment. No classification is proposed in accordance with the current EC classification directive.
No classification is proposed for this material.

Short-term toxicity

Table 5.8.1-2: Summary of short-term toxicity studies performed on SXX 0665.

Study / species / dose levels	NOAEL (mg/kg bw/day)	LOAEL[a] (mg/kg bw/day)	Effects at LOAEL	Reference
4-week dietary toxicity; Rat; 0, 100, 300, 1000ppm	< 11 (100 ppm)	11	Increased liver weight and fatty infiltration of liver	[redacted] & [redacted] (1992) M-008365-01-1
13-week dietary toxicity; Rat; 0, 30, 125, 500, 2000ppm	2.2 (30 ppm)	9.7	Liver enzyme induction and histopathology	[redacted] & [redacted] (1999) M-018496-01-1
13-week dietary toxicity; Mouse; 0, 40, 200, 1000, 5000ppm	< 12 (40 ppm)	12	Hepatic enzyme induction Hepatic hypertrophy	[redacted] (1999) M-023192-02-1
6-week dietary toxicity; Dog; 0, 10, 100, 1000, 5000ppm	0.37 (10 ppm)	3.7	Hepatic enzyme induction Liver histopathology	[redacted] & [redacted] (1999) M-008029-03-1
13-week dietary toxicity; Dog; 0, 40, 200, 1000ppm	7.8 (200 ppm)	38	Increased liver enzyme activities, weights and histopathology.	[redacted], [redacted] & [redacted] (2000) M-026972-01-1
30-week dietary toxicity; Dog; 0, 40, 300, 2000ppm	10 (males) (300 ppm)	40	Liver enzyme induction and associated cytoplasmic changes in both sexes	[redacted], [redacted] & [redacted] (2001) M-136735-01-1
Sub-acute inhalation toxicity; Rat; (range finder study) 0, 10.7, 54, 235mg/m^3	54mg/m^3	235mg/m^3	Elevated transaminase and alkaline phosphatase activity in plasma; Increased thromboplastin time	[redacted] (1991) M-008347-01-1
4-week inhalation toxicity; Rat; 0, 11.3, 47, 228 mg/m^3	> 228mg/m^3 (highest dose tested) (≡ 82mg/kg)	-	-	[redacted] (1992b) M-008343-01-1

Liver effects

The common target organ in all 3 species was the liver, and the LOAELS for the short-term studies were based on liver effects. Hepatic enzyme induction as measured by hepatic cytochrome P_{450}, deethylase and demethylate activities, and characteristic cytoplasmic changes in hepatocytes, were common to the rat and the dog. Further histological alterations occurred in the liver of the mouse and included fatty changes/vacuolation (signs compatible with the mode of action of SXX 0665), hypertrophy and single cell necrosis.

Other effects

It is noted that there were some small (<10%) but occasionally statistically significant effects on red blood cells parameter in rats and mice in the short term and chronic studies. However the exact parameters affected were not consistent between studies, and there were no other effects such as increased bilirubin or reticulocytes that would give an indication of the mechanism or origin of these effects. Platelet counts were also reduced with treatment in rats and mice but were not of toxicological significance. Given that the blood effects were isolated, not consistent, not seen in dogs and did not drive any NOAELs, they are not considered to be of toxicological relevance.

Other effects seen in the short term toxicity studies were considered sequalae of the liver effects above. The most notable effects were on rodent ovaries: As a result of disturbance of hormone levels (most likely a consequence of liver enzyme induction), ovary effects were apparent in rats and mice (although not in dogs). These effects were increased ovary weights in the rat 90 day study (the increased ovary weights in the 28 day study was more likely a result of an unusually low control value), and at the high doses in the 28 day rat study there was also an increased number of follicles and stromal cell oedema

(although there was no ovary histopathology seen in the 90 day rat study). In the 13 week mouse study, there was no effect on ovary weights, but haemorrhagic degeneration of corpora lutea was seen. See also '**Ovary effects seen in the short-term and chronic toxicity studies**' in the summary of the chronic toxicity studies (below Table 5.8.1-4).

Most sensitive species

Comparison of the NOAELs derived in these studies showed the rat is considered to be no less sensitive than the dog (13 week rat versus 13 week dog NOAEL and LOAEL). For the 13 week dog study there is an obvious inconsistency between the NOAEL mentioned several times in the DAR for the first Annex I inclusion (B.6.3.3′b), Table 6.24, Table B.6.67: 200 ppm, equivalent to **7.8 mg/kg bw/day**) and the EFSA Scientific Report (page 53; **1.6 mg/kg bw/day**). The reason for this is unclear but it might be related to the fact that the study report claims a "marginal elevation of ASAT" (aspartate aminotransferase) at 200 ppm (due to a marginally increased value in one single dog (no. 147, male) in week 13). But this assessment is clearly incorrect based on the following facts:

A) The comparably high week 13 value (31.9 U/l) in male no. 147 (compare: mean week 13 value for control males: 20.4 U/l) is due to the unusually high pretreatment value (26.4 U/l) in this dog (compare: mean pretreatment value for control males: 14.8 U/l).
B) The 20.8% increase of ASAT in male no. 147 from pretreatment to week 13 is less than the 37.8% increase of mean ASAT for control males from pretreatment to week 13.
C) The mean ASAT values for males in week 13 (20.4 – 23.7 – 25.4 – 23.4 U/l in ascending dose) demonstrate the absence of any treatment related effect on ASAT up to the highest tested dose of 1000 ppm (equivalent to 38 mg/kg bw/day).

Thus, the overall NOAEL for the 13 week dog study should be corrected in the EFSA Scientific Report from 1.6 to 7.8 mg/kg bw/day.

From the 2 year oncogenicity studies the mouse also did not appear to be a more sensitive species to SXX 0665 than the rat. The absence of data from a 52-week study in the dog. is considered not to compromise the human risk assessment. The 30 week dog NOAEL was similar to the 13 week dog LOAEL.

Inhalation toxicity studies in rats

In the inhalation studies conducted in rats the animals were not more sensitive to SXX 0665 than via the oral route. Only clinical chemistry effects of liver enzyme effects were apparent in the inhalation studies. These were similar to effects seen in the dietary toxicity studies.

Genotoxicity testing

Table 5.8.1-3: Summary of genotoxicity studies with JAU 6476-desthio

Test system; study	Concentration / dose levels	Result	Reference
***In vitro* studies**			
Bacterial point mutation assay (Ames test) in *S. typhimurium* strains (TA1535, TA100, TA1537, TA98)	Plate incorporation assay: 3 - 5000 µg /plate (±S9)	**Negative**	[redacted] (1990) [M-031136-01-1]
Bacterial point mutation assay (Ames test) in *S. typhimurium* strains (TA1535, TA100, TA1537, TA98, TA102)	Plate incorporation assay: 3 - 5000 µg /plate (±S9) Pre-incubation assay: 33 - 5000 µg/plate (±S9)	**Negative**	[redacted] (2017) [M-588632-01-1]

Test system; study	Concentration / dose levels	Result	Reference
Mammalian cell gene mutation assay *in vitro* (HPRT locus, V79 CHL cells)	Gene mutation assay: 12.5 - 250 µg/ml (-S9) 50 - 500 µg/ml (+S9)	**Negative**	[redacted] (1999) [M-009104-01-1]
Rat liver UDS assay *in vitro*	UDS assay: 5.0 – 60.0 µg/ml	**Negative**	[redacted] (1992) [M-031126-01-1]
Mammalian chromosomal aberration assay *in vitro* (Chinese hamster ovary (CHO) cells)	Chromosome aberration assay: 8 h harvest: 125 µg/ml (±S9) 24 h harvest: 5 - 125 µg/ml (±S9) 30 h harvest: 125 µg/ml (±S9)	**Negative**	[redacted] (1995) [M-031119-01-1]
***In vivo* studies**			
Micronucleus assay (*in vivo* mouse bone marrow)	350 mg/kg bw (i.p.)	**Negative** (PCE/NCE ratio altered)	[redacted] (1993) [M-031124-01-1]

JAU 6476-desthio was tested for gene mutation in two bacterial point mutation assays (Ames test), in an HPRT locus gene mutation assay in V70 CHL cells and an *in vitro* rat liver UDS assay. All assays gave negative results. Therefore it is concluded that JAU 6476-desthio is not a gene mutagen.

JAU 6476-desthio did not induce chromosome aberrations in Chinese hamster ovary cells and was negative in an in vivo mouse bone marrow micronucleus assay. Therefore it is concluded that JAU 6476-desthio is not clastogenic/aneugenic.

Report: KCA 5.8.1/15 [redacted] 1990; M-031136-01-1
Title: SXX 0665 - Salmonella microsome test
Report No.: 19539
Document No.: M-031136-01-1
Guideline(s): OECD 471 (1983), EEC Directive 84/449/EEC B.14
Guideline deviation(s): none
GLP/GEP: **yes**

Deviations: Deviations from the current OECD guideline (1997):
Only four strains of bacteria were used (instead of at least five), the number of analysable concentrations tested was not sufficient, and the composition of medium used and the number of cells per culture were not reported.
Due to those deviations a new study was conducted according to the current guideline which confirmed the results of this study from 1990.

Executive summary:

JAU 6476-desthio (batch No. 17005/89, purity 93.7 %) was tested in a bacterial point mutation assay (Ames test) on 4 histidine-auxotrophic strains of *Salmonella typhimurium*, TA 98, TA 100, TA 1535 and TA 1537. Two independent assays were performed by plate incorporation, both experiments with and without S9 rat liver fraction from rats previously induced with 'Arochlor 1254'. The highest concentration used in the second assay was based on the results of the first assay. The dose levels employed for the 1st assay were 0 (solvent), 8, 40, 200, 1000 and 5000 µg/plate with (30 % S9 mix) and without metabolic activation, and for the 2nd assay, 0 (solvent), 150, 300, 600, 1200 and 2400 µg/plate with either 10 or 30 % S9 mix or without metabolic activation.

JAU 6476-desthio produced a strain-specific bacteriotoxic effect at concentrations of ≥ 600 µg/plate in both assays and growth inhibition in all strains at ≥ 1200 µg/plate with and/or without metabolic activation. Precipitation on the plates was also apparent at concentrations of ≥1000 µg/plate. Therefore, plates exposed to 600 – 2400 µg/plate were of limited value for the assessment of mutagenicity and 5000 µg/plate could not be evaluated. None of the 4 strains showed a dose-related and biologically relevant increase in mutant counts over those of the negative controls, either with or without metabolic activation, in either of the independent assays. The positive controls produced satisfactory results.

However, considerable deviations from the current (1997) OECD guideline 471 occurred (see above). Therefore a new bacterial point mutation assay according to the current guideline was conducted (see 5.8.1/60, [M-588632-01-1]).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: JAU 6476-desthio

Synonym:	SXX 0665
Description:	Beige to brown powder
Batch No.:	17005/89
Purity:	93.7 %
Stability of the test compound:	The batch used was analysed prior to study initiation and approved for use during the test period.
Solvent used:	DMSO
Solvent/final concentration:	0.1 ml/plate

2. Control Materials:

Solvent control: DMSO (0.1 ml/plate)

Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 1535	Sodium azide (Na-azide)	DMSO	10 µg/plate
TA 100	Nitrofurantoin (NF)	DMSO	0.2 µg/plate
TA 1537	4-nitro-1,2-phenylene diamine (4-NPDA)	DMSO	10 µg/plate
TA 98			0.5 µg/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 1535	2-aminoanthracene (2-AA)	DMSO	3 µg/plate
TA 100			
TA 1537			
TA 98			

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism of the test substance.

Preparation: The S9 fraction was isolated from the livers of at least six adult male Sprague Dawley rats. For enzyme induction, the animals received a single intraperitoneal injection of Aroclor 1254, dissolved in corn oil, at a dose of 500 mg/kg body weight, five days prior to sacrifice and liver preparation (Preparation of S9 fraction: May 7, 1990; protein content: 28.6 mg/ml).
Prior to first use, the batch was checked for sterility and its metabolizing capacity.
The S9 mix was freshly prepared before use and used only on the same day. For this purpose, a sufficient amount of S9 fraction was slowly thawed and mixed with the cofactor solution.
70 ml of cofactor solution were composed as follows:

$MgCl_2 \times 6 H_2O$	162.6 mg
KCl	246.0 mg
glucose-6-phosphate, disodium salt	179.1 mg
NADP, disodium salt	315.0 mg
phosphate buffer	100.0 mM

The S9 mix comprised 70 % cofactor solution and 30 % S9 fraction. In the 2nd assay an S9 mix containing 10 % S9 fraction was also used (see Table 5.8.1/15-2). In this case, the 20 % of volume lacking were substituted by KCl (0.15 M).

4. Test organisms: *Salmonella typhimurium* strains TA1535, TA100, TA1537, and TA98.
All strains were checked for crystal-violet sensitivity (rfa) and all strains except TA 102 (not required for TA 102) were checked for UV sensitivity (uvrB). In each individual test, histidine dependence of the cultures was automatically checked by the accompanying negative controls. A special test for ampicillin resistance was not necessary since strains TA 100 and TA 98 were incubated on ampicillin containing nutrient agar and formed individual colonies. Consequently surviving bacteria were ampicillin resistant.

5. Test concentrations:
Plate incorporation assay: 0, 8, 40, 200, 1000, 5000 µg JAU 6476-desthio/plate

B. TEST PERFORMANCE

1. Dates of experimental work: June 08, 1990 – June 28, 1990

2. Bacterial point mutation assay (Ames test)

Two independent assays were performed by plate incorporation. For each assay and strain 1 ml portion of stock culture was thawed and 0.2 ml of the thawed culture was added to 10 ml nutrient broth. This culture was incubated overnight at 37°C and used only on the same day.

The bacterial suspensions were obtained from 17-hour cultures in nutrient broth, which had been incubated at 37°C and 90 rpm. These suspensions were used for the determination of mutant counts. No standardized procedure was employed to set the bacterial suspensions at a defined density of viable cells per millilitre, since the chosen method of incubation normally produces the desired density. However, the numbers of viable cells were established in parallel procedure when determining the titres.

Plate incorporation assay

To test tubes containing 2 ml portions of warm soft agar, 0.1 ml test substance solution or solvent, 0.1 ml bacterial culture, 0.5 ml S9 mix (in tests with metabolic activation) or 0.5 ml buffer (in tests without metabolic activation) were added. The mixture was placed in a waterbath at 45°C for max. 30 seconds. After mixing, the samples were poured onto petri dishes with solid agar. After incubation for 48 h at 37°C, his+ revertants were counted. Four test plates per concentration or per control incubation were included.

4. Statistics

Descriptive statistical methods were used to calculate means and standard deviations.

5. Evaluation criteria

Solubility: If observed, precipitation of the test substance would have been recorded.

Toxicity: The toxicity of the test substance was assessed in three ways:

- by gross appraisal of background growth on the plates for mutant determination
- by mutant count per plate (if marked and dose-dependent reduction in the mutant count per plate compared to the negative controls appeared, a toxic effect was assumed)
- by titre determination (To establish the number of viable cells total bacterial counts were taken on two plates for each concentration studied with S9 mix. The dilution of bacterial suspensions used for the determination of titres was 1:1,000,000. Titres were determined under the same conditions as were the mutations, except that the histidine concentration in the soft agar was increased fivefold to permit the complete growth of bacteria.)

Mutagenicity: A reproducible and dose-related increase in mutant counts of at least one strain was considered to be a positive result, if:

- for TA 1535, TA 100 and TA 98 this increase was about twofold compared to negative controls
- for TA 1537 this increase was at least threefold compared to negative controls

Otherwise, the result was evaluated as negative.

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

Analytical determinations verified that JAU 6476-desthio is stable in the solvent at room temperature at concentrations ranging from 0.08 mg/ml to 200 mg/ml for at least twenty-four hours.

Table 5.8.1/15_1: Analysis for stability of JAU 6476-desthio in the solvent at room temperature

Nominal value in mg/ml	Content in % after storage time	
	0 hrs	24 hrs
0.08	97.3	96.9
200	102	105

B. TOXICITY AND SOLUBILITY

The bacterial titres showed that JAU 6476-desthio produced a strain-specific bacteriotoxic effect at concentrations of ≥ 600 µg/plate in both assays and growth inhibition in all strains at ≥ 1200 µg/plate with and/or without metabolic activation. Precipitation on the plates was also apparent at concentrations of ≥ 1000 µg/plate. Therefore, plates exposed to 600 – 2400 µg/plate were of limited value for the assessment of mutagenicity and 5000 µg/plate could not be evaluated.

C. MUTATION ASSAY

None of the 4 strains showed a dose-related and biologically relevant increase in mutant counts over those of the negative controls, either with or without metabolic activation, in either of the independent assays. The positive controls produced satisfactory results.

Table 5.8.1/15- 2: Ames test with JAU 6476-desthio – Mean number of revertants

Strain	**TA 1535**		**TA 100**		**TA 1537**		**TA 98**	
Metabolic activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
1st assay (30 % S9 fraction in S9 mix)								
Negative control	13	20	59	76	8	8	21	26
Test substance (µg/plate)								
8	11	15	68	70	9	7	23	26
40	12	19	67	71	8	8	18	18
200	14	13	56	64	8	7	20	19
1000	10 [A]	14 [A, C]	36 [A, B]	43 [A]	8 [A, B]	5 [A, C]	14 [A]	19 [A]
5000	-- [B, C]	-- [B, C]	-- [C]	-- [C]	-- [C]	-- [B, C]	-- [B, C]	-- [B, C]
Positive control[#]	668	161	174	270	52	48	77	211
2nd Assay (30 % S9 fraction in S9 mix)								
Negative control	16	22	60	100	9	11	30	24
Test substance (µg/plate)								
150	14	23	71	94	9	13	24	25
300	13	19	70	99	10	11	23	24
600	18	20	68	76	9 [A]	8 [A]	26	23
1200	10 [A, B]	23 [A]	22 [A, B]	78 [A, B]	3 [A, B]	9 [A, B]	14 [A, B]	18 [A]
2400	-- [B, C]	11 [A, B, C]	-- [A, B, C]	-- [A, B, C]	-- [A, B, C]	-- [A, B, C]	-- [A, B, C]	7 [A, B, C]
Positive control[#]	889	174	277	786	56	45	134	547

Strain	**TA 1535**		**TA 100**		**TA 1537**		**TA 98**	
Metabolic activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
2nd Assay (10 % S9 fraction in S9 mix)								
Negative control		20		87		8		31
Test substance (µg/plate)	-		-		-		-	
150		24		69		6		31
300		25		83		8		31
600		17 A		100 A		8 A		31 A
1200		12 A		35 A, B		5 A, B		28 A
2400		8 A, B, C		-- A, B, C		-- A, B, C		16 A, B, C
Positive control#		273		1397		183		1523

A bacteriotoxic effect observed in titre determination at this concentration
B reduced background growth
C precipitation
see Material and Methods (I.A.2.) above for compound and concentrations

III. CONCLUSION

JAU 6476-desthio did not induce gene mutations in the strains of *S. typhimurium* used in the study at concentrations up to 2400 µg/plate.

Report: KCA 5.8.1/60 [redacted], 2017; M-588632-01-1
Title: AE 1194888, pure substance (JAU 6476-desthio): Salmonella typhimurium reverse mutation assay
Report No.: 1825400
Document No.: M-588632-01-1
Guideline(s): Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471: Bacterial Reverse Mutation Test, adopted July 21, 1997
Commission Regulation (EC) No. 440/2008 B13/14, dated May 30, 2008
EPA Health Effects Test Guidelines, OPPTS 870.5100, Bacterial Reverse Mutation Test EPA 712-C-98-247, August, 1998
Guideline deviation(s): none
GLP/GEP: **Yes**

Deviations: Deviations from the current OECD guideline (1997): none

Executive summary:

A new reverse mutation assay compliant with the current (1997) OECD guideline using *S. typhimurium* strains TA1535, TA100, TA1537, TA98 and TA102 was conducted to assess potential mutagenicity of JAU 6476-desthio (batch no. SES 10834-2-1, purity 98.3 %). Bacterial cultures were exposed to the test material (dissolved in DMSO) at concentrations between 3 and 5000 µg/plate in the presence and absence of an exogenous metabolic activation system (Phenobarbital/β-naphthoflavone-induced rat liver S9 mix). The results were confirmed in an independently repeated assay incorporating a pre-incubation step. Both assays were performed in triplicate.

Precipitation of JAU 6476-desthio was observed at concentrations ≥ 1000 µg/plate but had no influence on the data recording. Background growth was not affected at any concentration tested. Toxic effects, evident as a reduction in the number of revertants were only observed in the pre-incubation assay in strain TA 100 without S9 mix at 1000 µg/plate and in strain TA 1537 with S9 mix at 5000 µg/plate. There were no increases in mutation frequency induced by JAU 6476-desthio either with or without S9 mix for any strain. Positive control substances (sodium azide, 4-nitro-o-phenylene diamine, methyl methane sulfonate and 2-aminoanthracene) produced significant increases in the number of revertants in all strains tested.

JAU 6476-desthio was concluded to be non-mutagenic in this assay.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: JAU 6476-desthio
Synonym: AE 1194888
Description: White powder
Batch No.: SES 10834-2-1
Purity: 98.3 %
Stability of the test compound: All formulations were prepared freshly before treatment and used within two hours of preparation. The formulation was assumed to be stable for this period.
Solvent used: DMSO
Solvent/final concentration: 0.1 ml/plate

2. Control Materials:
Solvent control: DMSO (0.1 ml/plate)
Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 1535	Sodium azide (NaN_3)	Deionised water	10 µg/plate
TA 100			
TA 1537	4-nitro-o-phenylene diamine (4-NOPD)	DMSO	50 µg/plate
TA 98			10 µg/plate
TA 102	Methyl methane sulfonate (MMS)	Deionised water	2.0 µl/plate

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration
TA 1535	2-aminoanthracene (2-AA)	DMSO	2.5 µg/plate (10.0 µg/plate in TA 102)
TA 100			
TA 1537			
TA 98			
TA 102			

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism of the test substance.

Preparation: Phenobarbital/β-naphthoflavone induced rat liver S9 was used as the metabolic activation system. Each batch of S9 was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the Ames test. Furthermore for each S9 batch a sterility test and the determination of the protein concentration were performed. The protein concentration of the S9 preparation was 35.4 mg/ml in both experiments.
An appropriate quantity of S9 fraction was thawed and mixed with S9 cofactor solution, to result in a final concentration of approx. 10 % v/v in the S9 mix. Cofactors were added to the S9 mix to reach the following concentrations in the S9 mix:

$MgCl_2$	8 mM
KCl	33 mM
Glucose-6-phosphate, disodium salt	5 mM
NADP	4 mM
Sodium-ortho-phosphate buffer (pH 7.4)	100.0 mM

The S9 cofactor solution was prepared freshly and sterile-filtrated before the S9 supernatant was added. During the experiment, the S9 mix was stored in an ice bath.

4. Test organisms: *Salmonella typhimurium* strains TA1537, TA98, TA1535, TA100, and TA102.
Regular checking of the properties of the *Salmonella typhimurium* strains regarding the membrane permeability, ampicillin resistance, UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates was performed by the laboratory.

5. Test concentrations:

Plate incorporation assay: 0, 3, 10, 33, 100, 333, 1000, 2500, 5000 µg JAU 6476-desthio/plate

Pre-incubation assay: 0, 33, 100, 333, 1000, 2500, 5000 µg JAU 6476-desthio/tube

B. TEST PERFORMANCE

1. Dates of experimental work: March 17, 2017 – April 05, 2017

2. Salmonella/microsome test

The thawed bacterial suspension was transferred into 250 ml Erlenmeyer flasks containing 50 ml nutrient medium. A solution of 50 µl ampicillin (25 µg/ml) was added to the strains TA 98, TA 100, and TA 102. This nutrient medium contained per litre:

8 g Nutrient Broth
5 g NaCl

The bacterial cultures were incubated in a shaking water bath for 4 hours at 37° C. The optical density of the bacteria was determined by absorption measurement and the obtained values indicated that the bacteria were harvested at the late exponential or early stationary phase (10^8-10^9 cells/mL).

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

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

Plate incorporation assay

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

Pre-incubation assay

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

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

In parallel to each test a sterile control of the test item was performed and documented in the raw data. Therefore, 100 µl of the stock solution, 500 µl S9 mix / S9 mix substitution buffer were mixed with 2.0 ml overlay agar and poured on minimal agar plates.

4. Statistics

Descriptive statistical methods were used to calculate means and standard deviations.

5. Evaluation criteria

Solubility:	Precipitation of the test substance was recorded if observed.
Toxicity:	Toxic effects evident as reduction in the number of revertants below the indication factor of 0.5.
Mutagenicity:	A reproducible and dose-related increase in mutant counts of at least one strain was considered to be a positive result, if:
	- for TA 102, TA 100 and TA 98 this increase was about twofold compared to negative controls
	- for TA 1535 and TA 1537 this increase was at least threefold compared to negative controls
	A dose dependent increase in the number of revertant colonies below the threshold was regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However, whenever the colony counts remained within the historical range of negative and solvent controls such an increase was not considered biologically relevant.
	Otherwise, the result was evaluated as negative.

II. RESULTS AND DISCUSSION

A. TOXICITY AND SOLUBILITY

JAU 6476-desthio precipitated in the overlay agar in the test tubes from 2500 to 5000 µg/plate. Precipitation of JAU 6476-desthio in the overlay agar on the incubated agar plates was observed from 1000 to 5000 µg/plate. The undissolved particles had no influence on the data recording.

The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9 mix in all strains used. Toxic effects, evident as a reduction in the number of revertants

(below the indication factor of 0.5), were observed in the pre-incubation assay in strain TA 100 without S9 mix at 1000 µg/plate and in strain TA 1537 with S9 mix at 5000 µg/plate.

B. MUTATION ASSAY

No increase in revertant colony numbers of any of the five tester strains was observed following treatment with JAU 6476-desthio at any dose level, neither in the presence nor absence of metabolic activation (S9 mix). There was also no tendency of higher mutation rates with increasing concentrations in the range below the generally acknowledged border of biological relevance.

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

Table 5.8.1/60- 1: Mutation assay with JAU 6476-desthio – Mean number of revertants

Strain	TA 1535		TA 100		TA 1537		TA 98		TA 102	
Metabolic activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Plate incorporation assay										
Solvent control	16 ± 5	18 ± 3	196 ± 10	191 ± 16	10 ± 5	8 ± 3	29 ± 4	38 ± 2	535 ± 11	674 ± 19
Negative control	10 ± 1	17 ± 2	214 ± 10	205 ± 10	11 ± 4	11 ± 3	34 ± 3	36 ± 6	591 ± 36	628 ± 31
JAU 6476-desthio (µg/plate)										
3	13 ± 2	13 ± 1	210 ± 30	201 ± 15	11 ± 5	12 ± 3	28 ± 8	44 ± 4	621 ± 16	799 ± 84
10	14 ± 6	17 ± 2	192 ± 12	203 ± 23	9 ± 1	10 ± 3	24 ± 6	42 ± 6	635 ± 34	884 ± 20
33	13 ± 2	16 ± 4	206 ± 14	179 ± 16	7 ± 2	8 ± 1	29 ± 7	35 ± 4	617 ± 33	869 ± 2
100	18 ± 2	15 ± 3	210 ± 10	181 ± 15	11 ± 3	12 ± 5	34 ± 8	33 ± 6	634 ± 24	812 ± 66
333	14 ± 4	16 ± 5	176 ± 10	193 ± 25	12 ± 1	11 ± 0	23 ± 6	43 ± 2	608 ± 42	810 ± 46
1000	9 P ± 2	16 ± 5	160 P ± 23	204 P ± 1	11 P ± 3	5 P,M ± 2	25 P,M ± 7	42 P ± 8	646 P ± 8	889 P ± 11
2500	10 P ± 1	11 P, M ± 2	219 P,M ± 21	192 P ± 8	12 P M ± 2	5 P,M ± 1	21 P,M ± 2	27 P,M ± 7	667 P ± 2	884 P ± 14
5000	13 P, M ± 2	11 P M ± 4	215 P,M ± 12	167 P,M ± 12	12 P M ± 2	5 P,M ± 2	22 P,M ± 4	20 P,M ± 5	631 P ± 40	900 P ± 14
Positive control#	1316 ± 44	454 ± 26	2115 ± 88	5096 ± 103	94 ± 25	219 ± 20	540 ± 50	4658 ± 276	4383 ± 383	1423 ± 75
Pre-incubation assay										
Solvent control	9 ± 2	11 ± 1	181 ± 7	155 ± 21	10 ± 1	13 ± 3	23 ± 6	35 ± 8	533 ± 5	652 ± 5
Negative control	11 ± 4	17 ± 4	210 ± 20	187 ± 25	10 ± 1	16 ± 1	21 ± 1	47 ± 10	579 ± 28	670 ± 34
JAU 6476-desthio (µg/tube)										
33	13 ± 4	12 ± 3	172 ± 18	133 ± 12	10 ± 1	15 ± 5	29 ± 3	46 ± 1	581 ± 12	748 ± 19

Strain	TA 1535		TA 100		TA 1537		TA 98		TA 102	
Metabolic activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
100	11 ± 1	14 ± 2	167 ± 19	145 ± 17	11 ± 1	14 ± 0	22 ± 2	48 ± 9	586 ± 27	746 ± 14
333	9 ± 2	15 ± 4	90 ± 14	137 ± 14	10 ± 3	14 ± 2	20 ± 3	35 ± 8	561 ± 44	752 ± 17
1000	9 P ± 2	10 P ± 1	55 P ± 3	105 P ± 21	7 P ± 2	11 P ± 2	26 P ± 2	35 P ± 6	388 P ± 17	762 P ± 13
2500	11 P, M ± 3	11 P, M ± 3	125 P, M ± 5	93 P, M ± 5	7 P, M ± 2	9 P, M ± 2	23 P, M ± 5	29 P, M ± 6	640 P ± 20	773 P ± 28
5000	9 P, M ± 2	10 P, M ± 3	120 P, M ± 4	82 P, M ± 9	7 P, M ± 0	5 P, M ± 1	17 P, M ± 5	30 P, M ± 2	637 P ± 34	792 P ± 23
Positive control[#]	1460 ± 34	331 ± 20	2243 ± 159	3726 ± 411	114 ± 10	244 ± 21	384 ± 19	5335 ± 288	5953 ± 286	2048 ± 52

P Precipitation
M Manual count
see Material and Methods (I.A.2.) above for compound and concentrations

III. CONCLUSION

In conclusion, it can be stated that during the described mutagenicity test and under the experimental conditions reported, JAU 6476-desthio did not induce gene mutations by base pair changes or frameshifts in the genome of the strains used. Therefore, JAU 6476-desthio is considered to be non-mutagenic in this *Salmonella typhimurium* reverse mutation assay.

Report: KCA 5.8.1/16 [redacted]; 1999; M-009104-01-1
Title: SXX 0665 - Mutagenicity study for the detection of induced forward mutations in the V79-HGPRT assay in vitro
Report No.: 28965
Document No.: M-009104-01-1
Guideline(s): OECD 476 (1984), EEC Directive 87/302/EEC; US-EPA PB 84-233295 (1984)
Guideline deviation(s): none
GLP/GEP: **yes**

Deviations: The following minor deviations from the current OECD guideline (2016) occurred: The number of cells treated and cultured was lower than required according to the current guideline ($1.5x10^6$ cells instead of at least $2x10^6$ were cultured during the expression period; not 10 spontaneous mutants were maintained in every culture in all phases of the test). However, cell numbers were acceptable according to the contemporary guideline (1984) and differ only slightly from the ones required by the current guideline. Therefore those minor deviations are not considered to have influenced the study outcome.

Executive summary:

In a 1999 GLP study JAU 6476-desthio (batch no. 1717008/90, purity 92.7 - 93.1 % %) was investigated in a gene mutation assay in Chinese hamster lung cells (V79) using the HPRT locus. Duplicate flasks of exponentially growing cells (4 x 10^6 cells/flask) were exposed for 5 hours to JAU 6476-desthio (dissolved in DMSO) at concentrations of 12.5-250 µg/ml (without S9 mix) or 50-500 µg/ml (with S9 mix). Cells were incubated for 7 days to allow for expression of mutant phenotype and were then plated

in selective medium and incubated for 7 days before counting the number of 6-TG resistant colonies. Four and 3 valid assays, with and without metabolic activation, respectively, were evaluated. Cytotoxicity and cloning efficiency were also assessed. Appropriate negative, vehicle and positive controls were also used and gave acceptable results. The study was compliant with OECD Guideline 476 (1984) and, apart from minor deviations (see above), complied also with the 2016 Guideline.

Dose levels were based on a preliminary cytotoxicity test. Precipitation was recorded at 500 µg/ml and marked cytotoxicity was recorded without S9 mix at ≥300 µg/ml.

There were no increases in mutant frequency at any of the cultures treated in the absence or presence of S9 mix. When the results of the assays combined were analysed statistically (Dunnett's test), there was a statistically significant ($p<0.05$) increase in mutation frequency at 100 µg/ml in the absence of S9 mix. Since there was no relationship to dose, it was considered biologically not relevant.

JAU 6476-desthio is considered non-mutagenic in a V79-HPRT gene mutation assay.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material: JAU 6476-desthio
Synonym: SXX 0665
Description: Beige powder
Batch No.: 1717008/90
Purity: 93.1 %, 92.8 %, 92.7 % (analytical results dated Sept. 16, 1992, March 18, 1993, September 01, 1993, respectively)
Stability of the test compound: JAU 6476-desthio was checked analytically in advance and the batch used was shown to be stable for the treatment period.
Solvent used: Dimethylsulfoxide (DMSO)
Solvent/final concentration: ≤1 % (v/v) DMSO

2. Control Materials
Solvent control: 1 % (v/v) DMSO
Positive control -S9: Ethylmethanesulfonate (EMS), final concentration: 900 µg/ml
Positive control +S9: Dimethylbenzanthrazene (DMBA), final concentration: 20 µg/ml

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism.
Preparation: The S9 fraction was isolated from the livers of Aroclor 1254 induced Wistar rats. It was purchased from Cytotest Cell Research, Germany (protein content: 40.0/42.0 mg/ml). For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with the freshly prepared cofactor solution in a sodium phosphate buffer pH 7.4. The S9 mix was kept on ice and used on the same day.
Final concentrations in the S9 mix:

S9 fraction	40.0 % (v/v)
$MgCl_2$ x 6 H_2O	8 mM
KCl	33 mM
Glucose-6-phosphate (disodium salt)	5 mM
NADP (disodium salt)	1 mM

4. Test organism: Chinese hamster lung cells (V79)
5. Culture media:

Culture medium:	Hypoxanthine-free Eagle's minimal essential medium (MEM), supplemented with nonessential amino acids, 2 mM L-glutamine, MEM-vitamins, $NaHCO_3$-solution, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10 % heat inactivated foetal calf serum (FCS)
Treatment medium:	Culture medium with reduced serum content (2 % FCS)
Selection medium:	Culture medium with 10 µg/mL 6-thioguanine (6-TG)
6. Locus examined:	HPRT
7. Test concentrations:	
Pre-test for cytotoxicity:	0, 12.5, 25.0, 50, 100, 200, 300, 400, 500, 600 µg/ml (±S9)
Gene mutation assays:	0, 12.5, 25, 50, 100, 150, 200, 250 µg/ml (-S9) 0, 50, 100, 200, 300, 400, 500 µg/ml (+S9)

B. TEST PERFORMANCE

1. Dates of experimental work: June 14, 1993 – September 14, 1993

2. Test substance preparation

Stability of JAU 6476-desthio in the vehicle in a range from 0.08 mg/ml to 200 mg/ml was analytically approved for at least twenty-four hours (Table 5.8.1/16- 1). Test substance solutions were prepared in vehicle immediately prior to cell treatment.

Table 5.8.1/16- 1: Analysis for stability of JAU 6476-desthio in the vehicle at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 hrs	24 hrs
0.08	97.3	96.9
200	102	105

3. Pre-test for cytotoxicity

Initially 9 concentrations of JAU 6476-desthio in the range 12.5 - 600 µg/ml, plus vehicle control, with and without S9 activation were tested.

Exponentially growing V79 cells were plated in culture medium in a 250 ml flask ($4x10^6$ cells per flask, one culture per concentration). After attachment (16-24 hours later), cells were exposed to vehicle alone and to 9 concentrations of the test substance ranging from 12.5 µg/ml to 600 µg/ml for 5 hours in treatment medium, both in the presence and absence of S9 metabolic activation.

Thereafter, cell monolayers were washed with phosphate buffered saline (PBS), trypsinised and replated in culture medium at a density of 200 cells into each of 3 Petri dishes (60 mm). These dishes were incubated for 7 days to allow colony development. Thereafter, colonies were fixed with 95 % methanol, stained with Giemsa and counted either by eye, excluding colonies with 50 cells or less, or with an automatic counter. Cytotoxicity was expressed by comparison of colonies in treated cultures versus vehicle control cultures (relative cloning efficiency).

Following the determination of the cytotoxicity of JAU 6476-desthio, the concentration range of JAU 6476-desthio for the mutagenicity study was chosen ranging from approximately 0 % - 90 % reduction in colony forming ability.

4. Gene mutation assays

Four and three gene mutation assays, with and without metabolic activation were performed and evaluated as follows:

Cell treatment and expression period

Exponentially growing V79 cells were plated in culture medium in two 250 ml flasks per dose group ($4x10^6$ cells per flask). This was defined as day 0 of the assay. After attachment (16-24 hours later), the cells were exposed to each test substance concentration for 5 hours in treatment medium. For metabolic activation 5 % S9 mix were added for the treatment period. The corresponding controls were incubated under the same conditions.

An experiment without metabolic activation consisted of one negative control, one vehicle control, one positive control (EMS) and 7 test substance doses. An experiment with metabolic activation consisted of one negative control, one vehicle control, one positive control (DMBA) and 6 test substance doses.

Thereafter, cell monolayers were washed with PBS, trypsinised and replated in culture medium at a general density of $1.5x10^6$ cells in 250 ml flasks and at 200 cells into each of 3 Petri dishes (60 mm). The Petri dishes were incubated (as a rule for 7 days) to allow colony development and to determine the cytotoxicity associated with each test substance directly after treatment ("Survival to Treatment").

The large flasks were incubated to permit growth and expression of induced mutations. Cells were subcultured on days 3 and 6. At the first subculture the 2 cultures for each dose level and the controls were reseeded at generally $1.5x10^6$ cells into two 250 ml flasks each.

Seeding for selection and cloning efficiency

At the end of the expression period, the cultures from each dose level were reseeded at $3x10^5$ cells per dish (100 mm, a total of 8 dishes) in selection medium for selection of mutant cells. In addition, three dishes (60 mm) were seeded in culture medium at 200 cells/dish to determine the absolute cloning efficiency for each dose level. All dishes were incubated at 37 °C in a humidified atmosphere with about 5 % CO_2 for 7 days.

Colony counting and determination of colony size distribution

After incubation for 7 days, the colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the cloning efficiency dishes. Colonies with 50 cells or less were excluded.

Calculations and processing of the data

The data listed in the tables of results are calculated as follows:

Relative survival (%)	$\frac{\text{Average no. of colonies per treated culture}}{\text{Average no. of colonies per vehicle control dish}} * 100$
Absolute population growth	cell number day 4 $*$ cell number day 7 for each culture
Relative population growth (%)	$\frac{\text{Absol. Pop. Growth of treated culture}}{\text{Absol. Pop. Growth of corresponding vehicle control culture}} * 100$
Absolute cloning efficiency (CE) (%)	$\frac{\text{Average no. of viable colonies per dish}}{200} * 100$ The absolute CE is expressed by the average number of viable colonies per dish (200 cells/dish seeded).
Mutant Frequency	$\frac{\text{Total Number of Mutant Colonies} * 100}{\text{Number of Evaluated Dishes} * 3 * 10^5 * \text{CE}}$ The mutant frequency is expressed as 6-TG resistant mutants per 10^6 cloneable cells.

5. Statistics

The statistical analysis relies on the mutation frequencies rather than on individual plate counts which are submitted to a weighted analysis of variance as well as to a weighted recursive regression, both with Poisson derived weights ([redacted]., 1981; [redacted]., 1989). Mutation frequencies based on less than 5 plate counts are very uncertain estimates of the true mutation frequencies and provide no basis for a rational estimation of the variance. Therefore, such values are not included in the statistical analysis. If the relative population growth in an experiment falls below 10 %, the corresponding mutation frequency is discarded.

The two mutation frequency values obtained per group in the standard HPRT-assay are, although somewhat related, considered as independent measurements thus increasing the power of the statistical tests applied. Since the protocol of the HPRT assay requires at least two replicates, the overall analysis per type of metabolic activation is the most important one for classifying substances into mutagens and non-mutagens. However, separate analyses will be run for each assay in order to examine the consistency of the results.

All groups are included in the weighted analysis of variance followed by pairwise comparisons to the vehicle control on a nominal significance level of $\alpha = 0.05$ using the Dunnett test. The regression analysis part is performed on the basis of the actual dose levels thereby omitting the positive, negative and vehicle controls. If there is a significant increase of the mutation frequency with dose ($\alpha = 0.05$) in the main analysis the highest dose group will be dropped and the analysis will be repeated until $p > 0.05$. Dose levels eliminated in that way are flagged correspondingly.

6. Acceptability criteria

Normally, an assay is only considered acceptable for evaluation if the following criteria are met:

- The assay (with and without metabolic activation) is repeated at least once independently.

Cloning efficiency:

- The average cloning efficiency of the negative and vehicle controls should be at least 50 %. Assays below 50 % cloning efficiency will be unacceptable.

Cytotoxicity

- Cytotoxicity is determined after treatment with the test substance by the assay parameter "Survival to Treatment". The highest test substance concentration should produce a low level of relative survival (0 – 30 %) or should be the first concentration where insolubility occurs. The survival at the lowest concentration should approximate the negative control.

Mutant frequency

- The background mutant frequency (average value for vehicle controls) in a trial should not exceed 25×10^6 cells. Assays with higher spontaneous mutant frequencies are not necessarily invalid, however, if all other criteria are fulfilled.
- An experimental mutant frequency is considered acceptable only if the absolute cloning efficiency is 10 % or greater.
- Mutant frequencies for at least four concentrations of the test substance are routinely determined in each assay.
- Mutant frequencies are normally derived from sets of 8 dishes per parallel-culture of each dose level. To allow for contamination losses, an acceptable mutant frequency can be calculated from a minimum of 5 dishes.
- The positive control must induce an average mutant frequency of at least three times that of the vehicle control.

7. Evaluation criteria

An assay will be considered:

positive - if a dose-dependent, significant and in parallel cultures reproducible increase in mutant frequency is observed.
(It is desirable to obtain this dose-relationship for at least 3 doses. To be significant, the mutagenic response to the substance should be at least approximately two to three times that of the highest negative or vehicle control value observed in that trial.)
- if the result can be reproduced in a second assay
- if a reproducible increase greater than two times the minimum criterion is observed for a single dose near the highest testable concentration, the test substance is also considered mutagenic.
- if no significant change in osmolality compared to the vehicle control can be observed (otherwise, unphysiological culture conditions may be the reason for the positive result).

equivocal - if there is no dose-dependency but one or more doses induce a reproducible, significant mutant frequency in all assays.

negative - if none of the doses tested (for a range of applied concentrations which extends to sufficient toxicity) induces a reproducible mutant frequency which is considered significant.

II. RESULTS AND DISCUSSION

A. PRE-TEST FOR CYTOTOXICITY

Since precipitation of JAU 6476-desthio occurred on addition of medium, the preliminary cytotoxicity test was performed at concentrations up to 600 µg/ml. Based on marked cytotoxicity at 300 µg/ml without metabolic activation, 250 µg/ml was selected as the highest exposure concentration for the main mutagenicity assays. Based on the occurrence of precipitation at 500 µg/ml, 500 µg/ml was selected as the highest concentration for the main assays with metabolic activation.

Table 5.8.1/16- 2: Results of the pre-test for cytotoxicity

Dose (µg/ml)	Survival (% control)	Cloning efficiency (%)	Survival (% control)	Cloning efficiency (%)
± S9	**- S9**		**+ S9**	
0$	100.0	82.7	100.0	96.8
12.5	88.3		84.5	
25.0	110.1		109.6	
50.0	79.8		110.0	
100.0	77.0		89.0	
200.0	66.3		78.0	
300.0	-		30.3	
400.0	-		32.5	
500.0	- P		38.9 P	
600.0	-		- P	

$ solvent control; - no cell survival; P precipitation of the test substance

B. GENE MUTATION ASSAYS

The absolute cloning efficiencies for the vehicle controls in the mutagenicity assays varied from 61.0 - 83.8 % without activation and 61.8 - 130.2 % with activation, demonstrating good cloning conditions. Four and three valid assays, with and without metabolic activation, respectively, were evaluated. A dose-related decrease in relative survival and relative population growth occurred at higher concentrations, without metabolic activation (Table 5.8.1/16- 3). Immediately after exposure, almost all cells were lost due to cytotoxicity in one assay at 200 µg/ml and in all 3 assays at 250 µg/ml without activation. No biologically relevant and reproducible increase in the mutation frequency over the vehicle control frequency was evident in any of the three assays without activation, although a statistically significant increase in mutation frequency occurred at 100 µg/ml when all assays were jointly analyzed (Table 5.8.1/16- 5). Since there was no relationship to dose, it was considered biologically not relevant. The positive control, EMS, induced a clear, statistically significant mutagenic effect in all assays.

JAU 6476-desthio also elicited a dose-related decrease in the relative survival and relative population growth with metabolic activation, and precipitation was evident in two assays at 500 µg/ml (Table 5.8.1/16- 4). There were no biologically relevant increases in mutation frequency in any of the four assays, and there were no statistically significant increases in mutation frequency at any dose level in the joint statistical assessment of the four assays. The positive control, DMBA, induced a clear mutagenic effect in all assays, which was statistically significant in the joint analysis.

Table 5.8.1/16- 3: Results of the three gene mutation assays without metabolic activation

Dose (µg/ml)	Survival (% control)			Relative growth (% control)			Cloning efficiency (%)			Mutant frequency (x 10^{-6})		
	1	2	3	1	2	3	1	2	3	1	2	3
0	107.9	103.6	84.6	101.4	54.9	0.0	75.7	107.2	71.0	14.3	5.4	5.9
				93.2	74.4	112.4	73.3	94.3	61.0	8.0	0.9	10.2
0$	100.0	100.0	100.0	100.0	100.0	100.0	82.3	83.8	79.8	8.1	3.4	4.2
				100.0	100.0	100.0	78.2	77.0	61.0	12.3	3.2	5.5
12.5	99.6	88.9	101.5	111.9	93.3	133.1	71.8	79.2	70.8	27.9	9.5	3.5
				87.7	119.1	138.2	75.5	90.5	63.5	9.9	3.2	2.6
25	72.1	84.9	88.4	61.0	95.6	161.0	83.8	80.7	78.7	11.4	7.2	0.5
				125.5	92.6	145.6	62.8	59.0	70.3	16.6	6.4	9.5
50	90.6	114.0	50.1	119.0	88.4	100.4	78.3	74.7	94.8	4.3	6.1	9.2
				73.3	105.6	127.3	90.7	83.8	80.8	14.2	4.0	8.3
100	80.2	66.9	52.7	87.4	112.6	116.6	69.2	76.2	70.5	24.1	7.7	10.6
				70.8	76.8	74.2	76.7	93.7	71.8	14.7	6.7	3.5
150	95.9	38.3	44.8	69.7	36.3	91.8	61.0	79.5	73.5	9.6	6.3	6.8
				83.5	40.5	98.1	62.5	89.0	82.0	7.3	4.2	1.5
200	23.8	-	7.6	3.4	-	5.0	56.3	[n]	67.3	28.9	-	0.6
				4.9	-	7.5	61.5	[n]	61.2	10.2	-	2.7
250	1.4	-	-	-	-	-	[n]	[n]	[n]	-	-	-
				-			[n]	[n]	[n]	-	-	-
EMS	47.9	63.6	31.3	23.9	25.9	35.0	59.7	57.7	55.0	693.7	959.7	1086.4
				18.3	31.9	28.8	66.5	55.0	58.5	1017.5	881.8	1012.1

$ solvent control; [a] no calculation due to low cell number; [n] not cloned due to cytotoxicity

Table 5.8.1/16- 4: Results of the four gene mutation assays with metabolic activation

Dose	Survival (% control)				Relative growth (% control)				Cloning efficiency (%)				Mutant frequency (x 10^{-6})			
(µg/ml)	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	93.4	94.9	86.1	108.3	81.6	108.2	112.3	108.3	80.0	89.7	82.8	79.7	6.8	0.5	9.6	3.1
					65.8	97.8	106.0	100.7	84.2	102.3	64.2	71.7	2.0	4.1	5.8	4.1
0$	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	65.3	130.2	74.2	63.3	4.4	4.8	9.0	5.9
					100.0	100.0	100.0	100.0	71.3	80.2	61.8	90.2	3.5	3.1	6.1	3.7
50	95.7	78.0	56.8	89.6	70.2	109.0	88.5	98.0	77.0	77.2	76.0	59.5	4.3	5.9	5.5	2.1
					81.0	90.4	103.0	129.8	75.2	102.8	68.3	66.3	2.8	0.8	7.8	6.9
100	89.3	74.1	60.3	81.6	109.2	96.3	84.4	100.6	81.2	74.3	72.8	61.0	1.0	0.0	13.7	2.0
					61.7	109.2	95.3	108.7	76.3	93.5	65.2	71.2	4.9	1.8	12.1	2.9
200	47.0	69.5	70.3	67.4	37.1	70.3	68.2	82.8	75.2	69.2	74.5	59.5	12.8	4.2	11.7	5.6
					72.0	94.1	89.5	86.3	59.3	91.2	68.9	81.0	2.1	0.5	9.8	7.2
300	75.3	62.8	39.7	80.6	74.2	67.8	55.6	65.6	71.3	78.3	75.5	85.7	1.2	4.8	17.7	2.4
					30.1	59.8	48.9	59.5	82.0	79.0	57.3	76.3	0.0	2.1	5.1	3.8
400	51.5	43.8	41.9	91.0	33.5	65.8	56.7	60.2	81.0	60.8	80.0	69.2	1.0	6.9	12.0	0.6
					25.0	53.2	50.6	64.5	78.5	83.0	63.8	61.7	0.5	1.5	5.2	2.0
500	19.4 [P]	28.5 [P]	38.1 [P]	61.0 [P]	7.0	31.6	52.7	45.2	75.8	100.8	57.0	76.2	2.7	4.1	20.5	6.6
					23.6	25.7	41.5	45.4	76.5	75.7	64.5	64.2	1.6	8.3	2.6	5.8
DMBA	73.2	59.2	55.8	102.1	54.7	71.4	56.5	58.8	81.0	76.0	74.0	79.5	20.6	72.4	75.5	68.7
					59.5	66.1	77.8	60.9	68.2	82.8	71.3	90.3	26.3	93.6	55.5	50.8

$ solvent control; [a] no calculation due to low cell number; [n] not cloned due to cytotoxicity; [P] precipitation of the test substance

Table 5.8.1/16- 5: Weighted ANOVA and regression results

± S9	P values weighted		PC	Concentration of JAU 6476-desthio (µg/ml)								
	ANOVA	Regression		12.5	25	50	100	150	200	250	300	400
-	<0.001	0.541	*	-	-	-	*	-	m	m	nt	nt
+	<0.001	0.713	*	nt	nt	-	-	nt	-	m	nt	-

PC: positive control
* significant (α=0.05) increase relative to vehicle control using the Dunnett test
\- not significant
m: missing value due to cytotoxicity of the test substance
nt: not tested

III. CONCLUSION

JAU 6476-desthio was not mutagenic in the V79-HPRT forward mutation assay, based on the absence of dose-related and reproducible increases in mutation frequencies at assessable concentrations of up to 500 µg/ml.

Report: KCA 5.8.1/17 [redacted]; 2092; M-031126-01-1
Title: SXX 0665 - Mutagenicity test on unscheduled DNA synthesis in rat liver primary cell cultures in vitro
Report No.: 21187
Document No.: M-031126-01-1
Guideline(s): Directive 87/302/EEC; OECD 482 (1986); US-EPA PB 84-233295 (1984)
Guideline deviation(s): none
GLP/GEP: **yes**

Deviations: Deviations from the current OECD guideline (1986):
According to the guideline the result should be confirmed by an independent experiment. In the current study a sufficient number of analysable concentrations was tested from which no statistically significant results were obtained and no positive dose-response relationship in net nuclear grain count and/or proportion of cells in repair was observed. Therefore, despite that no independent repeat assay was conducted, the negative outcome of this study is considered to be reliable.

Executive summary:

JAU 6476-desthio (batch no. 17005/89, purity 93.7 %) was tested for its potential to induce Unscheduled DNA Synthesis (UDS) *in vitro* in primary rat hepatocyte cultures, at concentrations of 0 (vehicle), 5.0, 10.0, 20.0, 40.0, 50.0 and 60.0 µg/ml. The positive control was 0.5 µg/ml 2-acetylaminofluorene (2-AAF). 10µCi/ml ^{3}H-thymidine was added to the test cultures at the same time as the test materials to measure the UDS. The dose levels for this study were based on the results of a preliminary cytotoxicity test.

Precipitation occured in the medium at concentrations of ≥500 µg/ml. Excessive cytotoxicity, manifested at 125 µg/ml (preliminary cytotoxicity study) when relative cell viability dropped to 11.7 %, and moderate cytotoxicity at 62.5 µg/ml (relative viability 51.7 %) occurred. Thus, a concentration range of 5.0 - 60.0 µg/ml was selected for the UDS assay. In the main assay the test material was non-cytotoxic up to the highest concentration tested. However, hepatocytes incubated with 60 µg/ml JAU 6476-desthio showed a high amount of destroyed or altered cells and a reduced cell number on the

coverslips indicating a cytotoxic effect of the highest dose tested. There was no increase in net nuclear grains at any of the concentrations tested and the proportion of cells in repair was comparable to, or less than, the vehicle control group at all concentrations tested. The positive control produced satisfactory results.

JAU 6476-desthio did not induce unscheduled DNA synthesis in the *in vitro* UDS assay at concentrations up to those producing evidence of cytotoxicity, based on the absence of a statistically significant and dose-related increase in nuclear labelling.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	JAU 6476-desthio
Synonym:	SXX 0665
Description:	White powder
Batch No.:	17005/89
Purity:	93.7 %
Stability of the test compound:	The batch used was analysed prior to study initiation and approved for use during the test period.
Solvent used:	Dimethylsulfoxide (DMSO)
Solvent/final concentration:	1 % (v/v) DMSO
2. Control Materials	
Solvent control:	1 % (v/v) DMSO
Positive control:	2-Acetylaminofluorene (2-AAF); final concentration: 0.5 µg/ml (solvent: DMSO)
3. Test organism:	Primary rat hepatocytes from young adult male Sprague-Dawley rats
Preparation of cells:	A single animal was used for each trial. The cells were obtained by perfusing the rat liver in situ with a collagenase solution. Monolayer cultures were established on plastic coverslips in culture dishes and used on the same day to initiate the UDS assay.
Culture medium:	Williams Medium E supplemented with L-glutamine (2 mM), gentamycin sulfate (50 µg/ml) and 10 % heat-inactivated foetal calf serum (FCS).
Treatment medium:	Culture medium with a reduced serum content of 1 % FCS.
Culture conditions:	37°C in a humidified atmosphere containing approx. 5 % CO_2
4. Test concentrations:	
Pre-test for cytotoxicity:	0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, 1000, 1500, 2000 µg/ml
UDS assay:	0, 5.0, 10.0, 20.0, 40.0, 50.0, 60.0 µg/ml

B. TEST PERFORMANCE

1. Dates of experimental work: April 03, 1990 – June 05, 1990

2. Test substance preparation and solubility in culture medium

JAU 6476-desthio was dissolved in DMSO. A clear solution was obtained up to a concentration of 200 mg/ml but precipitation occurred in the medium at concentrations of ≥ 500 µg/ml. Solutions were prepared immediately prior to cell treatment.

The stability of JAU 6476-desthio in DMSO at room temperature at concentrations ranging from 0.08 mg/ml to 200 mg/ml for at least twenty-four hours was analytically verified (Table 5.8.1/17-1).

Table 5.8.1/17- 1: Analysis for stability of JAU 6476-desthio in DMSO at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 hrs	24 hrs
0.08	97.3	96.9
200	102	105

3. Dose selection

A preliminary cytotoxicity test was performed to evaluate the dose range of the test substance in the UDS assay. Treatments were initiated by replacing the culture medium on the hepatocyte cultures with treatment medium containing the test material in the desired concentrations. The experimental design of the cytotoxicity test was identical to the procedure described below for the UDS assay.

A series of 10 concentrations of JAU 6476-desthio ranging from 7.8 µg/ml to 2000 µg/ml was applied to the cells (two 60 mm Petri-dishes per dose group, 750000 living cells per dish with a viability of 77.1 % after isolation). After 18-24 hours, a viable cell count (trypan blue exclusion) was obtained. Cytotoxicity was expressed by comparison of viable cells in treated cultures versus vehicle control cultures (relative survival to treatment).

After determining the cytotoxicity of JAU 6476-desthio, the concentration range was chosen for the genotoxicity study, starting with the highest dose that resulted in a sufficient number of survivors with intact morphologies, and proceeding to successively lower doses.

4. Experimental design

Liver perfusion and preparation of rat hepatocytes

A single animal was used for each assay. The cells were obtained by perfusing the rat liver in situ with a collagenase solution. After perfusion, primary hepatocytes were prepared according to the protocol of Butterworth et al. (1987) under sterile conditions. Cell preparations used for the UDS assay were single-cell suspensions with good viability (determination of cell viability and cell concentration by trypan blue exclusion).

The cells were seeded as follows:

5. For determination of cytotoxicity: two 60 mm-Petri dishes ($7.5x10^5$ viable cells per dish) precoated with collagen were available for each dose group as well as for the positive and negative controls. Two additional dishes were seeded to determine cell viability, attachment rate and morphology about 1.5 hours after establishment of the cultures.
6. For determination of genotoxicity: a 25 mm round plastic coverslip precoated with collagen was placed into each well of 6-well culture dishes. Approx. $3.75x10^5$ viable cells were seeded per well (in 2.5 ml culture medium), whereby 3 wells per dose group including the control groups were established.

For cell-attachment all cultures were incubated for 90-150 min. in a 37°C incubator in a humidified atmosphere containing approximately 5 % CO_2.

Culture labelling and treatment

After the attachment period, the cultures were washed with Hanks solution to remove unattached cells. Cell number and viability of the cultures were determined by the method of trypan blue exclusion employing the two additional 60 mm-Petri dishes.

Test substance solutions were prepared in the vehicle and applied to the cells in two 60 mm dishes per dose group for determination of cytotoxicity. The medium in the 6-well dishes (cultures for determination of genotoxicity) was replaced by treatment medium containing the dissolved test chemical in the indicated concentrations and 10 µCi/ml ^{3}H-thymidine (18-20 Ci/mmole). The cultures were then placed in the incubator for 18-24 hours.

Thereafter, the culture medium was preplaced with a 1 % sodium citrate solution to swell the nuclei. The cells on the coverslips were then fixed, washed with deionized distilled water and air dried.

Determination of cytotoxicity

At 20-24 hours after initiation of treatment, viable cell counts (trypan blue exclusion) were determined in the two 60 mm-Petri dishes available for each treatment condition. Cell survival for the dose groups was estimated in relation to the negative controls.

Autoradiography and Staining

Air-dried coverslips were mounted cell-side-up on microscope slides and coated in the dark with NTB-2 photographic emulsion. The coated slides were stored in light-tight boxes in the presence of a drying agent for 4-10 days at +4°C. The photographic emulsion was then developed at temperatures below 15°C. The slides were rinsed afterwards with distilled water, fixed and air dried. Slides were then stained with haematoxylin and eosin.

Grain counting

Each slide was examined by counting 50 cells per slide, normally 3 slides per dose group (total of 150 nuclei for each treatment condition). Only cells viable at the time of fixation were scored; isolated nuclei and cells with abnormal morphology were excluded. A starting point was randomly selected on each slide, and cells were scored in a regular fashion by bringing new cells into the field of view, moving along the X-axis. If the total number of 50 cells had not been reached before coming to the edge of the slide, the stage was moved on the Y-axis, and counting resumed in the opposite X-direction, parallel to the first line.

UDS was measured by counting nuclear grains and subtracting the average number of grains in 3 nuclear-sized areas adjacent to each nucleus. This value was referred to as the net nuclear grain (NNG) count. The mean net nuclear grain count was routinely determined from triplicate coverslips. The number of cells in repair (nuclei with 5 or more net grains) was also determined.

5. Data presentation and assay evaluation

Net grains per nucleus:	Average no. of net nuclear grain counts on triplicate coverslips (total of 150 cells)
Mean cytoplasmic grain count:	Average no. of cytoplasmic grain counts (3 areas per cell) on triplicate coverslips
% Nuclei with 5 or more grains:	(No. of cells with 5 or more net nuclear grain counts per dose/ no. of evaluated cells per dose) x 100
Survival (%):	No. of viable cells relative to vehicle control.

1. For each of the 50 cells on each slide, the number of nuclear grains (NG) was scored, as well as numbers of three cytoplasmic grain counts from nuclear-sized areas adjacent to each nucleus.

2. A number of 5 net nuclear grains or more was chosen as a conservative estimate as to whether a particular cell is responding (cell "in repair").

3. A minimum of 4 - 5 dose levels were analysed for NNG. Repeat trials need only augment the number of analysed dose levels in the first trial to achieve a total of five different concentrations.

4. Only cells viable at the time of fixation and with nuclei evenly coated with emulsion were scored. Cells with abnormal morphology, such as those with pyknotic or lysed nuclei, were not counted. Isolated nuclei not surrounded by cytoplasm were not counted.

5. S-phase cells having dense NG were excluded; however, the number of S-phase cells was noted.

6. Statistics

The mean cytoplasmic count from each evaluated cell was subtracted from the nuclear count to derive the NNG. For each slide, the mean and standard deviation for NNG was calculated, as was the percentage of cells in repair.

An evaluation was made of the percentage of cells in repair per dose group compared to the negative control using a one-sided 2x2-chi^2-test corrected for continuity. To assess the statistical significance of a result, the square root of the test statistic was compared to the upper 95 % quantile (P = 0.05) of the normal standard distribution.

7. Acceptance and assessment criteria

An assay is normally considered acceptable for evaluation only if the following criteria are satisfied. However, these criteria may be overruled by good scientific judgment.

Viability

- The viability of the hepatocytes collected by this process normally exceeds 70 %, although values between 50 % and 70 % viability can also be acceptable. Trials below 50 % would have been considered unacceptable, to avoid the possible use of a damaged cell population.
- The viability of the monolayer cell cultures used for the UDS assay had to be 80 % or greater. Normally, the viability of attached cells is about 90 %.
- The number of viable cells in the negative (vehicle) control cultures was supposed to remain reasonably stable over the experimental time period because rapidly declining (dying) cultures may not respond in a representative manner to the test substance treatments. Therefore, the number of viable cells in the negative control cultures had to be 60 % or greater after 16-24 hours.

Nuclear grain counts

- Grain count data obtained for a given treatment were acceptable as part of the evaluation if obtained from at least two replicate cultures and at least fifty cells per culture.
- The highest analysed dose had to approach an excessive cytotoxicity of about 50 % or result in test material insolubility, or reach the highest applicable dose of 5 mg/ml.
- The average NNG in the negative control cultures was supposed to range between -8 to +1. No more than 10 % of the cells should have been be in repair.
- The positive control 2-AAF was used to demonstrate the responsiveness of the cell population employed and the adequacy of the method for the detection of UDS. For the positive control 2-AAF (0.5 µg/ml), one might expect mean values of 10-25 NG with 70-100 % of the cells with greater than or equal to 5 NNG.
- An experiment was considered invalid if cytoplasmic background counts of control cultures exceeded 30 grains per nuclear-sized area.

For the conditions described a response was considered positive if a chemical yielded +5 NNG or more (population average) and more than 20 % of the cells responding.

A population average of between +1 NNG and +5 NNG was considered a marginal response. A positive dose-response relationship in both NNG and the percentage of cells in repair was required as additional information to confirm a positive response for counts below +5 NNG. In this case, an additional experiment might have been necessary.

II. RESULTS AND DISCUSSION

A. PRELIMINARY CYTOTOXICITY ASSAY

The test material was excessively toxic at a concentration of 125 µg/ml (11.7 % relative survival; Table 5.8.1/17- 2). Moderate cytotoxicity was observed at 62.5 µg/ml (51.7 % rel. survival). Dose levels at and below 31.3 µg/ml were non-toxic. Therefore, 6 dose groups (5 µg/ml to 60 µg/ml) covering a good range of toxicity (51.5 % to 90.9 % relative survival) were selected for the UDS assay.

Table 5.8.1/17- 2: Cytotoxicity assay for dose selection

Dose (µg/ml)	Dish No.1 (Cells x10^6)	Dish No.2 (Cells x10^6)	Average number of cells (x10^6)	Viable cells (%)	Relative survival (%)[a]
0$	0.85	0.90	0.88	77.5	100.0
7.8	1.31	0.92	1.12	67.6	87.2
15.6	0.78	1.95	1.37	69.9	90.1
31.3	1.41	0.91	1.16	70.5	90.9
62.5	1.45	1.18	1.32	40.1	51.7
125.0	0.41	0.78	0.60	9.1	11.7
250.0	0.38	0.42	0.40	0	0
500.0	I	I			
1000.0	I	I			
1500.0	I	I			
2000.0	I	I			

[a] relative to solvent control; $ solvent control;
I insoluble material present; if it takes place in both dishes no more values are available for that concentration

B. UNSCHEDULED DNA SYNTHESIS ASSAY

Cytotoxicity

Viability after isolation and attachment was 75.7 % and 83.2 % respectively, which is acceptable.

The test material was non-cytotoxic up to the highest concentration tested (60 µg/ml: 86.6 % relative survival) after trypan blue exclusion. However, hepatocytes incubated with 60 µg/ml JAU 6476-desthio showed a high amount of destroyed or altered cells and a reduced cell number on the coverslips resulting in only one coverslip being evaluable. This is indicating a cytotoxic effect of the highest dose tested.

Therefore, 5 dose groups were available for analysis of nuclear labelling. The positive control (2-AAF; 0.5 µg/ml) was moderately toxic in this assay.

UDS assay

JAU 6476-desthio did not produce an increase in NNG at any of the concentrations tested (Table 5.8.1/17- 3 and Table 5.8.1/17- 4) and the proportion of cells in repair was comparable to, or less than,

the vehicle control group at all concentrations tested. Although the NNG of + 0.93 for the 60 µg/ml group, derived from one coverslip only, was slightly higher than the vehicle control group (NNG -1.02), it was not statistically significant. Furthermore this value was still in the proposed range of -8 to +1 NNG which is considered appropriate for negative controls. The positive control produced satisfactory results.

Table 5.8.1/17- 3: Rat liver UDS assay – mean values per slide

Concentration (µg/mL)	Net grains per nucleus ± SD	Mean grains per nucleus ± SD	Mean cytoplasmic grain count ± SD	Mean cells in repair (%)
0 (vehicle)	- 3.07 ± 2.13	3.9 ± 2.51	6.84 ± 2.40	0
	- 2.06 ± 2.39	4.1 ± 2.77	6.12 ± 2.61	0
	- 0.30 ± 2.61	4.8 ± 2.82	5.04 ± 2.20	2.0
5.00 [a]	- 0.41 ± 2.56	5.9 ± 3.33	6.36 ± 2.78	2.0
	x	x	x	x
	- 1.63 ± 2.49	5.9 ± 3.22	7.51 ± 2.22	2.0
10.0	- 0.13 ± 2.78	6.0 ± 3.76	6.13 ± 2.82	2.0
	- 1.65 ± 2.57	6.1 ± 3.46	7.77 ± 2.29	0
	- 1.62 ± 3.01	8.4 ± 4.30	10.06 ± 3.02	0
20.0 [a]	x	x	x	x
	2.44 ± 2.41	3.8 ± 2.73	6.20 ± 2.55	0
	- 1.81 ± 2.46	5.1 ± 2.88	6.87 ± 1.96	0
40.0 [a]	- 0.61 ± 2.93	5.4 ± 3.06	6.03 ± 2.35	4.0
	x	x	x	x
	- 0.63 ± 2.35	4.7 ± 3.01	5.25 ± 2.66	2.0
50.0	- 0.27 ± 1.84	4.0 ± 1.99	4.35 ± 1.46	0
	- 0.41 ± 2.64	5.6 ± 4.14	6.03 ± 3.06	4.0
	- 0.72 ± 2.48	4.9 ± 3.23	5.58 ± 2.15	2.0
60.0 [b]	x	x	x	x
	0.93 ± -	4.7 ± 2.58	3.74 ± -	2.0
	x	x	x	x
2-AAF [a]	7.86 ± 4.70	14.1 ± 2.83	6.24 ± 2.76	72.0
0.5 µg/mL	x	x	x	x
	7.25 ± 4.29	13.5 ± 4.82	6.26 ± 1.81	72.0

SD standard deviation; [a] one slide not evaluable; [b] two slides not evaluable; x slide not evaluable; - standard deviation could not be calculated

Mean values per slide can be found in a letter submitted in 2002 to the Pesticides Safety Directorate, attached as a supplemental document to the study report.

Table 5.8.1/17- 4: Rat liver UDS assay – group mean data

Concentration (µg/mL)	Net grains per nucleus ± SD	Mean grains per nucleus ± SD#	Mean cytoplasmic grain count ± SD	Mean cells in repair (%)	Relative Survival (%)
0 (vehicle)	- 1.81 ± 1.40	4.27 ± 0.47	6.00 ± 0.91	0.7	100
5.00 [a]	- 1.02 ± 0.86	5.90 ± 0	6.93 ± 0.82	2.0	108.8
10.0	- 1.13 ± 0.87	6.83 ± 1.36	7.98 ± 1.97	0.7	88.4
20.0 [a]	- 2.13 ± 0.45	4.45 ± 0.92	6.54 ± 0.47	0	91.4
40.0 [a]	- 0.62 ± 0.01	5.05 ± 0.5	5.64 ± 0.55	2.0	84.6
50.0	- 0.47 ± 0.23	4.83 ± 0.80	5.32 ± 0.87	2.0	77.5
60.0 [b]	0.93 ± -	4.66 ± -	3.74 ± -	2.0	86.6
2-AAF [a] 0.5 µg/mL	7.56 ± 0.43	13.80 ± 0.42	6.25 ± 0.01	72.0*	72.4

SD standard deviation; [a] one slide not evaluable; [b] two slides not evaluable; * $p < 0.05$; - standard deviation could not be calculated

Values of mean grains per nucleus can be found in a letter submitted in 2002 to the Pesticides Safety Directorate, attached as a supplemental document to the study report.

III. CONCLUSION

JAU 6476-desthio did not induce unscheduled DNA synthesis in the in vitro UDS assay at concentrations up to those producing evidence of cytotoxicity, based on the absence of a statistically significant and dose-related increase in nuclear labelling.

Report: KCA 5.8.1/18 [redacted]; 1995; M-031119-01-1
Title: SXX 0665 - In vitro mammalian chromosome aberration test with chinese hamster ovary (CHO) cells
Report No.: 24457
Document No.: M-031119-01-1
Guideline(s): OECD 473 (1983), EEC Directive 92/69/EEC B.10 (1992); US-EPA 'In vitro mammalian cytogenetics' (1986)
Guideline deviation(s): none
GLP/GEP: yes

Deviations: The following deviations from the current OECD guideline (2016) were noted:
Cytotoxicity: In this assay the Mitotic Index (MI) was used to detect cytotoxicity. According to the current guideline Relative Population Doubling (RPD) or Relative Increase in Cell Count (RICC) are recommended as appropriate methods for the assessment of cytotoxicity in cell lines in cytogenetic tests while the MI should be used to determine cytotoxicity in primary cultures of lymphocytes. However, the determination of the MI was required by the contemporary guideline of 1983 and the MI is a valid method to assess cytotoxicity.
Treatment: According to the current guideline in addition to the 3-6 h exposure period with and without metabolic activation, continuous exposure without metabolic activation is required. In the 1983 guideline the duration of exposure was not specified. Therefore the assay was conducted with a 4 h exposure period only (with and without metabolic activation). According to the current guideline cell sampling should occur at a time equivalent to about 1.5 normal cell cycle lengths

after the beginning of treatment. The 24-hour harvest interval fulfils this requirement.
Scoring of metaphases: The number of metaphases to be scored was not defined in the 1983 guideline. 200 metaphases were scored (instead of 300 required by the current guideline).
Those deviations are not considered to diminish the results of this mammalian chromosomal aberration assay. The *in vivo* micronucleus assay (see 5.8.1/19) further confirmed that JAU 6476-desthio has no clastogenic or aneugenic effects.

Executive summary:

The potential clastogenicity of JAU 6476-desthio (batch no. 1717008/90, purity 93.1 %) was investigated in Chinese hamster ovary cells (CHO). Duplicate cultures were exposed to the test substance (dissolved in ethanol) in the presence and absence of an exogenous metabolic activation system. Colcemid was used to arrest mitosis two hours prior to the end of the incubation period of 8, 24 and 30 hours. Chromosomes of 100 metaphases per culture (200 metaphases per concentration) were scored for aberrations. Mitotic index was determined from 1000 cells per culture. The stability of the test substance in the solvent was confirmed by analysis. The study was compliant with OECD guideline 473 (1983). Deviations from the current (2016) guideline are noted above.

Based on the results of an initial cytotoxicity test, 125 µg/ml was used in the chromosome aberration assay with a 4 hour exposure period and 8, 24, and 30 hour harvest times. For the 24 hour harvest time, 5 and 25 µg/ml was tested in addition. Without metabolic activation, the relative mitotic indices of cells exposed at the highest concentration of 125 µg/ml were reduced for cells harvested at 24 and 30 hours. A similar, but slightly more marked, effect occurred at 25 µg/ml in cells harvested at 24 hours. Exposure of cells with metabolic activation resulted in reduced relative mitotic indices at 5 and 25 µg/ml in cells harvested at 24 hours, but not at 125 µg/ml. There was no reduction of mitotic indices of cells harvested at 8 hours (with and without metabolic activation).

There were no statistically significant or biologically relevant increases in the incidence of aberrant metaphases at any exposure concentration and harvest time without metabolic activation. Isolated and incidental increases in the number of aberrant metaphases including and/or excluding gaps occurred at 125 µg/ml with metabolic activation at the 8-hour harvest interval and at the lowest concentration, 5 µg/ml, with metabolic activation at the 24-hour harvest interval. However, the percentage of cells with aberrations including and excluding gaps were within the range of the laboratory historical control data for ethanol solvent and are considered not to be biologically relevant. There were no other statistically significant increases at any exposure concentration or harvest interval. The positive controls, mitomycin C and cyclophosphamide produced clear, statistically significant increases in the incidence of aberrant metaphases at the 24-hour harvest interval, indicating the sensitivity of the assay system.

JAU 6476-desthio is considered not to be clastogenic to CHO cells *in vitro*, based on the absence of a statistically significant and biologically relevant increase in the incidence of aberrant metaphases. This result is also confirmed by an *in vivo* micronucleus test (see 5.8.1/19).

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	JAU 6476-desthio
Synonym:	SXX 0665
Description:	Beige powder
Batch No.:	1717008/90
Purity:	93.1 %

Stability of the test compound:	The batch used was analytically examined prior to study initiation and was approved for use for the test period.
Solvent used:	Ethanol
Solvent/final concentration:	1 % (v/v)

2. Control Materials

Negative control:	Culture medium (only for 24 h harvest time)
Solvent control:	1 % (v/v)
Positive control -S9:	Mitomycin C (MMC; solvent: Hanks' balanced salt solution), concentration in the culture medium: 2 µg/ml
Positive control +S9:	Cyclophosphamide (CP; solvent: Hanks' balanced salt solution), concentration in the culture medium: 10 µg/ml

3. Metabolic activation: S9 mix was used to simulate the mammalian metabolism.

Preparation: The S9 fraction was isolated from the livers of Aroclor 1254 induced Wistar rats. It was purchased from CCR, Roßdorf, Germany (protein content: 42.0 mg/ml).
For use, frozen aliquots of the S9 fraction were slowly thawed and mixed with a cofactor solution (4:6). The S9 mix contained 40 % (v/v) S9 fraction and was kept on ice and used on the same day.

Cofactor solution per 100 ml S9 mix:

Sodium phosphate buffer (pH 7.4)	60.0 ml
S9 fraction	40.0 ml
$MgCl_2$ x 6 H_2O	162.6 mg
KCl	246.0 mg
Glucose-6-phosphate (disodium salt)	152.0 mg
NADP (disodium salt)	78.8 mg

4. Test organism: Chinese hamster ovary cells (CHO)

Culture medium: Ham's F12 medium supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 5 % or10 % heat inactivated foetal calf serum (FCS)

Treatment medium: Culture medium with reduced serum content (2 % FCS)

Culture conditions: 37 °C in a CO_2-incubator (5 % CO_2)

5. Test concentrations:

Pre-test for cytotoxicity:

1st	10, 25, 50, 100, 200, 300, 400 µg/ml (±S9, 4 h treatment, 24 h harvest time)
2nd	100, 120, 140, 160, 180, 200 µg/ml (±S9, 4 h treatment, 24 h harvest time)
	1, 10, 50, 100, 150 µg/ml (-S9, continuous treatment, 24 h harvest time)
3rd	10, 20, 30, 40, 50 µg/ml (-S9, continuous treatment, 24 h harvest time)

Chromosome aberration assay:

8 h harvest time:	0, 125 µg/ml (±S9)
24 h harvest time:	0, 5, 25, 125 µg/ml (±S9)
30 h harvest time:	0, 125 µg/ml (±S9)

B. TEST PERFORMANCE

1. Dates of experimental work: December 15, 1992 – June 15, 1993

2. Test substance preparation and solubility in culture medium

For JAU 6476-desthio, ethanol was selected as solvent. The stability of JAU 6476-desthio in the solvent ethanol at room temperature at concentrations ranging from 0.1 mg/ml to 200 mg/ml for at least twenty-four hours was analytically approved (Table 5.8.1/18- 1).

Table 5.8.1/18- 1: Analysis for stability of JAU 6476-desthio in ethanol at room temperature

Nominal value in mg/ml	Content as % of nominal value after storage time	
	0 hrs	24 hrs
0.1	100	101
200	100	99.8

3. Culturing of CHO cells

Chinese hamster ovary (CHO) cells can be kept in culture as established cell lines with a generation time of approximately fourteen hours. The cells were normally grown in 20 ml medium and 75 cm^2 flasks and incubation of the cells was always performed at 37°C in a CO_2-incubator (5% CO_2). The karyotype of the CHO cells (modal chromosomes number: 21) was confirmed. There was no evidence of mycoplasma contamination.

4. Pre-tests for cytotoxicity and determination of cytotoxicity in the main study

Cytotoxic effects were assessed in three pre-tests with 24 h harvest time after 4 h treatment at concentrations of 10-400 µg/ml with and without S9 mix and after continuous treatment at concentrations of 1-150 µg/ml without S9 mix. Both cell survival and mitotic index were determined in the presence and absence of S9 mix. The mitotic index was also determined within the main study as indicator of cytotoxic effects. The selection of the concentrations used for the main study was based on the results of the cytotoxicity pre-tests.

Survival of the cells was assessed by counting of cells in individual flasks after treatment with the test substance. At the end of the treatment period cells of all cultures were trypsinised, and an appropriate dilution was counted using a haemocytometer. The survival index was determined by comparing cell numbers of solvent control and treatment groups. The mitotic index was determined after the preparation of metaphases from treated and untreated (solvent control) cells. The number of mitotic cells among a total of 1000 cells per culture was determined. Duplicate cultures were evaluated in the pre-test and in the main study. All cells which were not in interphase were defined as mitotic.

5. Chromosome aberration assay

All cultures were set up in duplicate. The chromosome aberration assays was performed as follows:

Cell treatment

The Chinese hamster ovary (CHO) cells were passaged on the day prior to treatment. Approximately 1 x 10^6 cells were seeded in 20 ml of medium per 75 cm^2 flasks and incubated. All cultures were set up in duplicate. Immediately before treatment with test substance, the culture medium was removed from the cultures. For the treatment without metabolic activation, 0.2 ml test substance solution and 20 ml treatment medium was added. For treatment with metabolic activation 0.2 ml test substance solution, 1 ml S9-mix and 19 ml treatment medium was added.

The cells were incubated for 4 hours at 37°C. After this treatment period, the medium was removed, the cells were washed with pre-warmed phosphate buffered saline (PBS) (about 37°C), 20 ml of fresh culture medium was added to the flasks and the flasks were placed in a CO_2-incubator for the remaining

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

Positive controls and solvent controls (0.2 ml solvent per culture), and negative controls (no addition of solvent) were set up in parallel and handled as described for JAU 6476-desthio-treated cultures.

Chromosome preparations

The medium was removed from each flask and cells were removed from the bottom of the flask by trypsinisation and suspended in medium. Cells were pelleted by centrifugation, the supernatant was removed and hypotonic solution (0.56 % KCl; 37°C) was added. The cells were resuspended, centrifuged again, and after removal of the supernatant cold fixative was added. The mixture was incubated at room temperature for 20-30 minutes, cells were pelleted by centrifugation as before and the supernatant was discarded. Cells were again resuspended in fixative as before and centrifuged. Pelleted cells were resuspended carefully in a small volume of fresh fixative. This suspension was dropped onto clean slides which had been cooled in demineralized water previously.

The slides were allowed to dry for at least 2 hours. Thereafter, they were stained for about 5 minutes in 5 % Giemsa solution. Slides were rinsed in water twice. The slides were allowed to dry completely and covered. Alternatively, slides were submerged in pure methanol for 3 minutes and stained for 20-30 minutes in 5 % Giemsa solution. Slides were rinsed twice in water and once in acetone and were then kept in xylene for about 30 minutes. The slides were allowed to dry completely and covered. At least two slides were generated per culture. All solutions used during this preparation were freshly prepared each time. The Giemsa solution was filtered before usage.

6. Evaluation criteria

Coded slides were evaluated using a light microscope.

Mitotic index: The mitotic index was determined by counting 1000 cells per culture. The numbers of mitotic and non-mitotic cells were noted. Duplicate cultures were processed and examined.

Chromosome aberrations: Chromosomes of approximately 200 metaphases per concentration, 100 metaphases from each of two parallel cultures, were examined. Only metaphases containing the modal chromosome number (21) were analysed unless exchanges were detected. In this case, metaphases were evaluated even if the chromosome number was not equal to 21. The classes of structural chromosome damage were defined and recorded. Both chromatid and chromosome-type aberrations were assessed. Chromatid-type aberrations are clastogenic effects restricted to one of the two corresponding chromatids. Chromosome-type aberrations are defined as changes expressed in both corresponding sister chromatids at the same locus. The distinction between chromatid and chromosome type aberrations was not made for exchanges.

The different classes of aberrations are characterized as follows:

Gap: A gap is an achromatic lesion within a chromatid arm without obvious dislocation of the chromatid end(s). Gaps are found on one chromatid ("gap") or on both chromatids at apparently identical sites ("isogap"). The biological relevance of gaps of both types is not clear.

Break: A break is defined as a discontinuity of one chromatid ("break") or both chromatids, at apparently the same locus ("isobreak"), with dislocation of the chromatid ends. The dislocated chromatid end(s) has (have) to be present within the respective metaphase.

Fragment:	Fragments are parts of chromosomes without centromer. A fragment is the result of a break. The corresponding defective chromosome is not detectable among the chromosomes of the same metaphase. Fragments can be derived from one chromatid ("fragment") or from both corresponding chromatid regions of a chromosome ("isofragment").
Deletion:	A deletion occurs as the result of a break. In case of a deletion, one chromatid ("deletion") or both corresponding terminal chromatid parts of a chromosome ("isodeletion") are missing within the metaphase under assessment.
Exchange:	This is an exchange of chromatid parts between different chromosomes (interchange) or within the same chromosome (intrachange).
Multiple aberration:	A cell was assessed as to contain "multiple aberrations" when five or more structural changes (excluding gaps) occur within one metaphase.

In addition to these aberrations, metaphases showing chromosome disintegration as an indication of a cytotoxic effect were also recorded if they were observed. They were counted separately and were not included among the cells that were assessed for aberrations. "Chromosome disintegration" was recorded if fewer than half of the chromosomes reveal characteristic structural features within a given metaphase. Additionally observed polyploid metaphases were recorded.

7. Statistics

The statistical analysis was performed by pair-wise comparison of JAU 6476-desthio-treated and positive control groups to the respective solvent control group.

Statistical test	Parameter
Fisher's exact test	- number of metaphases with aberrations (including and excluding gaps) - number of metaphases with exchanges (provided that these data superseded the respective negative control)

A difference was considered to be significant if the probability of error was below 5 %.

8. Assessment and acceptability criteria

A test was considered positive if there was a relevant and statistically significant increase in the aberration rate. An increased incidence of gaps of both types without concomitant increase of other aberration types was not considered as indication of a clastogenic effect. A test was considered negative if there was no such increase at any time interval. A test was considered equivocal if there was an increase which was statistically significant but not considered relevant, or if an increase occurred, which was considered relevant, but which was not statistically significant.

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

II. RESULTS AND DISCUSSION

A. PRE-TEST FOR CYTOTOXICITY

Precipitates were observed in the 1st pre-test in the cultures for the two highest concentrations of the test substance with S9 mix. Cell survival was drastically decreased at ≥ 200 µg/ml.

In the 2nd pre-test after 4 h treatment, a marked decrease in relative cell survival occurred at 140 µg/ml both without (26.0 %) and with (16.9 %) metabolic activation, whereas relative mitotic indices were 84.3 % and 87.8 %, respectively. Minimal cytotoxicity was evident at 120 µg/ml without (relative cell survival 74.8 %) and with (relative cell survival 84.2 %) metabolic activation.

When the cells were continuously exposed (24 h), relative cell survival was decreased at 150 µg/ml in the 2nd pre-test and minimally at all concentrations (10-50 µg/ml) in the 3rd pre-test, whereas the mitotic indices were reduced at ≥10 µg/ml in both pre-tests.

A high concentration of 125 µg/ml for 4 hours exposure and harvests at 8, 24 and 30 hours was selected for the main assay.

Table 5.8.1/18- 2: Cell survival and mitotic index in the pre-tests for cytotoxicity

Exposure period (h)	Harvest time (h)	Dose (µg/ml)	Survival index (%) [a]		Mitotic index (%) [a]	
			-S9	+S9	-S9	+S9
1st pre-test						
4	24	10	128.4	128.1	80.7	98.9
		25	95.4	130.3	71.1	86.0
		50	109.2	102.2	86.0	110.8
		100	70.6	91.0	91.2	119.4
		200	6.2	0.7	0	0
		300	1.3	1.4 [P]	0	0 [P]
		400	0.7	0.7 [P]	0	0 [P]
2nd pre-test						
4	24	100	67.7	142.1	93.4	81.3
		120	74.8	84.2	98.4	87.0
		140	26.0	16.9	94.3	87.8
		160	1.4	6.5	30.6	32.5
		180	0.8	2.5	0	0
		200	3.0	2.7	0	0
24	24	1	215.3	np	100	np
		10	242.2	np	65.6	np
		50	127.5	np	3.1	np
		100	103.4	np	6.3	np
		150	17.3	np	0	np
3rd pre-test						
24	24	10	86.5	np	83.3	np
		20	67.4	np	54.2	np
		30	73.1	np	49.0	np
		40	85.1	np	22.9	np
		50	73.1	np	15.6	np

[a]: relative to solvent control cells; [P]: precipitation; np: not performed

B. CHROMOSOME ABERRATION ASSAY

On the basis of the results of the pre-test for cytotoxicity, the assay was performed using concentrations of 0-125 µg/ml, with a 4 hour exposure period and 8, 24 and 30 hour harvest times.

Cytotoxicity

The relative mitotic indices of cells exposed at the highest concentration, 125 µg/ml without metabolic activation, were reduced to 76.0 and 73.5 % for cells harvested at 24 and 30 hours, respectively (Table 5.8.1/18- 3). A similar, but slightly more marked, effect occurred at 25 µg/ml without metabolic activation in cells harvested at 24 hours. Reduced relative mitotic indices also occurred at 5 and 25 µg/ml with metabolic activation in cells harvested at 24 hours, but not at 125 µg/ml. There was no reduction of mitotic indices of cells harvested at 8 hours.

Chromosome aberrations

The approximate generation time of CHO cells is 14 hours. Thus the appropriate harvest time according to the current guideline, which requires sampling at a time equivalent to about 1.5 normal cell cycle length, is the 24-hour interval. At the 8-hour interval only few cells have completed cell cycling, which makes this time point appropriate for determination of cytotoxicity rather than mutagenicity.

There were no statistically significant or biologically relevant increases in the incidence of aberrant metaphases at any exposure concentration and harvest time without metabolic activation. A statistically significant increase in aberrant metaphases, including and excluding gaps, occurred at 125 µg/ml with metabolic activation at the 8-hour harvest interval. This increase is not considered biologically relevant since the concurrent negative control was exceptionally low (zero for all parameters determined) and there was clearly no effect at this dose level at the relevant time point of harvest after 24-hours. There was also no effect at 125 µg/ml at the 30 hours harvest interval. Thus, the statistically significant increase in aberrant metaphases at 125 µg/ml is considered to be incidental. An increased incidence of aberrant cells including gaps also occurred at the lowest concentration (5 µg/ml) with metabolic activation at the 24-hour harvest interval. There was no increase at higher dose levels at this harvest interval with metabolic activation and no increase at any dose level without metabolic activation. Furthermore the percentage of aberrant metaphases including gaps was within the range of the laboratory historical control data for ethanol solvent and is considered not to be biologically relevant.

There were no other statistically significant increases at any exposure concentration or harvest interval. The positive controls, MMC and CP, produced clear, statistically significant increases in the incidence of aberrant metaphases at the 24-hour harvest interval, indicating the sensitivity of the assay system.

Table 5.8.1/18- 3: Mitotic indices and incidences of cells with aberrations

Exposure / harvest (hours)	±S9	Dose (µg/ml)	Mitotic index (%) [a]	Number of cells with structural chromosome aberrations							
				Gaps	Chromatid-type		Chromosome-type		Others[b]	Aberrant metaphases (%)		
				g + rg	b	f + d	ib	if + id		-g	+g	x
4 / 8	-	0$	100	3	1	1	1	1	0	1.5	2.5	0.0
		125	162.5	0	0	0	0	5	0	2.0	2.0	0.0
	+	0$	100	0	0	0	0	0	0	0.0	0.0	0.0
		125	133.7	3	1	0	0	5	0	3.0*	4.0*	0.0

Exposure / harvest (hours)	±S9	Dose (µg/ml)	Mitotic index (%) [a]	Number of cells with structural chromosome aberrations							
				Gaps	Chromatid-type		Chromosome-type		Others [b]	Aberrant metaphases (%)		
				g + ig	b	f + d	ib	if + id		-g	+g	±
4 / 24	-	0$	100	2	2	0	0	3	0	2.5	3.5	0.0
		0	115.0	0	0	1	1	1	0	1.5	1.5	0.0
		5	93.0	2	1	1	1	1	0	2.0	2.5	0.0
		25	60.0	0	0	0	1	5	0	3.0	3.0	0.0
		125	76.0	2	2	0	2	1	0	2.5	3.5	0.5
		MMC	63.0	7	8	0	8	10	12	25.0**	27.0**	14.0*
4/24	+	0$	100	0	0	0	0	2	1	1.0	1.0	0.5
		0	88.4	1	0	0	2	2	0	1.5	2.0	0.0
		5	68.1	2	1	0	0	6	0	3.5	4.5*	0.0
		25	65.2	0	0	0	0	3	1	1.5	1.5	0.5
		125	111.6	0	0	0	0	0	0	0.5	0.5	0.0
		CP	102.9	4	14	0	18	17	21	28.5**	30.0**	8.5**
4 / 30	-	0$	100	1	0	0	0	4	0	2.0	2.5	0.0
		125	73.5	0	3	0	1	7	0	5.0	5.0	0.0
	+	0$	100	0	0	0	1	2	0	1.0	1.0	0.0
		125	81.9	0	2	0	0	1	0	2.0	2.0	0.0
Historical control range for 1993-1994												
4 / 8	-	0 (ethanol)								1.5-3.0	2.0-3.5	0.0-1.0
										0.0-1.5	0.0-1.5	0.0-0.5
4 / 24	-									0.5-5.0	0.5-5.5	0.0-0.5
	+									0.5-4.5	0.5-5.5	0.0-0.5
4 / 30	-									0.0-2.5	0.5-3.5	0.0-1.5
										1.0-4.0	1.0-4.0	0.0-0.5

$: solvent control; [a] relative to solvent control; [b] includes exchanges, multiple aberrations, multiple aberrations + exchanges, and cell disintegrations; * $p < 0.05$; ** $p < 0.01$;
gaps/isogaps (g/ig); breaks/isobreaks (b/ib); fragments/ isofragments (f/if); deletions/isodeletions (d/id); exchanges (x)

III. CONCLUSION

JAU 6476-desthio is considered not to be clastogenic to CHO cells *in vitro*, based on the absence of a statistically significant and biologically relevant increase in the incidence of aberrant metaphases.

Report:	KCA 5.8.1/19[redacted],; 1993; M-031124-01-1
Title:	SXX 0665 - Micronucleus test on the mouse
Report No.:	22089
Document No.:	M-031124-01-1
Guideline(s):	OECD 474 (1983), EEC Directive 84/449/EEC B.12.; US-EPA PB 84-233295 (1983)
Guideline deviation(s):	none
GLP/GEP:	**yes**

Deviations: The following deviations from the current OECD guideline (2016) occurred:
Treatment and sampling: As required by the 1983-guideline, which was valid at the time of the study conduct, the test substance was administered only once. The way of administration was not specified in the 1983-guideline and intraperitoneal injection of JAU 6476-desthio was chosen to maximise systemic exposure (instead of oral gavage as recommended nowadays). Intraperitoneal injection was still one of the suggested routes of exposure in the 1997 guideline. Only a single dose level was used as required by the 1983 guideline, whereas the current guideline specifies a minimum of 3 dose levels. However, the maximum tolerated dose (MTD) was reached in the current study, which is also required by the current guideline. The method of euthanasia was not reported.
Overall, the treatment schedule, route of exposure, and dose selection are considered acceptable and do not diminish the study results.
Observations: 1000 polychromatic erythrocytes (PCE) per animal were scored for micronuclei as required by the 1983 guideline. According to the current guideline a minimum of 4000 PCE/animal should be scored for the incidence of micronuclei. Nonetheless, since the number of micronucleated PCE was clearly within the historical control range in the current study, the result obtained is still considered valid.

Executive summary:

JAU 6476-desthio (batch no. 1717008/90; purity 93.1 %), was tested for a possible clastogenic effect on the chromosomes of bone marrow erythroblasts of NMRI mice. Three groups of 5 male and 5 female NMRI mice were treated with 10 ml/kg JAU 6476-desthio in 0.5 % aqueous Cremophor at a dose level of 350 mg/kg by intraperitoneal injection. Five animals/sex were killed to prepare femoral marrow 16, 24 or 48 hours after treatment. Two further groups of 5 animals/sex, one treated with vehicle only and the other treated with 20 mg/kg cyclophosphamide (CP) in water, and killed 24 hours after treatment, acted as concurrent vehicle and positive controls, respectively. One thousand polychromatic erythrocytes (PCE) per animal were scored for the presence of micronucleated cells. In addition the number of normochromatic erythrocytes (NCE) per 1000 erythrocytes and the incidence of micronucleated NCEs were also determined. The study was conducted according to OECD guideline 474 (1983) and was compliant to GLP. Deviations from the current (2016) OECD guideline are noted above.

Animals treated with JAU 6476-desthio at 350 mg/kg showed apathy, staggering gait, roughened fur, lateral/sternal recumbency, spasm, uncoordinated movement and difficulty in breathing until necropsy, indicating relevant systemic exposure. Furthermore the ratio of NCE to PCE cells was altered at all 3 sampling intervals also demonstrating bone marrow exposure. No increase in micronucleated cells after treatment with JAU 6476-desthio was observed. No test has been performed to identify the nature of the micronuclei in order to determine whether the mechanism of micronucleus induction is due to clastogenic and/or aneugenic activity. However, the fact that JAU 6476-desthio did not cause any increase in micronuclei formation implies that JAU 6476-desthio did exhibit neither clastogenic nor aneugenic activity in this assay. The positive control material, cyclophosphamide, did not affect the NCE/PCE ratio but produced a very marked and statistically significant increase in the incidence of micronucleated PCE cells relative to the vehicle control group, and thus demonstrated the sensitivity of the test.

JAU 6476-desthio was not clastogenic or aneugenic in this *in vivo* test system in male and female mice.

I. MATERIAL AND METHODS

A. MATERIALS

1. Test Material:	JAU 6476-desthio
Synonym:	SX 0665
Description:	Light-brown powder
Batch No.:	1717008/90
Purity:	93.1 %
Stability of the test compound:	The batch used was analyzed prior to study initiation and approved for use during the test period.
Vehicle used:	0.5 % aqueous Cremophor emulsion
2. Control Materials:	
Vehicle control:	0.5 % aqueous Cremophor emulsion
Positive control:	cyclophosphamide (CP); solvent: deionized water
3. Test animals:	
Species:	Mouse
Strain:	Bor:NMRI
Sex:	Males and females (virgin)
Age:	8-12 weeks
Weight:	28-42 g
Source:	[redacted] Germany
Acclimation period:	At least one week
Number of animals per dose	5/sex/group
Diet:	Altromin 1324 Standard Diet (Altromin GmbH, Lage, Germany) *ad libitum*
Water:	Tap water, *ad libitum*
Housing:	The females were kept in groups of a maximum of three mice in Makrolon type I cages. Males were kept singly in type I cages. Bedding of soft wood granules was used.
Environmental conditions:	
Temperature:	22±1.5 °C
Humidity:	40-70 %
Air changes:	About 10 times per hour
Photo period:	12 h of electrical lighting daily
4. Test compound doses	
Micronucleus assay:	0, 350 mg JAU 6476-desthio/kg bw JAU 6476-desthio was administered once by intraperitoneal injection.

B. TEST PERFORMANCE

1. Dates of experimental work: November 02, 1992 – November 25, 1992

2. Dose selection

The selection of the JAU 6476-desthio dose was based on a range-finding study, in which groups of five animals, including both males and females, were treated once, by intraperitoneal (i.p.) injection, with JAU 6476-desthio at dose levels of 350, 500 (2 groups) and 750 mg/kg and observed for up to 5 days post-treatment. Mortalities were 1/5, 6/10 and 4/5 animals at 350, 500 and 750 mg/kg, respectively. Apathy, staggering gait, lateral / sternal recumbency, spasm, uncoordinated movement, eye-lids stuck

together and difficulty in breathing occurred at 350 mg/kg. As a result 350 mg/kg was selected as the dose level for the main study.

3. Micronucleus assay

Treatment and sampling

Each group comprised ten mice, five males and five females. They were divided into groups by a randomization plan. Each respective substance was administered once.

Table 5.8.1/19- 1: Treatment overview

	Vehicle control	JAU 6476-desthio			Positive control
					CP
Dose (mg/kg bw)	**0**	**350**	**350**	**350**	**20**
Volume (ml/kg bw)	10				
Route of application	intraperitoneal application (i.p.)				
No. of applications	1	1	1	1	1
No. of animals treated/sex	5	5	5	5	5
Time of sacrifice	24	16	24	48	24

The femoral marrow of all groups was prepared 24 hours after the last administration. At least one intact femur was prepared from each sacrificed animal. The femur was separated from all soft tissue. The proximal end of the femur was opened at its extreme end and the bone marrow was flushed out in a tube with foetal calf serum and subsequently centrifuged at approximately 1000 rpm for five minutes. The supernatant was discharged leaving only a small remainder. The sediment was mixed to produce a homogeneous suspension.

Slide preparation

One drop of the viscous suspension was placed on a well cleaned slide and spread with a suitable object to allow proper evaluation of the smear. The slides were then dried overnight. After drying the smears were stained automatically with an Ames Hema-Tek Slide Stainer from the Miles Company. The slides were then "destained" with methanol, rinsed with deionized water, and left to dry. Following this treatment, the smears were transferred to a holder. A cuvette was filled with xylene, into which the holder was immersed for approximately ten minutes. The slides were removed singly to be covered and a small amount of covering agent was applied to the coated side of the slide. A cover glass was then placed in position without trapping bubbles. The slides were not evaluated until the covering agent had dried.

Slide evaluation

In general, 1000 polychromatic erythrocytes (PCEs) were counted per animal. The incidence of cells with micronuclei was established by scanning the slides in a meandering pattern.

The number of normochromatic erythrocytes (NCE) per 1000 polychromatic ones was noted to establish the ratio of polychromatic to normochromatic erythrocytes, which is expedient for two reasons:

5. Individual animals with pathological bone-marrow depressions may be identified and excluded from the evaluation.
6. An alteration of this ratio may show that the test compound actually reaches the target.

In addition to the number of normochromatic erythrocytes per 1000 polychromatic ones, the number of normochromatic erythrocytes showing micronuclei was also established. This information is useful in two ways:

5. It permits the detection of individuals already subject to damage before the start of the test.
6. Combined with the number of micronucleated polychromatic erythrocytes, it permits a representation of the time-effect curve for positive substances.

4. Statistics

The number of polychromatic erythrocytes with micronuclei and the number of normochromatic erythrocytes of the dose groups and the positive control were analysed by Wilcoxon's non-parametric rank sum test. A variation was considered statistically significant if its error probability was below 5 % and the treatment group figure was higher than that of the negative control.

The rate of normochromatic erythrocytes containing micronuclei was examined if the micronuclear rate for polychromatic erythrocytes was already relevantly increased. In this case, the group with the highest mean was compared with the negative control using the one-sided chi²-test. A variation was considered statistically significant if the error probability was below 5 % and the treatment group figure was higher than that of the negative control.

In addition, standard deviations (as ranges) were calculated for all the means.

5. Evaluation criteria

The assay was considered positive, negative or equivocal if the following criteria applied:

positive
- if there was a relevant and significant increase in the number of polychromatic erythrocytes showing micronuclei in comparison to the negative control.

negative
- if there was no relevant or significant increase in the rate of micronucleated polychromatic erythrocytes.
- if there was a significant increase in that rate which, according to the laboratory's experience, was within the range of negative controls.

equivocal
- if there was an increase of micronucleated polychromatic erythrocytes above the range of attached historical negative controls (provided the increase was not significant and the result of the negative control was not closely related to the data of the respective treatment group)

II. RESULTS AND DISCUSSION

A. ANALYTICAL DETERMINATIONS

The stability of JAU 6476-desthio in vehicle was confirmed by analysis. The analytical determinations verified that JAU 6476-desthio is stable in the vehicle at room temperature at concentrations ranging from 1 mg/ml to 100 mg/ml for at least twenty-four hours.

Table 5.8.1/19- 2: Analysis for stability of JAU 6476-desthio in the solvent at room temperature

Nominal value in mg/ml	Content in % after storage time	
	0 hrs	24 hrs
1	97.8	101.0
100	94.5	101.0

B. MICRONUCLEUS ASSAY

Clinical findings

No deaths occurred in any group following treatment, but the animals treated with JAU 6476-desthio at 350 mg/kg showed apathy, staggering gait, roughened fur, lateral/sternal recumbency, spasm, uncoordinated movement and difficulty in breathing until necropsy.

Micronucleus assay results

There was an increase in the ratio of NCE to PCE cells at all 3 sampling intervals, although statistical significance was only apparent at the 48-hour sampling interval (Table 5.8.1/19- 3). Nevertheless, since the NCE/PCE ratios were outside the laboratory historical control range, the effect is considered to be treatment-related.

There were no biologically relevant effects on the incidence of micronucleated PCE cells at any sampling interval, although the incidence at the 24-hour sampling interval was statistically significantly higher than the vehicle control value. Since the value of 1.9 micronucleated PCEs/1000 was within the laboratory historical control range of 1.1 - 2.8, the incidence in the vehicle group (0.8) was particularly low in this study (lower than the historical control range), and all individual values were within the expected range of 0 - 3 micronucleated PCEs/1000 (in accordance with the laboratory's experience and/or the available literature data), the difference is considered not to be related to treatment with JAU 6476-desthio.

The incidences of micronucleated NCE cells were comparable to the vehicle control group at all sampling intervals.

Table 5.8.1/19- 3: Group mean PCE/NCE ratios and incidences of micronucleated PCE and NCE

Treatment group / sampling interval	No. of animals	Total no. PCE scored	No. NCE / 1000 PCE ± SD	No. micronucleated cells/1000 ± SD	
				NCE	PCE
Vehicle control / 24 h	10	10,000	978 ± 212	1.4 ± 1.3	0.8 ± 0.8
JAU 6476-desthio 350 mg/kg / 16 h	10	10,000	1297 ± 419	1.8 ± 1.0	1.6 ± 1.2
JAU 6476-desthio 350 mg/kg / 24 h	10	10,000	1266 ± 386	1.4 ± 1.1	1.9* ± 0.9
JAU 6476-desthio 350 mg/kg / 48 h	10	10,000	1828** ± 581	1.2 ± 1.6	0.9 ± 1.3
CP 20 mg/kg / 24 h	10	10,000	1034 ± 274	1.3 ± 1.9	20.6** ± 6.8
Historical control range[a] / 24 h		-	576 - 1222	0.5 - 2.0	1.1 - 2.8

* $p < 0.05$; ** $p < 0.01$;
PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes
[a] mean values from 12 studies performed during 1990 - 1991 using 0.5 % Cremophor vehicle; sampling based on a total number of 10,000 PCE scored per study

The positive control material, cyclophosphamide, did not affect the NCE/PCE ratio but produced a very marked and statistically significant increase in the incidence of micronucleated PCE cells relative to the vehicle control group.

III. CONCLUSION

The study recorded a negative result. Though the study was not fully compliant with the current OECD guideline, it was compliant with the contemporary guideline. JAU 6476-desthio did not induce increased incidences of micronucleated polychromatic erythrocytes while relevant systemic exposure was demonstrated by an altered ratio of polychromatic to normochromatic erythrocytes and clinical signs.

Therefore it can be concluded that JAU 6476-desthio had no clastogenic or aneugenic effects in this *in vivo* test system.

[redacted]-term toxicity and carcinogenicity

Table 5.8.1-4: Summary of chronic toxicity and carcinogenicity studies performed on SXX 0665.

Study / species / dose levels	NOAEL (mg/kg bw/day)	LOAEL[a] (mg/kg bw/day)	Effects at LOAEL	Reference
2-year chronic toxicity and carcinogenicity study in the rat; 0, 20, 140, 980 ppm	1.1 (20 ppm)	8.0 (140 ppm)	Liver histopathology. There was no evidence of [redacted] carcinogenic potential.	[redacted] (1999) M-027339-01-1
2-year carcinogenicity study in the mouse; 0, 12.5, 50, 200 ppm	3.1 (12.5 ppm)	13 (50 ppm)	Liver histopathology. (fine vesicular vacuolation, periacinar fat accumulation) There was no evidence of carcinogenic potential.	[redacted] and [redacted] (2000) M-044458-02-1

As in the short-term studies the liver was the target organ in both rodent species in the chronic toxicity and carcinogenicity studies. In rats and mice liver weights were increased and there were also histopathological changes in the liver in both species. The NOAELs for both studies were set using liver effects.

The endocrine system was also affected in rats leading to reductions in thyroid hormone (T4), ovary weights which indicated reduced normal ovarian atrophy with age, and histopathology in the adrenal cortex. These effects are likely to be related to enhanced endocrine hormone clearance by enzymes induced in the liver.

Other effects seen in these studies were similar to those seen in the short term studies.

The test material was not carcinogenic in either of these studies.

Ovary effects seen in the short-term and chronic toxicity studies

In the short-term studies in rats, the changes in the ovaries were considered by the applicant to be incidental and not related to the treatment because they may have been due to natural variations during the stages of the oestrus cycle and there were no consistent histomorphological correlates.

However given the likely effects of liver enzyme induction on the endocrine system (e.g. enhanced clearance of endocrine hormones) ovary histopathology in rats is considered to be related to treatment.

In the 13 week mouse study a reduced number of mature corpora lutea and an increased incidence of blood-filled (haemorrhagic) centres of corpora lutea were observed at the very highest dose. Because no such findings were seen in the oncogenicity study at a lower dose level after 12 or 24 months, the applicant concluded that the origin of the 13 week study ovarian findings was unclear. The applicant also stated that in mice, the ovaries are known to exhibit more changes of the vascular system than in rats ([redacted] 1996[19]). Especially in B6C3F1-mice, the strain used, vascular changes including haemorrhagic cysts, acute and chronic haemorrhage with thrombosis, and angiectasis are common features [redacted] 1987[20]). Based on the subchronic and the oncogenicity study (absence of ovarian findings at interim and terminal sacrifice), a NOEL for ovarian findings could be established in mice.

[19] [redacted] (1996): Changes in the ovary. In: [redacted].: Pathobiology of the aging mouse. ILSI Press, Washington DC, pp 451 – 467.

[20] [redacted] (1987): Nonneoplastic lesions of the ovary in Fischer 344 rats and B6C3F1 mice. Environ Health Perspect 73, 53 – 75.

Reproductive toxicity

Table 5.8.1-5: Summary of reproductive toxicity studies performed on JAU 6476-desthio

Study / species / dose levels	NOAEL (mg/kg bw/day)	LOAEL[a] (mg/kg bw/day)	Target organs / main effects	Reference
Pilot dietary reproductive toxicity; Rat; 0, 10, 50, 1000, 1500ppm	10ppm (all effects) 50ppm (reproductive)	50ppm 1000ppm	Increased liver weight; decreased litter size at birth, decreased pup viability, pup growth retardation, liver discoloration	[redacted] & [redacted] (1992) M-031146-01-1
2-generation dietary study; Rat; 0, 40, 160, 640ppm	2.7 (male) 11.0[a] (female) (all effects) 11.0[a] (reproductive) 18.6[b] (neonatal)	10.4 45.4 45.4 72.6[b]	Increased liver weight, liver necrosis, decreased food intake Dystocia, Decreased pup viability, pup growth retardation, low incidence cleft palate	[redacted] & [redacted] (2001) M-036230-01-1
Exploratory gavage embryotoxicity; Rat; 0, 100mg/kg bw/day	100 (maternal) < 100 (reproductive)	- 100	Enlarged placentae, increase in skeletal variants, increase in malformed fetuses mainly limb dysplasia, macroprossia, cleft palate	[redacted] (1990) M-031129-01-2
Embryotoxicity (gavage); Rat; 0, 10, 30, 100mg/kg bw/day	30 (maternal) < 10 (fetal)	100 10	Increased liver weight, liver histopathology increased incidence of 14th rib, 100 mg/kg bw/day caused also increased resorption, decreased litter size, cleft palate, decreased fetal weight gain	[redacted] *et. al.* (1991) M-026431-01-1
Supplementary embryotoxicity (gavage); Rat; 0, 1, 3mg/kg bw/day	> 3 (maternal) (fetal)	- -		[redacted] & [redacted] (1991) M-026445-01-1, [redacted], 2004 M-063046-01-1
Embryotoxicity study (gavage) on postnatal development; Rat; 0, 30mg/kg bw/day	> 30 (maternal) < 30 (fetal)	- 30	Increased in 14th rib, cleft palate, delayed ossification, 15th and 16th ribs (rudimentary). Reversibility of 14th rib extensive but not complete	[redacted] (1992b) M-008329-01-1
Embryotoxicity (gavage); Rabbit; 0, 2, 10, 30mg/kg bw/day	2 (maternal) 2 (fetal)	10 10	Liver histopathology Increase in fetuses arthrogryposis, and fetuses with multiple abnormalities.	[redacted] (1992) M-008334-01-1

Study / species / dose levels	NOAEL (mg/kg bw/day)	LOAEL[a] (mg/kg bw/day)	Target organs / main effects	Reference
Embryotoxicity (dermal) range-finder; Rabbit; 0, 100, 300, 1000mg/kg bw/day	> 1000 (maternal) > 1000 (fetal)	- -	- -	[redacted] & [redacted] (1991) M-031115-01-1
Embryotoxicity (dermal); Rat; 0, 100, 300, 1000mg/kg bw/day	> 1000 (maternal) < 100 (fetal) 300 (developmental toxicity)	- 100 1000	- Increased rudimentary 14th rib Increased incidences cleft palate, microglossia, hydrocephaly	[redacted] (1992a) M-008322-01-1
Supplementary embryotoxicity (dermal); Rat; 0, 10, 30mg/kg bw/day	> 30 (maternal) > 30 (fetal)	- -	- -	[redacted] (1991) M-008327-01-1

[a] mean value of achieved dose level during pre-mating and gestation periods
[b] mean achieved dose level during days 6-14 of lactation
[c] PRAPeR experts agreed on a NOAEL for developmental toxicity of 1 mg/kg bw/day, based on an increase in supernumerary rudimentary ribs at 3 mg/kg bw/day (EFSA Scientific Report (2007), Conclusion on the peer review of prothioconazole)

Reproductive toxicity studies in the rat comprised a pilot dietary study and a full 2-generation dietary study. In addition to gavage developmental studies, dermal developmental toxicity studies were performed in the rat and a dermal range-finding study in the rabbit. The purpose of the dermal studies was to establish any developmental toxicity by the dermal route and establish NOAELs that may be required for operator risk assessment. Where appropriate, supplementary studies were performed to clarify NOAEL values. A postnatal developmental study was also performed to elucidate the nature of supernumerary ribs. A summary of the reproductive toxicity studies performed is shown in Table 5.8.1-5 ~~B.6.62'~~.

Reproductive effects of JAU 6005 in rats ([redacted] & [redacted] (1992, M-031146-01-1) ~~B.6.6.1' (a)~~ and [redacted] & [redacted] (2001, M-036130-01-1) ~~B.6.6.1' (b)~~) comprised reduced litter size, reduced pup viability, pre-weaning growth retardation and a low incidence of cleft palate. In the main 2-generation study, a number of P and F1 generation females exhibited dystocia at the highest dose level and this drove the reproductive NOAELs. P and F1 generation maternal livers showed hepatocyte vacuolation. Some females also showed slight to moderate liver necrosis. These liver effects and decreased food intake drove the maternal NOAEL. There was also good correlation between dams exhibiting dystocia and the occurrence of liver necrosis ([redacted] & [redacted] (2001, M-036130-01-1) ~~B.6.6.1' (b)~~). In both the pilot and main study the parental NOEL values were comparable to, or lower than, NOEL values for reproductive and developmental effects.

In the developmental studies the prime effects were increased incidences of cleft palate in the rat and rabbit and supernumerary 7th ribs in the rat only. Where frank malformations and fully formed supernumerary 14th ribs tended to occur at higher dose levels, rudimentary supernumerary 14th ribs occurred at lower doses. The supplementary rat oral study at [redacted] & [redacted] (1991, M-026445-01-1) ~~B.6.6.2.1(c) (i)~~ established an oral developmental NOAEL in the rat based on this skeletal variant. Rudimentary supernumerary 14th ribs may have been considered indicative of a developmental toxicity [25] in these studies as they occurred in the absence of maternal toxicity in the supplementary oral study in rats ([redacted] & [redacted] (1991, M-026445-01-1)) ~~(B.6.6.2.1' (c) (i))~~. Hence further work was required to ascertain the nature of supernumerary 14th ribs in the absence of maternal toxicity and hence clarify the NOAEL for this study. To provide the necessary clarification, a review of archived material was carried out ([redacted] (2004, M-063046-01-1)) ~~(B.6.6.2.1' (c) (ii))~~ for the Main and Supplementary

developmental toxicity studies performed. The review ascertained what proportions of the supernumerary rib tally were fully-formed- or rudimentary- supernumerary ribs. Comparing each rib tally of treated groups in the Supplementary study against control values from the Main study, showed that there was no actual increase in fully-formed or rudimentary supernumerary 14th rib in the supplementary study. All supernumerary 14th rib incidences in the supplementary study were also within historical control ranges. The Supplementary and Main studies were performed 16 months apart at the same laboratory using the same strain of rat and conducted by the same key personnel and criteria for definition of rudimentary and fully formed, so this comparison is considered acceptable.

SXX 0665 administered dermally to rats at high dose level (1000 mg/kg bw/day) also elicited increased incidences of cleft palate, macroglossia and hydrocephaly, and rudimentary 14th rib ([redacted] (1992a, M-008322-01-1)) ~~(B.6.6.2.2′ (a))~~. The dermal developmental NOAEL for the rat studies was based again on the formation of rudimentary supernumerary ribs. This NOAEL occurred at non-maternally toxic dermal doses. No adverse effects, developmental or otherwise, were observed in the pilot rabbit dermal developmental study.

Overall, SXX 0665 was fetotoxic in rats via the oral and dermal routes at non-maternally toxic dose levels. In rabbits the oral NOAEL for maternal toxicity coincided with that for fetotoxicity and malformations.

[25] Traditionally, their occurrence is frequently considered as a common skeletal variant because the background incidence in rodents is high, their occurrence is often associated with maternal stress and toxicity, and typically tend to disappear post-natally. [redacted] (1992a, M-008322-01-1) ~~B.6.6.2.2 (a)~~ quotes a spontaneous incidence of supernumerary 14th rib of 18%.

Neurotoxicity studies in rodents

Table 5.8.1-6: Summary of neurotoxicity studies in rodents performed on JAU 6476-desthio

Study	NOAEL (mg/kg bw/d)	LOAEL	Findings at LOAEL	Reference
Developmental Neurotoxicity Study 0, 40, 160 and 500 ppm	Maternal / reproductive toxicity: 15.1	Maternal / reproductive toxicity: 43.3	Dystocia, increased gestation length	[redacted], 2007 M-060384-02-1
	Neonatal toxicity: 3.6	Neonatal toxicity: 15.1	Deviated snout, malocclusion of the incisors and associated skull findings	
	Developmental neurotoxicity: 43.3	Developmental neurotoxicity: ---	No effects on neurobehavioral and learning and memory parameters, on brain weight, brain morphometry and on neuropathology parameters	

JAU 6476-desthio caused no developmental neurotoxicity in rats; no effects on neurobehavioral and learning and memory parameters, on brain weight, brain morphometry and on neuropathology parameters were observed at the highest dose tested. Maternal / reproductive toxicity (dystocia and increased gestation length) occurred at the highest dose tested.

Skull mid-line suture effects (deviated snout, malocclusion of the incisors and associated skull findings (ulceration of the dorsal palate, missing dorsal incisors and/or nasal bone fracture)) were observed in pups from the mid- and high dose groups. These effects are expressions of an insult on mid-line skull sutures that is less severe than (but interrelated with) the formation of cleft palate which had been observed in previously conducted developmental and reproductive toxicity studies with JAU 6476-desthio. A comparison with the results from the reproductive toxicity study conducted with Prothioconazole-desthio reveals that the overall NOAEL for this type of finding is 10 mg/kg bw/day. This comparison is considered valid since both studies were conducted in the same laboratory and followed the same clinical observation procedures (as laid down in respective laboratory standard

operating procedures specifically listing “malocclusion - imperfect closure of the teeth” as a possible finding to be investigated). Further confirmation of this 10 mg/kg bw/d NOAEL is derived from the results of the oral gavage developmental toxicity study in rats ([redacted] et. al., 1991; M-026431-01-1).

Report:	KCA 5.8.1/59[redacted],;[redacted];[redacted];[redacted]; 2007; M-060384-02-1
Title:	A developmental neurotoxicity screening study with technical grade SXX 0665 in Wistar rats
Report No.:	200958
Document No.:	M-060384-02-1
Guideline(s):	US-EPA OPPTS 870.6300 (1998)
Guideline deviation(s):	yes, see report
GLP/GEP:	**yes**

Material and methods: Technical grade JAU 6476-destbo (= SXX 0665) (batch no. RUX76-105-1E, purity: 99.1 – 99.4%) was administered via the diet from gestation day (GD) 6 through lactation day (LD) 21 to groups of approximately 30 mated female Wistar rats (strain: Wistar Hannover Crl:WI (GlxBRL/Han) IGS BR) at concentrations of 0, 40, 160 and 500 ppm. The dietary concentrations were equal to doses during gestation of 0, 3.6, 15.1 and 43.3 mg/kg bw/day, and to doses during lactation of 0, 8.1, 35.7 and 104.6 mg/kg bw/day. The in-life phase of the study lasted from January to April 2003.

On postnatal day (PND) 4, litters with a minimum of eight pups, including at least three per sex, were culled to yield, as closely as possible, four males and four females. Subsets of surviving offspring, representing at least 20 litters per level, were subjected to evaluation using the following observations and measurements: Detailed clinical observations and a functional observational battery (FOB), preputial separation or vaginal patency, body weight, automated measures of activity (figure-eight maze), acoustic startle habituation, learning and memory (passive avoidance after weaning and a water maze task beginning on PND 60±2 days) and an ophthalmic examination. Neural tissues were collected from 10/sex/dietary level (representing approximately 20 litters) on PND 21 (brain only) and at study termination (approximately 75 days of age) for microscopic examination and morphometry. The neural tissues from high-dose animals were examined relative to those from the control groups. If no treatment-related lesions were evident, further analysis was generally not performed. In response to a specific Authority request, the peripheral nerves from low- and mid-dose groups were examined for axonal degeneration, and morphometric measures were taken of the frontal cortex (adult females) and of the corpus callosum (PND21 male pups) in control and all treated groups.

Findings:

a) Maternal toxicity: No treatment-related clinical signs were observed, mortality was not affected by treatment. Three high-dose dams were sacrificed on GD 22 due to dystocia. Body weights, body weight gain and food consumption were not affected. FOB investigations revealed no treatment-related findings.

b) Reproductive toxicity: 500 ppm caused a decreased fertility index, an increased gestation length, fetal deaths, and three dams in dystocia with dead fetuses on GD 22.

Table 5.8.1-7: Affected reproductive parameters

Dose [ppm]	Fertility Index [%]	Mean Gestation Length [days]
0	96.7	21.5
40	90.0	21.6
160	90.0	21.8
500	73.3	22.1*

* $p \leq 0.05$

c) Neonatal toxicity

General observations: In addition to the aforementioned dead pups in three high-dose females during late pregnancy, the number of stillborn pups was slightly increased in the high-dose group (0, 0, 2 or 3 stillborn pups occurred in control, low-, mid- or high-dose group). The occurrence of two stillborn pups at the mid dose is not ascribed to treatment as it is within the range of historical controls (a DNT study, study number 01-D72-CI, was conducted from January to April 2001 in the same laboratory using the same strain of rats as in the present study and revealed an incidence of stillborn pups of 2, 1, 1 or 0 in control, low-, mid- or high-dose groups).

Pup birth weight, body weight gain and terminal body weight were not affected.

Detailed clinical observations revealed the development of a deviated snout with associated malocclusion of the incisors in mid-and high-dose pups. This finding became apparent after weaning, as the snout elongated with maturation and lateral deviation from the midline became progressively more severe. Deviated snout and associated malocclusion are considered less severe manifestations related to improper development of the palate, while cleft palate seen in previously conducted studies with JAU 6476-desthio (i.e., developmental and reproductive toxicity studies) is regarded as a more severe effect. Deviated snout and cleft palate are therefore assessed as interrelated expressions of an insult on skull mid-line sutures. This assessment is confirmed by the published literature (see DNT study report). Associated findings, like lacrimation and lacrimal stain, were also observed in some of the animals exhibiting deviated snout / malocclusion in the present study. Gross necropsy examination identified additional mid- and high-dose animals with associated skull findings (e.g., ulceration of the dorsal palate, missing dorsal incisors, nasal bone fracture). The total number of affected animals, based on in-life and necropsy findings, is compiled in the table below.

Table 5.8.1-8: Number of pups exhibiting malocclusion or deviated snout

Dose [ppm]	Total Number of Pups Examined	Malocclusion	Deviated Snout	Total / % Affected (includes animals with related necropsy findings)
Males				
0	69	0	0	0
40	68	0	0	0
160	68	1	0	1 / 1%
500	63	3	1	4 / 6%
Females				
0	69	0	0	0
40	69	0	0	0
160	67	4	1	4 / 6%
500	61	7	2	9 / 15%

Developmental landmarks (sexual maturation): There were no compound-related effects on preputial separation or vaginal opening at any dietary level.

Neurobehavioral and learning and memory parameters: Functional observational battery (FOB) investigations confirmed the malocclusion and associated findings in mid- and high-dose animals that had also been observed during clinical observations. All other investigated parameters (motor / locomotor activity, acoustic startle habituation, passive avoidance, water maze task, pupil constriction, ophthalmology) were not affected at any dietary level.

Brain weight, brain morphometry, neuropathology: On PND 21 and/or at study termination, there were no treatment-related effects evident on brain weight, gross or microscopic brain measurements, or on neuropathological parameters (microscopic investigations on brain, neural tissues, skeletal muscle).

Conclusions:

a) NOAELs

The NOAEL for maternal reproductive toxicity was established at 160 ppm (equal to 15.1 mg/kg bw/day during gestation), based on dystocia and increased gestation length observed at 500 ppm (equal to 43.3 mg/kg bw/day during gestation).

The NOAEL for neonatal toxicity was established at 40 ppm (equal to 3.6 mg/kg bw/day during gestation), based on the occurrence of deviated snout, malocclusion of the incisors and associated skull findings (ulceration of the dorsal palate, missing dorsal incisors and/or nasal bone fracture) at 160 ppm (equal to 15.1 mg/kg bw/day during gestation). These effects are assessed as expressions of an insult on mid-line skull sutures that are less severe than (but interrelated with) the formation of cleft palate which had been observed in previously conducted developmental and reproductive toxicity studies conducted with Prothioconazole-desthio.

The NOAEL for developmental neurotoxicity was established at 500 ppm (equal to 43.3 mg/kg bw/day during gestation), based on the absence of effects on neurobehavioral and learning and memory parameters, on brain weight, brain morphometry and on neuropathology parameters at the highest dose tested.

b) Comparison of DNT Study Results with Results from the Reproductive Toxicity Study with JAU 6476-desthio

The DNT study confirmed the pattern of maternal toxicity as observed in the rat reproductive toxicity study conducted with JAU 6476-desthio ([redacted] & [redacted] (2001), M-036130-01-1).

The absolute and percent incidences of the interrelated findings deviated snout, malocclusion and cleft palate in both the rat DNT study and the rat reproductive toxicity study are compared in the following table. This comparison is made on the basis of the respective dose levels (in mg/kg bw/day) that were established during the gestation phase of each study. This comparison is considered valid since both studies were conducted in the same laboratory and followed the same clinical observation procedures (as laid down in respective laboratory standard operating procedures specifically listing "malocclusion - imperfect closure of the teeth" as a possible finding to be investigated).

Table 5.8.1-9: Absolute and percent incidence of pups (both sexes combined) exhibiting deviated snout, malocclusion or cleft palate in the DNT or reproductive toxicity (2-G) study conducted with JAU 6476-desthio

Finding	Dose Level During Gestation [mg/kg bw/day]							
	0 (2-G)	0 (DNT)	2.5 (2-G)	3.6 (DNT)	10.0 (2-G)	15.1 (DNT)	41.2 (2-G)	43.3 (DNT)
Deviated Snout	-	-	-	-	-	1 (1%)	-	3 (2%)
Malocclusion	3 (50%)#	-	-	-	-	5 (4%)	-	10 (8%)
Cleft Palate	-	-	-	-	-	-	3 (5%)##	-

- not observed
\# malocclusion due to missing dorsal incisors; refers to a total of six >21 day old F1a weanlings not selected for the subsequent generation
\## percent value related to 0-3 day old pups

Based on the results from both studies, it can be concluded that the interrelated findings deviated snout, malocclusion and cleft palate occurred only in the upper dose range with an overall LOAEL of 15.1 mg/kg bw/day. The respective overall NOAEL for this type of finding is 10 mg/kg bw/day.

The observation of malocclusion in the control group of the reproductive toxicity study, involving missing dorsal incisors, is the same type of finding as observed in some of the affected animals of the DNT study. This demonstrates that the investigations in the reproductive toxicity study were appropriate to detect a respective treatment-related effect. The absence of any respective findings in treated F1-pups or F1-adults of the reproductive toxicity study supports the overall NOAEL for „skull mid-line suture effects" of 10 mg/kg bw/day. Further confirmation of this 10 mg/kg bw/day NOAEL is derived from the results of the oral gavage developmental toxicity study in rats ([redacted] et. al., 1991; M-026431-01-1).

c) Impact on Human Risk Assessment for Prothioconazole-desthio

The results of the DNT study neither affect the previously submitted risk assessment nor change the overall hazard assessment of JAU 6476-desthio. The ARfD, systemic AOEL and ADI for Prothioconazole-desthio are set at 0.01 mg/kg bw/day. This provides a 1000-fold margin of safety over the overall NOAEL (10 mg/kg bw/day) for „skull mid-line suture effects". This margin of safety is considered acceptable.

JAU 6476-sulfonic acid (M02)

This metabolite was found in the straw of rotational wheat crops. JAU 6476-sulfonic acid ~~SA~~ was moderately acutely toxic (M-020192-02-1), was not mutagenic in an Ames test (M-041306-01-1), and in a 13-week dietary toxicity study (M-081053-01-1) NOAEL values of 34 and 163 mg/kg bw/day (top dose) were established in males and females, respectively. The NOAEL in the male is based on transitional cell hyperplasia in the urinary bladder at a dose level of 136 mg/kg bw/day. This finding is in common with the findings in a 52-week oral toxicity study of prothioconazole ~~JAU 6476~~ in the rat in which urothelial hyperplasia occurred. JAU 6476-sulfonic acid ~~SA~~ did not produce toxicologically significant hepatic microsomal enzyme induction in the rat 90 day study. In contrast to JAU 6476-sulfonic acid ~~SA~~, JAU 6476-desthio (M04, SXX 0665) had a NOAEL of 2.2 mg/kg bw/day in the rat 13-week dietary study, based on liver alterations, many of which indicate hepatic enzyme induction. JAU 6476-sulfonic acid ~~SA~~ does not produce adverse toxicological effects in the liver at oral dose levels up to the highest tested dose of 136 (males) and 163 (females) mg/kg bw/day in the rat. With respect to the very sensitive endpoint of supernumerary rib formation in the rat, JAU 6476-sulfonic acid ~~SA~~ does not produce an increased incidence of this skeletal variant at 750 mg/kg bw/day or any skeletal, visceral or external abnormalities (M-034925-01-1, M-058857-01-1). Effects are confined to reduced fetal weight gain associated with retarded ossification at 750 mg/kg bw/day. Thus, the NOAEL is 150 mg/kg bw/day. In comparison, the lowest NOAEL in a developmental toxicity study with prothioconazole-desthio (M04, SXX 0665) is 1 mg/kg bw/day and supernumerary rib formation is induced at a maternal dose level of 3 mg/kg bw/day.

Overall, the data on JAU 6476-sulfonic acid ~~SA~~ suggest a toxicological profile that is similar to that of prothioconazole ~~JAU 6476~~ but different and less toxic than prothioconazole-desthio. In addition, a SAR analysis (DEREK 17.1 test) of JAU 6476-sulfonic acid ~~SA~~ (M-035374-01-1) did not produce any significant structural alerts for mutagenicity, carcinogenicity or reproductive toxicity, supporting the contention of a low toxicological risk for JAU 6476-sulfonic acid ~~SA~~. However, there was a structural alert for potential skin sensitisation, but since JAU 6476-sulfonic acid ~~SA~~ may be formed within wheat, a possible skin sensitising property is considered not relevant for human risk assessment. Based on this information JAU 6476-sulfonic acid ~~SA~~ is not considered a toxicologically relevant metabolite, and is not included in the residue definition.

JAU 6476-triazolinone (M03), JAU 6476-desthio-α-hydroxy (M18), JAU 6476-desthio-α-acetoxy (M19), JAU 6476-benzylpropyldiol (M09)

None of the metabolites were mutagenic in the Ames test either with or without metabolic activation (M-043413-01-1, M-043536-01-1, M-041437-01-1, M-029692-01-1) and the acute oral toxicity studies produced LD50 values of >2000 mg/kg (M-044271-01-1, M-044287-01-1, M-044212-01-1, M-035102-01-1). Furthermore, SAR analyses (DEREK test) of each metabolite (M-035374-01-1) did not produce any significant structural alerts for mutagenicity, carcinogenicity or reproductive toxicity, and are not considered to be toxicologically relevant at the levels identified in wheat straw.

1,2,4-triazole, triazole alanine, triazole lactic acid, triazole acetic acid

The toxicological evaluation , including setting of reference values, of these potential soil, ground water, plant and/or livestock metabolites is currently ongoing as a separate process in the EU. Several new respective toxicological studies were recently sponsored and submitted by the Industry task force TDMG (Triazole Derivatives Metabolite Group - comprised of BASF SE, Bayer CropScience AG, Dow AgroSciences LLC, Isagro S.p.A., Syngenta Crop Protection AG). The UK CRD acts as the respective EU Rapporteur.

CA 5.8.2 Supplementary studies on the active substance

AE 1344264

Although the summary for this impurity in technical active substance was not part of the original DAR for the first Annex I inclusion it is nevertheless provided here in grey letters (standing for original information) since the studies and detailed study summaries had been provided already in the EFSA process for original Annex I listing.

AE 1344264 has no acute oral toxicity in rats, is non-mutagenic in the Salmonella microsome test, is not clastogenic for mammalian cells in vitro and is non-mutagenic in the V79-HPRT forward mutation assay. AE 1344264 is slightly skin sensitizing in the guinea pig maximization test according to Magnusson and Kligman. Developmental toxicity of AE 1344264 was investigated in a pilot study in rats. In general, maternal and developmental toxicity were comparable with those of prothioconazole, considering both the toxicological profile and the dose-effect relationship.

Table 5.8.2-1: Summary of studies with AE 1344264 (impurity in technical active substance)

Study	Concentration range or dose level tested	Result	Author, Year Reference
Acute oral, rat, female	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted], 2003 M-087359-01-1
Salmonella/ microsome test	Up to 5000 µg/plate (+/- S9 mix) Bacteriotoxic (not assessable) >500 µg/plate	Negative (with and without metabolic activation)	[redacted], 2003 M-083542-01-1
Mammalian chromosome aber-ration test, V79 cells	60 – 300 µg/mL (+/- S9 mix)	Negative (with and without metabolic activation)	[redacted], 2003 M-083364-01-1
V79-HPRT mammalian cell mutation test	Up to 440 µg/ml	Negative (with and without metabolic activation)	[redacted], 2003 M-103094-01-1
Skin sensitization, Guinea pig (Magnusson and Kligman)	Intradermal: 2.5% Topical: 50% Challenge: 50%	Slightly sensitising	[redacted], 2002 M-063236-01-1
Pilot developmental toxicity study in rats (gavage)	0, 40, 200 and 1000 mg/kg bw/day	Maternal toxicity: NOAEL 40 mg/kg bw/day, based on decreased feed intake and impaired body weight development observed at the LOAEL of 200 mg/kg bw/day. The top dose of 1000 mg/kg caused in addition initial body weight loss, increased water intake in all dams and increased urination. The effects on water intake and urination had also been observed for prothioconazole at 1000 mg/kg and indicate that also AE 1344264 causes severe disturbances of the kidney function and systemic water and electrolyte homeostasis. Fetal- and developmental toxicity: NOAEL 40 mg/kg bw/day, based on a possible reduction of fetal weight at the LOAEL of 200 mg/kg bw/day. The top dose of 1000 mg/kg caused in addition variations and retardations, indicating a retarded fetal development, as well as an increased overall number of unspecific common spontaneous malformations which are considered to be related to maternal toxicity. A similar profile had been observed for prothioconazole. For both prothioconazole and AE 1344264, there is no evidence for a specific embryotoxic or teratogenic potential.	[redacted], 2002 M-073983-01-1

AE 1344254

Although the summary for this impurity in technical active substance was not part of the original DAR for the first Annex I inclusion it is nevertheless provided here in grey letters (standing for original information) since the studies and detailed study summaries had been provided already in the EFSA process for original Annex I listing.

AE 1344254 has no acute oral toxicity in rats, is nonmutagenic in the Salmonella/microsome test, non-irritating to skin and irritating to eyes. AE 1344254 is slightly skin sensitizing in the guinea pig maximization test according to Magnusson and Kligman.

Table 5.8.2-2: Summary of studies with AE 1344254 (impurity in technical active substance)

Study	Concentration range or dose level tested	Result	Author / Reference
Acute oral, rat, male and female	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted],2001 M-090387-01-1
Salmonella/ microsome test	Up to 5000 µg/plate (+/- S9 mix) Bacteriotoxic (not assessable) >448 µg/plate	Negative (with and without metabolic activation)	[redacted], 2002 M-066978-01-1
Eye irritation rabbit	100 mg	Irritating	[redacted], 2001 M-089952-01-1
Skin irritation rabbit	500 mg, moistened with water	Non-irritating	[redacted], 2001 M-089949-01-1
Skin sensitization, Guinea pig (Magnusson and Kligman)	Intradermal: 2.5% Topical: 50% Challenge: 25%	Slightly sensitising	[redacted], 2002 M-070499-01-1

AE 1344265

Although the summary for this impurity in technical active substance was not part of the original DAR for the first Annex I inclusion it is nevertheless provided here in grey letters (standing for original information) since the studies and detailed study summaries had been provided already in the EFSA process for original Annex I listing.

AE 1344265 has no acute oral toxicity in rats and is nonmutagenic in the Salmonella/microsome test.

Table 5.8.2-3: Summary of studies with AE 1344265 (impurity in technical active substance)

Study	Concentration range or dose level tested	Result	Author / Reference
Acute oral, rat, male and female	2000 mg/kg bw	LD_{50} > 2000 mg/kg bw	[redacted],2001 M-084436-01-1
Salmonella microsome test	Up to 5000 µg/plate (+/- S9 mix)	Negative (with and without metabolic activation)	[redacted], 2001 M-087794-01-1

CA 5.8.3 Endocrine disrupting properties

It should be noted that to date no clear criteria are available in the EU to define endocrine disrupting properties.

The toxicological profile of prothioconazole does not meet the EU interim criteria for endocrine disrupting properties.

Prothioconazole caused no tumors in rats and mice and caused no effects on the following range of endocrine tissues: pineal gland, pituitary gland, hypothalamus, pancreas, ovaries, testes, parathyroid gland, adrenal glands.

The apical toxicological studies with prothioconazole as assessed already for Annex I inclusion give some indications for possible endocrine effects. These are changes in thyroid hormones in rats and dogs as well as changes in some reproductive and progeny parameters in the rat reproductive toxicity study. These minor effects can be explained as follows.

Regarding thyroid-related hormone changes, the following relevant findings were observed:

Rat 28 day study:
At the highest tested dose (1033 mg/kg bw/d), only females showed decreased T4 and increased TSH, T3 was unaffected. UDP-GT activity was increased in both sexes at approx. 150 mg/kg bw/d and above. Thyroid histology was not affected up to the highest tested dose.

Rat 90 day study:
At the highest tested dose (500 mg/kg bw/d), UDP-GT activity was increased in males. Thyroid-related hormones (T3, T4, TSH) and thyroid histology were not affected up to the highest tested dose.

Rat one year study:
At the highest tested dose (750 mg/kg bw/d), T3 was decreased in males only, and T4 and TSH were decreased in both sexes. UDP-GT activity was not measured (but was increased in the rat 28-day and 90-day studies – see above). Thyroid histology was not affected up to the highest tested dose.

Rat two year study:
At the mid dose (50 mg/kg bw/d), T4 was slightly decreased in both sexes. At the highest tested dose (750 mg/kg bw/d, due to overt toxicity reduced to 625 mg/kg (females, week 56) and to 500 mg/kg (males, week 84)). Additionally, T3 was decreased in both sexes, and TSH was decreased in males and increased in females. UDP-GT activity was not measured, but was increased in the rat 28-day and 90-day studies (see above). Thyroid histology was not affected up to the highest tested dose.

Mouse 90 day study:
UDP-GT activity was increased in both sexes at 100 mg/kg bw/d and above. Thyroid-related hormones were not measured. Thyroid histology was not affected up to the highest tested dose.

Dog 90 day study:
At the highest tested dose (300 mg/kg bw/d), T4 was decreased in both sexes, and TSH was decreased in females only. UDP-GT activity was not affected, but relative liver weights were increased in males (high dose only, relative) and in females (mid and high dose, absolute and relative). Thyroid histology was not affected up to the highest tested dose. These changes on T4 and TSH were not confirmed in the one year dog study up to the highest tested dose of 125 mg/kg bw/d.

It is concluded that the observed high-dose changes in thyroid-related hormones in rats and dogs, in the absence of any histological thyroid changes and thyroid tumors, indicate a compensated thyroid status secondary to increased thyroid hormone excretion due to increased liver enzyme induction (UDP-GT), but without adverse effect on the thyroid itself and, thus, no indication for direct endocrine activity of prothioconazole. This is especially true for humans since humans are much less susceptible to this mechanism due to species differences in the toxicokinetics of the thyroid hormone system.

This assessment is confirmed by the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015. Under point 3.9.2.5.3. this ECHA guidance states: *"Certain chemicals cause induction of liver enzymes and are interfering with the regulation of thyroid hormones. An increase in the activity of hepatic UDPG-transferase results in increased glucuronidation of thyroid hormones and increased excretion. It is known that rodents are highly sensitive to a reduction in thyroid hormone levels (T4), resulting in thyroid toxicity (e.g. hypertrophy, hyperplasia) after repeated stimulation / exposure of this organ. This in turn is related to an increase in the activity of hepatic UDPG-transferase. Humans, unlike rodents, possess a T4 binding protein that greatly reduces susceptibility to plasma T4 depletion and thyroid stimulation. Thus, such a mechanism/effect cannot be directly extrapolated to humans, i.e. these thyroid effects observed in rodents caused by an increase in hepatic UDPG-transferase are therefore considered of insufficient concern for classification."*

Regarding changes in some reproductive and progeny parameters in the rat reproductive toxicity study, the following relevant findings were observed:

As already stated above (CA 5.6), maternal general systemic toxicity was observed at the mid dose of 100 mg/kg bw/d and was very strong, even sublethal, at the highest tested dose of 750 mg/kg bw/d, based primarily on kidney dysfunction and resulting dehydration, with related mortalities at doses between 500 and 1000 mg/kg bw/d in other repeated dose rat studies and a drastically increased water consumption (up to >170% of control) at a dose of 750 mg/kg bw/d in a rat developmental toxicity study.

Secondary to this strong maternal toxicity, there were reproductive effects recorded at the highest tested dose of 750 mg/kg bw/d, including disruption of the oestrous cycle, increased time to insemination, reduced implantation site number and slightly increased duration of gestation. However, these effects did not result in effects on mating, fertility or gestation indices.

Secondary to this slightly increased duration of gestation at the highest tested dose (750 mg/kg bw/d), high-dose male and female F2 pups were slightly heavier at birth and, thus, had a slightly increased anogenital distance. This apparent increase in anogenital distance is not relevant to endocrine pathways, as an endocrine-related increase in anogenital distance would be expected to occur in only one sex and at an unchanged body weight.

High-dose male F1 pups demonstrated a slight delay in preputial separation (+2.5 days / +5.7%). Though the birth weight of these pups was not affected, their further body weight development was strongly retarded (secondary to maternal toxicity), resulting in 15.5% decreased body weights at the end of lactation at post partum (PP) day 21. At PP day 44 (day of preputial separation in controls), high dose pup body weights were still decreased by 13.4%. At their respective individual day of preputial separation high dose pups had almost reached (-4%) the respective body weight of controls at their day of preputial separation. Based on these data, it is concluded that the slight delay in preputial separation in F1 pups at the high dose is secondary to the clearly retarded growth subsequent to maternal toxicity. This pattern of data is clearly distinct from what would be expected for an endocrine-mediated effect; a primary endocrine effect would delay in preputial separation in the presence of higher body weight at the day of preputial separation, due to continuous growth of the pup over time.

Overall, it can be concluded that, based on a complete toxicological data set, there is no evidence for direct endocrine activity of prothioconazole.

Further, the following two publications describing *in vitro* investigations of the effects of prothioconazole on estrogen receptor and androgen receptor transactivity, aromatase enzyme activity, thyroid hormone function, and aryl hydrocarbon receptor transactivity were identified in the open literature and are described and assessed below.

Report:	KCA 5.8.3/01 [redacted],; [redacted]; [redacted]; 2013; M-476775-01-1
Title:	Currently used pesticides and their mixtures affect the function of sex hormone receptors and aromatase enzyme activity
Report No.:	M-476775-01-1
Document No.:	M-476775-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

Test material:

Name:	Prothioconazole (Sigma-Aldrich, Denmark)
Description:	no information provided
Lot/Batch no:	no information provided
Purity:	>99%
Stability:	no information provided
Vehicle:	dissolved in dimethyl sulfoxide (DMSO) into 50 mM stock solution and stored in the dark at room temperature

Positive controls:

ER transactivation assay:

High-affinity ER ligand 17β-estradiol (E2, Sigma-Aldrich, Denmark), dissolved in 96% ethanol (Merck, Germany) into a 100 mM stock solution.

AR transactivation assay:

AR agonists methyltrienolone (R1881, Perkin Elmer, Denmark, dissolved in 96% ethanol into a 10 mM stock solution) and dihydrotestosterone (DHT, Sigma-Aldrich, Denmark; dissolved in DMSO, Thermo Scientific, Denmark) into a 10 mM stock solution).

AR antagonist hydroxyflutamide (HF, MikroMol GmbH, Germany, dissolved in 96% ethanol into a 20 mM stock solution).

Aromatase assay:

Aromatase inhibitor 4-androsten-4-ol-3,17-dione (4-AOD, Sigma-Aldrich, Germany), dissolved in DMSO into a 50 mM stock solution.

Aromatase substrates [1β-^{3}H] 4-androstene-3,17-dione (250 mCi, 9.25 MBq, Perkin Elmer, Denmark) and unlabelled 4-androstene-3,17-dione (Riedel-de Haën, Germany, dissolved in 96% ethanol into a 35 nM stock concentration).

B. Study design and methods

ER transactivation: The estrogenic and antiestrogenic activities of prothioconazole were assessed using the stably transfected MVLN cell line, derived from the human breast adenocarcinoma MCF-7 cell line, carrying an estrogen response element luciferase reporter vector. The MVLN cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) with supplements [4 mM L-glutamine, 6 g/l insulin, 64 mg/l hexamycin, 1 mM sodium pyruvate, 20 mM HEPES] and 5% fetal calf serum.

MVLN cells were seeded in white 96-well microtiter plates with a density of approximately 4×10^4 cells per well in DMEM with supplements and 1% charcoal/dextran-treated fetal calf serum (CD-FCS). The cells were cultured for 24 h and subsequently exposed to serial dilutions of prothioconazole, prepared

in DMEM with supplements and 0.5% CD-FCS, followed by incubation for 24 h. The measured luciferase data were corrected for cell density as the protein content in each well was determined by addition of 50 µl/well of fluorescamine, diluted in acetonitrile (500 mg/l), followed by fluorometric measurements in aWallac VICTOR2 at 355/460 nm wavelength, according to a standard curve of bovine serum albumin (BSA).

Prothioconazole was analyzed alone and upon co-exposure with 25 pM E2, corresponding to approximately E2-EC_{65} (i.e., the concentration that induces 65% of the maximum E2 effect) in this study. Prothioconazole was assessed alone in various concentrations within the range of 1×10^{-10} to 1×10^{-4} M, whereas E2 co-exposure experiments were carried out with prothioconazole concentrations ranging from 1×10^{-10} to 1×10^{-5} M. As positive control, the E2 concentration–response relationship (3.1–300 pM) was analyzed in parallel in each assay. Additionally, the E2-EC_{65} and E2-EC_{100} (i.e., the maximum E2 effect concentration, corresponding to 100 pM) served as positive controls at each 96-well microtiter plate.

AR transactivation: The androgenic and antiandrogenic activities of prothioconazole were assessed using the Chinese hamster ovary cell line CHO-K1. The CHO-K1 cells were transiently co-transfected with the MMTV-LUC reporter vector and the human AR expression plasmid pSVAR0. The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 with phenol red (DMEM/F-12, no glutamine) with supplements [2 mM L-glutamine, 64 mg/l hexamycin] and 10% FCS.

24 h before transfection, CHO-K1 cells were seeded in white 96-well microtiter plates (Perkin Elmer) with a density of approximately 8000 cells per well in DMEM/F-12 with supplements and 10% CD-FCS. The transfection was carried out for 5 h in DMEM/F-12 without supplements and serum, using 0.3 µl per well of the transfection reagent FuGene and 150 ng cDNA per well of the AR expression plasmid pSVAR0 and the MMTV-LUC reporter vector in a ratio of 1:100. Subsequently, cells were exposed to serial dilutions of pesticides prepared in DMEM/F-12 with double the amount of supplements (4 mM L-glutamine, 128 mg/l hexamycin) and CD-FCS (20%), and incubated for 20 h. Prothioconazole was tested in various concentrations within the range of 1×10^{-10} to 1×10^{-5} M, without removal of the transfection reagent and cDNA. The measured luciferase data were corrected for cell density as the protein content in each well was determined by fluorometric measurements as described for the ER transactivation assay.

Prothioconazole was tested alone and upon co-exposure with 25 pM of one of the AR agonists R1881 or DHT (corresponding to approximately the half maximum effect concentration). Initially, the synthetic AR agonist R1881 was used for co-treatment analyses and as positive control; however, R1881 was replaced by DHT during the course of the study as US export of R1881 was prohibited. According to the test laboratory similar and comparable data are obtained by the use of the two AR ligands (data not shown). An R1881 or DHT concentration–response control (1–250 pM or 5–500 pM, respectively) was performed in parallel in each assay, and additionally 25 pM of R1881 or DHT served as positive control at each 96-well microtiter plate. A HF concentration–response control (0.5–500 nM) was included in each assay to serve as an inhibitor control.

Aromatase activity: Effects of prothioconazole on aromatase activity were assessed using the human choriocarcinoma JEG-3 cell line (ATCC no. HTB-36). The cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Minimum Essential Medium with phenol red (MEM, NEAA, no glutamine) with supplements [2 mM L-glutamine, 64 mg/l hexamycin, 1 mM sodium pyruvate] and 10% FCS.

JEG-3 cells were seeded in 24-well microtiter plates with a density of approximately 4×10^4 cells per well in MEM with supplements and 10% FCS. The cells were cultured for 48 h at 37°C in a humidified atmosphere of 5% CO_2. During this period, a medium change was performed once. Subsequently, the culture medium was aspirated, and the cells were exposed to serial dilutions of prothioconazole prepared in serum-free MEM and incubated for 18 h. The aromatization process was performed with serum-free MEM (500 µl/well) containing 0.2 µCi [1β-^{3}H] 4-androstene-3,17-dione and 10 mM unlabeled 4-androstene-3,17-dione. After termination of the 2 h aromatization process, 300 µl of the culture mediumwas extractedwith 750 µl chloroform, and 150 µl of the aqueous phase was treated with 150 µl dextran-charcoal in PBS (5%). Finally, an aliquot of 150 µl of the treated water phase was mixed with 4 ml Hionic Fluor in a 6 ml vial for scintillation, and the samples were assayed for radioactivity (Wallac liquid scintillation counter). The measured aromatase activities were subtracted background level, corrected to cell protein concentration, and related to the solvent control (set to 100%).

For determination of protein concentrations, the left over culture medium was removed, and cells were lyzed with 500 µl lysis buffer per well. Subsequently, a 100 µl aliquot (in replicate) from each well was transferred to a white 96-well microtiter plate and added 50 µl fluorescamine (diluted in acetonitrile (500 mg/l) per well. Finally, fluorometric measurements (Wallac VICTOR2) at 355/460 nm wavelength were performed according to a standard curve of bovine serum albumin (BSA).

Prothioconazole was tested at various concentrations within the range of 1×10^{-9} to 1×10^{-4} M. In each assay, the aromatase inhibitor 4-AOD was analyzed in parallel at two concentrations, 1×10^{-6} M and 1×10^{-5} M, corresponding to approximately 4-AOD-EC_{50} and 4-AOD-EC_{100}, respectively (data not shown).

Cytotoxicity: In parallel with the bioassays, the cytotoxicity of prothioconazole was measured using the Cytotoxicity Detection Kit (LDH) from Roche (Denmark). As a positive control, cells in triplicate were lyzed by Triton (X-100 from Sigma, final concentration of 1%), corresponding to a maximum release of lactate dehydrogenase (LDH). Culture medium from cells exposed to solvent control was used as a negative control.

II. Results

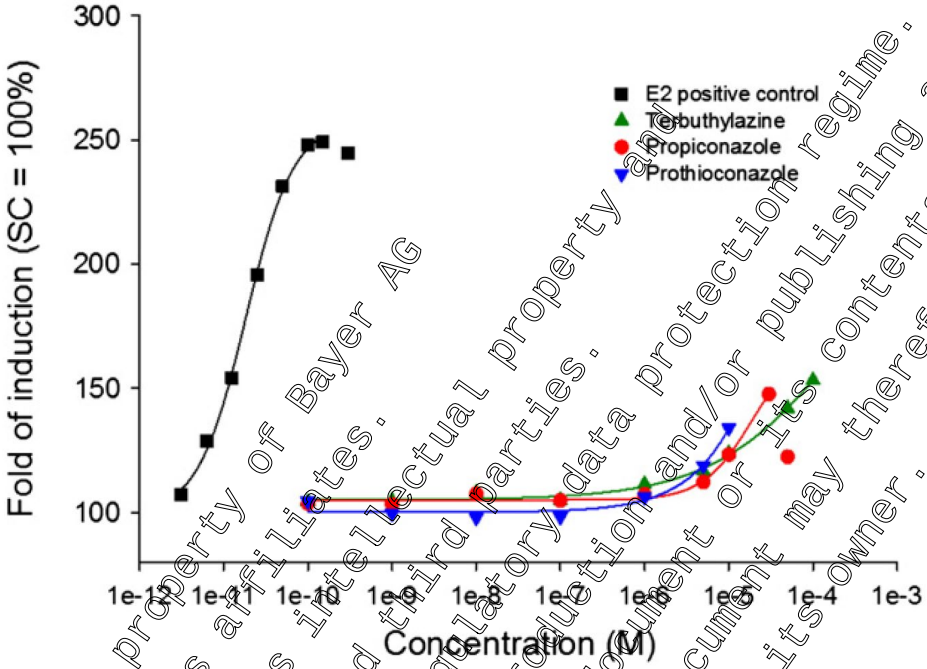
ER transactivation: Cytotoxicity

Prothioconazole displayed a cytotoxic response to MVLN cells at concentrations $\geq 5 \times 10^{-5}$ M.

Estrogenic activity

Prothioconazole exerted a weak estrogenic activity, with a maximum response of 134% compared to the solvent control (compare positive control E2: 248% of solvent control). As evident from the EC_{50} values, the relative potency of prothioconazole was more than 10^5-fold less than the positive control E2.

(The figure below is a respective extract from the original publication.)



Antiestrogenic activity

Prothioconazole exerted no antiestrogenic activity (data not shown).

AR transactivation: Cytotoxicity

Prothioconazole displayed no cytotoxic response to CHO-K1 cells up to the highest tested concentration of 1×10^{-5} M.

Androgenic activity

Prothioconazole elicited no agonistic AR effect (data not shown).

Antiandrogenic activity

Upon co-treatment with 25 nM of a potent AR agonist (R1881 or DHT), prothioconazole exerted no antagonistic effects on the agonist-induced AR transactivity (data not shown).

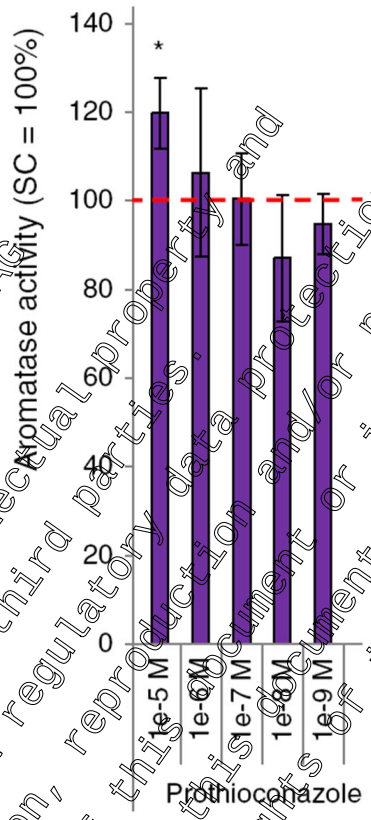
Aromatase activity: Cytotoxicity

Prothioconazole displayed a cytotoxic response to JEG-3 cells at concentrations $\geq 1 \times 10^{-4}$ M.

Aromatase activity

Prothioconazole weakly induced the aromatase activity with a maximum effect of 120% compared to the solvent control (set to 100%).

(The figure to the right is a respective extract from the original publication.)



* Statistically significant ($p < 0.05$) relative to solvent control (SC)

III. Discussion and conclusion

It is known that azole fungicides, as a consequence of their fungicidal mode of action, may inhibit mammalian aromatase, an enzyme responsible for the physiologic balance of androgens and estrogens. However, the present publication does not confirm such an effect for prothioconazole, but claims that prothioconazole weakly induces aromatase activity. However, considering the high variability between groups (the 1e-8 M group had an approx. 15% lower value than control) it is questionable whether the approx. 20% increase of the 1e-5 M group reflects a real treatment-related effect.

Furthermore, the present publication claims that prothioconazole is a weak estrogen receptor agonist. However, "marginal" is considered to be a more appropriate description of the effect observed for prothioconazole since:

- the maximum response increase over control caused by prothioconazole (34%) was only less than a quarter (23%) of the respective maximum response increase over control caused by the positive control E2 (148%)
- the relative potency of prothioconazole was more than 10^5-fold less than that of the positive control E2 (as evident from comparing the EC_{50} values).

Based on their test results, the authors demonstrate the absence of antiestrogenic, androgenic and antiandrogenic activity of prothioconazole.

As the authors state themselves, the effects they observed occurred at relatively high concentrations compared to pesticide exposure levels of the general population.

The authors also state that the findings of their in vitro assays have limitations and might not reflect the in vivo situation where bioaccumulation and metabolism can influence the intracellular concentration

of prothioconazole. The authors thus conclude that *„in vivo studies are needed to further elucidate the endpoint effects of the suspected pesticides."*

Such apical in vivo studies are available for prothioconazole as part of the comprehensive regulatory package of toxicological studies conducted under GLP and according to international test guidelines. As already outlined above, this complete data package demonstrates that there is no evidence for endocrine disrupting properties of prothioconazole.

Report:	KCA 5.8.3/02 [redacted]; 2015; M-530846-01-1
Title:	Effects of currently used pesticides and their mixtures on the function of thyroid hormone and aryl hydrocarbon receptor in cell culture
Report No.:	M-530846-01-1
Document No.:	M-530846-01-1
Guideline(s):	not applicable
Guideline deviation(s):	not applicable
GLP/GEP:	**no**

I. Materials and methods

A. Materials

Test material:

Name: Prothioconazole (Sigma-Aldrich, Denmark)

Vehicle: Dissolved in dimethyl sulfoxide (DMSO) into 50 mM stock solution and stored in the dark at room temperature. The stock solution was diluted with appropriate culture medium immediately before use to give less than 0.1% (v/v) solvent.

Positive controls:

T-screen assay:
L-3,5,3′-Triiodothyronine (T3) (Sigma-Aldrich, Denmark) dissolved in 1M NaOH to produce a 1.5 mM stock solution.

AhR transactivation assay:
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, 98%) (Cambridge by Bie & Berntsen, Denmark), dissolved in DMSO.

Cytotoxicity measurements:
Triton X-100 (Sigma, final concentration of 1%)

B. Study design and methods

T-screen assay: T-Screen assay is a bioassay based on the thyroid hormone-dependent cell proliferation of a rat pituitary tumor cell line, GH3 that is mediated by intracellular expressed TRs. GH3 cells were maintained in phenol-red Dulbecco's MEM culture medium (DMEM) (Sigma-Aldrich) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco, UK), 2 mM glutamin, 1 mM sodium pyruvate, 64 mg/L Garamycin (Schering-Plough, Brussels, Belgium) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. GH3 cells were seeded in 96-well clear plates (Nucleon Delta, Denmark) at an initial concentration of 3000 cells/well in hormone-free medium (Bio-Rad AG-8 resin treated CD-FCS). On the following day, the medium was replaced with hormone-free medium containing prothioconazole to be tested (dissolved in DMSO); each prothioconazole concentration was analyzed in at least triplicate in the range of 10^{-10} to 5×10^{-5} M. The final DMSO concentration in the assay medium did not exceed 0.1% (v/v). The assay was terminated on day 6 by removing the medium and staining the fixed cells with sulforhodamine-B dye.

In order to mimic the physiological processes and assessing the ability of prothioconazole to compete with the endogenous thyroid hormones, prothioconazole was tested in the presence of T3. This setup allows possible detection of antagonistic or potentiating activities. The same concentrations of prothioconazole (10^{-10} to 5×10^{-5} M) were applied in the presence of 0.5 nM T3 inducing half of the maximal response (T3-EC_{50}). In every 96-well plate, a vehicle solvent control (0.1% DMSO) and a positive reference control (T3-EC_{50}) were included. For each assay, a complete T3 standard curve ranging from 10^{-11} to 10^{-8} M was included as quality control. At least three independent experiments were carried out. The average intra and inter-assay coefficient of variation (CV) of the samples were below 7% and 10% respectively. The proliferative effect (PE) was calculated as the percentage ratio between the cell growth obtained with prothioconazole versus the cell growth in the vehicle solvent control (SC, 0.1% DMSO). The relative proliferative effect (RPE), which measures the ratio between the maximal cell growth (E_{100}) achieved by prothioconazole and that of T3-EC_{100}, was calculated as $RPE = \frac{PE\ (prothioconazole) - PE\ (control)}{PE\ (T3) - PE\ (control)} \times 100\%$. Concentration response curves and EC_{50} calculations of T3 and prothioconazole were performed in Sigma Plot using the sigmoid Hill model.

AhR transactivation assay:

The cell culture based AhR (Aryl hydrocarbon receptor) transactivation reporter gene bioassay is a technique that can detect compounds that can activate or inhibit the AhR and thus AhR-dependent gene expression. This assay has proven to be a quick and sensitive assay to detect the AhR mediated potential of chemicals, environmental matrices and biological samples. The Hepa1.12cR cells, stably transfected with the PAH/HAH-inducible luciferase expression vector pGudLuc1.1 that respond in a time-, dose-, and AhR-dependent manner, were used for determination of effects on the AhR transactivation. The Hepa1.12cR cells were maintained with alpha minimal essential medium (α-MEM), supplemented with 10% FCS (Gibco, UK), 64 mg/L Garamycin (Schering-Plough, Brussels, Belgium) and 400 mg/L geneticin (G418, Sigma-Aldrich) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The Hepa1.12cR cells were seeded into 96-well white microtiter plates at 6×10^4 cells per well and cultured in supplemented α-MEM for 24 h. Media was replaced by test compounds or controls following incubation for 4 h. Finally, cells were lysed and the luciferase activity in the lysates was measured. The luciferase data were corrected for cell density by measuring the cell protein in each well and expressed as relative light unit per µg protein (RLU/µg protein). In order to detect agonistic and antagonistic effects prothioconazole was tested alone or in combination with 60 pM TCDD (TCDD-EC_{50}), respectively. Prothioconazole was tested in the range of 10^{-10} to 10^{-4} M in triplicate in at least three independent experiments. In each plate, the solvent control (SC, 0.1% DMSO) and 60 pM TCDD were included as vehicle and positive control, respectively. In each experiment, a set of serial dilutions of TCDD (0.002–5 nM) was included for further calculation of AhR relative potency (AhR-REP) of prothioconazole. The average intra CV was 10.5% and the inter CV of samples was 13.3%. The EC_{50} of TCDD was obtained by fitting dose-response data to a three-parameter sigmoidal Hill curve using Sigma Plot.

Cytotoxicity:

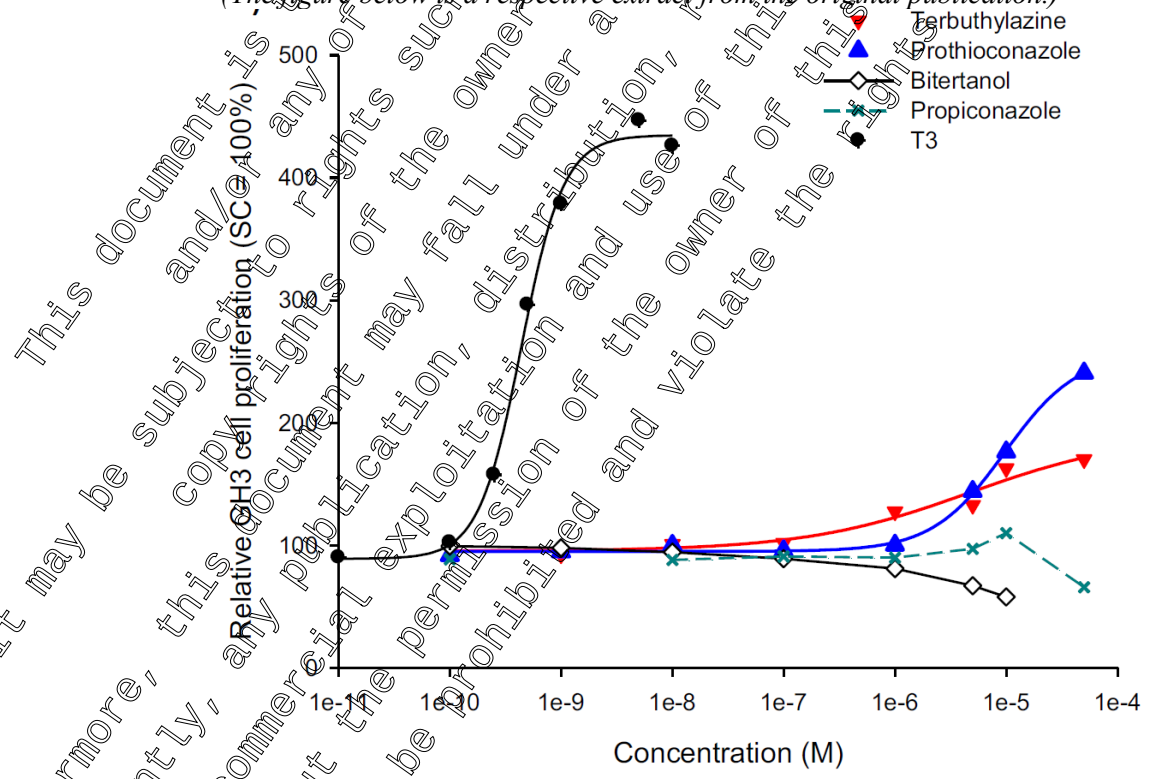
In parallel with the T-screen and AhR transactivation assays, the cytotoxicity of prothioconazole was measured using the LDH Cytotoxicity detection kit

(lactate dehydrogenase) (Roche, Denmark). In addition, the viability of Hepa1.12cR cells after prothioconazole exposure was analyzed using MTT assay. Following exposure (identical to AhR transactivity assay), cells where washed with PBS and incubated with 0.5 mg/mL MTT in cell culture medium (without FCS) for 4 h at 37 °C. MTT solution was then discarded, DMSO (100 µL/well) was added and the plate was shaken for 10 min. The optical density was read on a microplate reader at 570 nm, with a reference at 655 nm (EL800, Bio-Tek Instruments, Inc.). Only data at non-cytotoxic concentrations are reported.

II. Results

Cytotoxicity: Prothioconazole was not cytotoxic.

T-screen assay: <u>Agonistic effect (GH3 cells treated with prothioconazole in the absence of T3):</u>
Prothioconazole significantly affected the basal growth of GH3 cells with a RPE of 38% (compared with the maximal 100% response induced by T3). The LOEC / MOEC of prothioconazole was 5 x 10^{-6} M / 5 x 10^{-5} M, the LOEC / MOEC of T3 was 10^{-10} M / 5 x 10^{-9} M.
As evident from the LOEC / MOEC values, the relative potency of prothioconazole was more than 10^3-fold less than the positive control T3.
(The figure below is a respective extract from the original publication.)



<u>Competitive effect on the T3-induced GH3 cell proliferation in the presence of 0.5 nM T3 (=T3-EC_{50}):</u>
Prothioconazole significantly inhibited the T3-induced GH3 cell growth to 79% of the T3-EC_{50} control. The LOEC / MOEC of prothioconazole was 5 x 10^{-6} M / 5 x 10^{-5} M.

AhR transactivation assay:

Agonistic effect (Hepa1.12cR cells treated with prothioconazole in the absence of TCDD):
Prothioconazole had no agonistic effect but - only at the highest tested concentration (10^{-4} M) - decreased the basal AhR transactivity to 59% of the solvent control. The positive control TCDD increased the basal AhR transactivity to 7050% of the solvent control.

Potentiating or antagonistic effect on the TCDD-induced AhR transactivation (exposure of Hepa1.12cR cells to prothioconazole in the presence of 60 pM TCDD (TCDD-EC_{50})):
Prothioconazole had no potentiating effect but - only at the highest tested concentration (10^{-4} M) - inhibited the TCDD-induced AhR transactivation to 50%.

III. Discussion and conclusion

AhR transactivation assay

The authors assume that the observed effect of prothioconazole in the AhR transactivation assay is not biologically relevant for humans since it was seen only at a very high concentration (10^{-4} M).

T-screen assay

The authors claim that prothioconazole induced the GH3 growth demonstrating an agonistic potential and, upon co-exposure with T3, prothioconazole showed an antagonistic effects on thyroid hormone (TH) action by inhibiting the T3-mediated GH3 cell proliferation. The authors state that triazole fungicides were shown to affect the activity of a number of CYP450s and also of UDP-GT in the liver. The authors state that the T-screen assay is generally useful for the assessment of agonistic and antagonistic properties of compounds but it cannot distinguish cell proliferation through thyroid hormone receptor (TR) and non-TR-mediated mechanisms. Therefore, to document that the effect in the T-screen assay is TR-mediated, cells should be treated with a TR antagonist. A commercially available TR antagonist from Sigma (1-850; CAS 251310-57-3) was used by the authors but did not work in their T-screen assay.

The authors state that the observations in their in vitro study have limitations and do not reflect the in vivo situation where bioavailability, bioaccumulation, metabolism and elimination may influence the intracellular concentration of prothioconazole. Therefore, the authors see a challenge in interpreting the in vitro data to in vivo for potential human toxicity and risk assessment purposes.

This challenge can be overcome by using the available apical in vivo studies that are available for prothioconazole as part of the comprehensive regulatory package of toxicological studies conducted under GLP and according to international test guidelines. As already outlined above, this complete data package demonstrates that the observed high dose changes in thyroid-related hormones in rats and dogs, in the absence of any histological thyroid changes and thyroid tumors, indicate a compensated thyroid status secondary to increased thyroid hormone excretion due to increased liver enzyme induction (UDP-GT) but no adverse effect on the thyroid directly and, thus, no indication for endocrine disrupting properties of prothioconazole. This is especially true for humans since humans are much less susceptible to this mechanism.

The assessment of these publications does not change the overall conclusion that, based on a complete toxicological data set, there is no evidence for endocrine disrupting properties of prothioconazole.

CA 5.9 Medical data

Report:	KCA 5.9/01 [redacted],; 2015; M-531709-01-1
Title:	Summary of medical data known for prothioconazole
Report No.:	M-531709-01-1
Document No.:	M-531709-01-1
Guideline(s):	EU Regulation 1107/2009, 283/2013
Guideline deviation(s):	none
GLP/GEP:	**no**

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

Confidential data. Please refer to document J-CA.

CA 5.9.2 Data collected on humans

No cases of overexposures or intoxications with Prothioconazole have been reported in literature.

CA 5.9.3 Direct observations

No cases of overexposures or intoxications with Prothioconazole have come to the attention of Bayer CropScience.

CA 5.9.4 Epidemiological studies

No epidemiological studies have been published.

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

There are no reports on human poisoning cases.

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

First Aid:

- Remove patient from exposure/terminate exposure.
- Thorough skin decontamination with copious amounts of water and soap, if available with polyethyleneglykol 300 followed by water.
  Note: Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethyleneglykol 300 is not required.
- Flushing of the eyes with lukewarm water for 15 minutes
- Induction of vomiting does not seem to be required in regard of the low toxicity. It should only be considered if a large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious.
- Induced vomiting can remove maximum 50% of the ingested substance. *Note:* Induction of vomiting is forbidden, if a formulation containing organic solvents has been ingested.

Treatment:

- Gastric lavage does not seem to be required in regard of the low toxicity.
- The application of activated charcoal and sodium sulphate (or other carthartic) might be considered in significant ingestions.
- As there is no antidote, treatment has to be symptomatic and supportive.

CA 5.9.7 Expected effects of poisoning

No persisting effects of poisoning are expected.

Appendix I - Proposed reference values for JAU 6476 and JAU 6476-desthio

The following ADI, ARfD and AOEL values for JAU 6476 and JAU 6476-desthio are proposed; these deviate partially from the currently established EU reference values (see footnotes [A)] and [B)]):

	Compound	Value (mg/kg bw/d)	Study	Uncertainty factor
Acceptable Daily Intake (ADI)	JAU 6476	0.05	rat – oncogenicity	100
	JAU 6476-desthio	0.01	rat – oncogenicity	100
Acute Reference Dose (ARfD)	JAU 6476	0.8[A)]	Rat & rabbit developmental tox	100
	JAU 6476-desthio			
	- females 13+	0.01[B)]	Supplementary rat developmental tox	100
	- general population	0.022[B)]	Rat 90 day tox	100
Acceptable Operator Exposure Level (AOEL)	JAU 6476	0.25[A)]	Mouse & dog 90 day tox	100
	JAU 6476-desthio			
	- females 13+	0.01[B)]	Supplementary rat developmental tox	100
	- general population	0.022[B)]	Rat 90 day tox	100

[A)] At the PRAPeR 04 Meeting (September 2006), the experts defined a "combined" NOAEL of 20 mg JAU 6476/kg bw/day for the formation of rudimentary 14th ribs in rats, considering the results of both the original and the new supplemental developmental toxicity studies in rats. Consequently, the experts set the ARfD and AOEL for JAU 6476 at 0.2 mg/kg bw/day. As outlined in this dossier (see CA 5.6), and supported by a new benchmark dose analysis (see M-531958-01-1), this "combined" NOAEL of 20 mg/kg bw/day for rudimentary 14th ribs is not considered appropriate and should be replaced by a conservatively set NOAEL of 80 mg/kg bw/day for the original JAU 6476 rat developmental toxicity study (as meanwhile also done by PMRA Canada, US EPA, UK HSE as ECB-Rapporteur, FAO/WHO and EFSA PPR Panel (for references see CA 5.6)).
Consequently, based on this revised NOAEL and the equivalent NOAELs observed in the supplemental rat developmental toxicity study and in the rabbit developmental toxicity study, the ARfD for JAU 6476 should be set at 0.8 mg/kg bw/day.
Furthermore, The AOEL for JAU 6476 should be set at 0.25 mg/kg bw/day, based on the NOAEL of 25 mg/kg bw/day established in the 90-day toxicity studies conducted in mouse and dog.

[B)] For JAU 6476-desthio the current ARfD and AOEL of 0.01 mg/kg bw/day is based on the NOAEL of 1 mg/kg bw/day for rudimentary 14th ribs derived from the JAU 6476-desthio supplementary rat developmental toxicity study. This is considered appropriate for women of childbearing age but not for the general population. For the general population a non-developmental NOAEL should be selected as the basis for the ARfD and AOEL; the NOAEL of 2.2 mg/kg bw/day from the JAU 6476-desthio rat 90 day study is considered appropriate. In this context, an inconsistency regarding the NOAEL for the JAU 6476-desthio 90 day dog (7.8 mg/kg bw/day in the DAR for the first Annex I inclusion and 1.6 mg/kg bw/day in the EFSA Scientific Report) should be resolved – 7.8 mg/kg bw/day is regarded to be the correct study NOAEL for the reasons provided in chapter CA 5.8.1 of this dossier.
The proposal to define an ARfD / AOEL for women of childbearing age based on a developmental NOAEL and a different ARfD / AOEL for the general population based on a non-developmental NOAEL is generally in line with the respective proposal of the FAO/WHO (Joint Meeting on Pesticide Residues, Report 2008, 193: 265, p. 271-272) who proposed for JAU 6476-desthio an ARfD of 0.01 mg/kg bw/day (based on the NOAEL of 1 mg/kg bw/day for rudimentary 14th ribs derived from the JAU 6476-desthio supplementary rat developmental toxicity study) and an ARfD of 1 mg/kg bw/day for the general population (based on the acute oral LD_{50} studies in rat and mouse).
A similar approach of setting two different reference values for two different parts of the population has also

been used before by EFSA for the ARfD of glufosinate (EFSA Scientific Report (2005) 27, 1-81, Conclusion on the peer review of glufosinate): *"Two acute reference doses (ARfD) values are set; the first is based on the NOAEL from the rabbit developmental study for women of child bearing potential …. The second is based on the NOAEL … from the 1- year dog study, … to be used for the general population."*

Appendix II - Proposed toxicological classification of JAU 6476 against the CLP criteria

This appendix provides a detailed comparison of potentially classification-relevant toxicological findings of JAU 6476 with the respective applicable CLP criteria (following the Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015). As an outcome of this exercise, proposals for classification / non-classification are made for acute toxicity, skin irritation, eye irritation, skin sensitization, germ cell mutagenicity, carcinogenicity, reproductive toxicity, STOT-SE, STOR-RE.

ACUTE TOXICITY, SKIN IRRITATION, EYE IRRITATION, SKIN SENSITISATION

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, the results of the acute toxicological studies (oral LD_{50} rat >6200 mg/kg bw (M-012312-01-1), dermal LD_{50} rat >2000 mg/kg bw (M-009688-01-1), inhalation LC_{50} rat >4990 mg/m³ (M-008846-01-1), no skin irritation (M-009890-02-1), minimal eye irritation (M-009893-02-1), no skin sensitization (M-009898-03-1, M-291490-01-1)) do not trigger any respective classification. Furthermore, JAU 6476 does not show a phototoxic potential (M-498655-01-1).

GERM CELL MUTAGENICITY

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, a classification for germ cell mutagenicity Category 2 is based on:

A) Positive somatic cell mutagenicity tests in vivo, in mammals; or

B) Other positive in vivo somatic cell genotoxicity tests which are supported by positive results from in vitro mutagenicity assays; or

C) Positive in vitro mammalian mutagenicity assays for substances which also show chemical structure activity relationship to known germ cell mutagens.

A summary of available mutagenicity studies conducted with JAU 6476 is provided in the following table:

Study	Result		Reference
	+ S9	- S9	
Bacterial point mutation assay (Ames test) in *S. typhimurium* strains	Negative	Negative	M-012254-01-1
Clastogenicity *in vitro* (V79 CHL cells)	Positive	Positive	M-012277-01-1
Mammalian cell mutation assay (V79 CHL cells – HPRT-locus)	Negative	Negative	M-012273-01-1
Rat liver UDS assay (*in vitro*)	Equivocal		M-012317-01-1
Rat liver UDS assay (*in vivo*)	Negative		M-007155-01-1
Micronucleus assay (*In vivo* mouse bone marrow)	Negative		M-012265-01-1
Micronucleus assay (*In vivo* mouse bone marrow)	Negative		M-102790-01-1

Based on these results, the aforementioned classification criteria A) and B) are not met. Classification criterion C) is also not met since JAU 6476 does not show a chemical structure activity relationship to known germ cell mutagens. This is also convincingly demonstrated by the fact that the structurally closely related JAU 6476 metabolite JAU 6476-desthio was not mutagenic in a battery of assays as summarised in the table below.

Summary of available mutagenicity studies conducted with JAU 6476-desthio:

Study	Result		Reference
	+ S9	- S9	
Bacterial point mutation assay (Ames test) in *S. typhimurium* strains	Negative	Negative	M-031136-01-1
Clastogenicity *in vitro* (CHO cells)	Negative	Negative	M-031119-01-1
Mammalian cell mutation assay (V79 CHL cells – HPRT locus)	Negative	Negative	M-009104-01-1
Rat liver UDS assay (*in vitro*)	Negative		M-031126-01-1
Micronucleus assay (*In vivo* mouse bone marrow)	Negative		M-031124-01-1

Based on these data it can be concluded that no classification for germ cell mutagenicity is applicable for JAU 6476 according to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015.

CARCINOGENICITY

In a two year rat study, the MTD was exceeded at the high dosage, the most notable effects were in the liver and kidneys. Despite these treatment-related findings, there were no notable neoplastic findings in the liver, kidneys or urinary bladder, nor in any other organs and tissues. The overall incidence of tumour-bearing animals, the time of occurrence and the pattern of neoplastic findings did not indicate a carcinogenic effect.

In an 18 month mouse study, the adverse bodyweight effects and a high incidence of histopathological findings in the kidneys suggests that the high dose level was close to the MTD. Consistent with rats, the liver and kidneys were the target organs in mice. There was no increase in neoplastic findings in the liver or kidneys, and the pattern of neoplastic findings in all organs and tissues did not indicate a treatment-related effect.

It can be concluded that JAU 6476 is not carcinogenic in rats or mice and, thus, that no classification for carcinogenicity is applicable.

REPRODUCTIVE TOXICITY

As detailed in the following tables for the respective individual animal studies, JAU 6476 caused only non-specific developmental or reproductive toxicity secondary to very strong maternal toxicity; this does not warrant any reproductive toxicity classification.

Concerns on a possible specific developmental or reproductive toxic potential of JAU 6476 may arise from the fact that some other triazole fungicides are suspected of having a specific developmental or reproductive toxic potential. Furthermore, such concerns might be based on the fact that the structurally closely related JAU 6476 metabolite JAU 6476-desthio, which exhibited developmentally toxic effects,

is stated in the original Draft Assessment Report to be a significant (3-18% of the administered dose) animal metabolite and might, thus, theoretically have contributed to the developmental or reproductive effects seen in the studies conducted with JAU 6476.

In fact, JAU 6476-desthio was detected in significant amounts (3-18% of the administered dose) only in the faeces. Only a small portion (ca. 0.5% of the dose) was found in the urine and bile of rats. This demonstrates that JAU 6476-desthio is only a minor systemic animal metabolite of JAU 6476. This correlates well with the very low plasma concentrations of JAU 6476-desthio (2-4% of the respective JAU 6476 plasma concentrations) that were detected in the subchronic rat study conducted with JAU 6476 (M-011757-01-1). JAU 6476-desthio has therefore no impact on the results of the JAU 6476 developmental toxicity studies.
The overall most abundant metabolite in the rat in vivo ADME studies was the S-glucuronide (M06) of the parent compound, ocurring in the bile (ca. 45-47%) and in the urine (up to ca. 8% of the administered dose). This compound was identified unambiguously as the S-glucuronide by NMR-spectroscopy after the submission of the original Annex I dossier.

It can thus be stated that the sometimes observed triazole-related developmental or reproductive toxicity is not observed for JAU 6476 due to the chemical modification of the triazole moiety to a triazolinethione. The sulphur "handle" is readily "gripped" by the ubiquitous detoxification agent glucuronic acid, forming the S-glucuronide conjugate of JAU 6476 (M06) which is rapidly excreted. After this conjugation, the sulphur is protected against cleavage and no relevant amount of JAU 6476-desthio can be formed in animals. These metabolic features are considered as the **"mechanism of detoxification"** of JAU 6476, are reflected in the fact that it was possible to apply very high top doses in the toxicological studies (i.e., 500 – 750 mg/kg bw/d in the rat and mouse chronic studies, 750 – 1000 mg/kg bw/d in the rat reprotoxicity studies) and, also in the favourable developmental and reproductive toxicity profile and distinguish JAU 6476 from JAU 6476-desthio and some other triazole fungicides.

This "principle of detoxification" was also verified for humans based on the results of a new comparative hepatocyte metabolism study in rats and humans (M-534556-02-1) which confirms that the rat is a suitable animal model for the assessment of the metabolism of JAU 6476 in humans and that the same **"mechanism of detoxification"** prevails in both species.

2-generation study in rats (gavage): 0, 10, 100 and 750 mg/kg bw/d, [redacted], 2001a (M-036206-01-1)

Parental effects: NOAEL: 10 mg/kg bw/d

Effects at LOAEL (100 mg/kg bw/d): Lower body weight gains (mainly males), decreased thymus weights, increased liver weights.

Additional effects at highest tested dose (750 mg/kg bw/d): Increased feed intake, urine stain, salivation, increased kidney weights, hepatocytomegaly, multifocal cortical nephrosis.

Assessment of severity of parental toxicity at highest tested dose (750 mg/kg bw/d): The maternal toxicity is considered to be very strong, even sublethal, based primarily on the kidney dysfunction and resulting dehydration, which in other repeated dose rat studies (14 week, 1 year, 2 year rat, pilot developmental tox rat) even caused mortalities at doses between 500 and 1000 mg/kg bw/d. Dehydration of pregnant dams at 1000 mg/kg bw/d caused 25% mortality or, at 750 mg/kg bw/d, could not be fully compensated even by a drastically increased (up to >170% of control) water consumption (as determined in the pilot developmental toxicity study or the new main developmental toxicity study (both conducted in the same rat strain as the 2-generation study, see M-067839-01-1)).

Reproductive effects:	NOAEL: 100 mg/kg bw/d Effects at LOAEL (750 mg/kg bw/d): Reduced number of oestrous cycles / increased cycle length, slight effects on time to insemination, reduced number of implantation sites, increased duration of gestation (but these effects did not result in effects on mating, fertility or gestation indices). In the F1 generation, the number of pre-antral follicles and F1 testicular sperm count deviated in all dose groups from the concurrent controls. Since these changes were not dose-related, were not affected in the P-generation, were not confirmed by other corresponding parameters (epididymal sperm count, spermatology, testes histology, reproductive outcome) and since the dose group values are within the new historical control data (see supplementary information, M-525951-01-1), they are not considered as a treatment-related effect.
Offspring effects:	NOAEL: 100 mg/kg bw/d Effects at LOAEL (750 mg/kg bw/d): Decreased pup weight gain, decreased pup spleen weights and delayed preputial separation. The delayed preputial separation is secondary to the clearly retarded growth (e.g., on post partum day 21 body weight was decreased by 15.5% in high dose male F1 pups). At the respective individual day of preputial separation, the high dose pups had reached a practically identical (-4%) body weight as the controls. This data constellation is clearly different from that to be expected for an endocrine-mediated effect – in that case a delay in preputial separation would be connected with a higher body weight at the day of preputial separation due to continuous growth over time (see supplementary information, M-524357-01-1).
Classification proposal:	Parental effects started at 100 mg/kg bw/d; at 750 mg/kg bw/d, maternal toxicity was very strong, even sublethal, based primarily on kidney dysfunction and resulting dehydration. In other studies (pilot or new main developmental toxicity) conducted in the same laboratory and rat strain as the present study, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25% mortality or, at 750 mg/kg bw/d, could not be fully compensated even by a drastically increased (up to >170% of control) water consumption (see M-067839-01-1). Reproductive and offspring effects occurred only at the highest tested dose of 750 mg/kg bw/d, and are assessed as a secondary non-specific consequence of the disrupted maternal water homeostasis at the same dose. According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, this constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. The classification criteria for a Category 2 classification (see 3.7.2.2. of the ECHA Guidance) are not met: *"... the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects."*

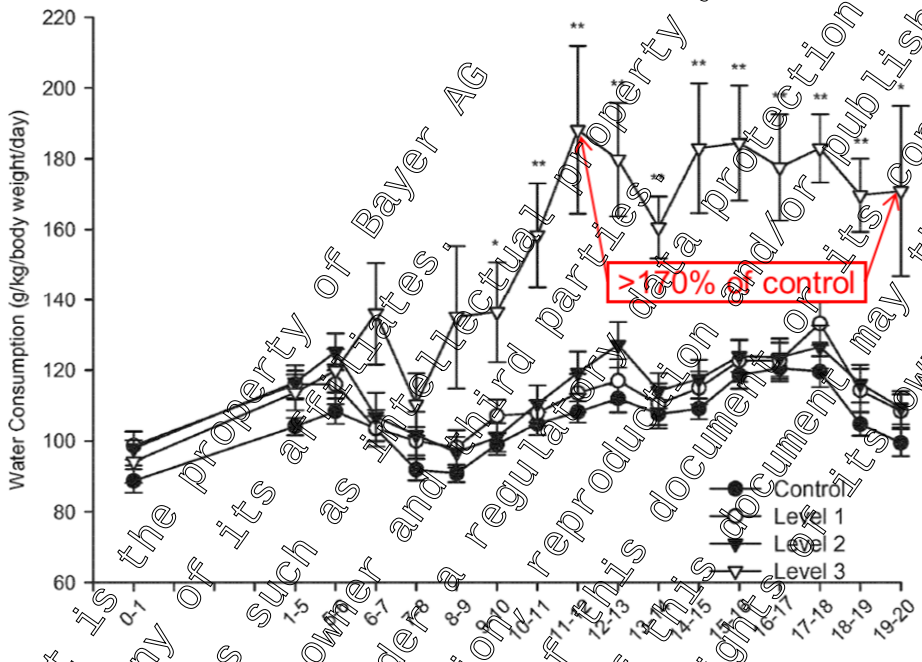
Special developmental toxicity study in rats (gavage) using a strain with a virtually zero incidence of microphthalmia, 0, 20, 80 and 750 mg/kg bw/day, [redacted], 2004 (M-067839-01-1)

This second special study was required by, and the design of this study was agreed with, the UK Expert Committee on Pesticides (previously Advisory Committee on Pesticides) in order to clarify the findings microphthalmia and rudimentary supernumerary rib observed in the original rat developmental toxicity study ([redacted], 1997, M-012279-01-1). Although this second special study was conducted after the original first study, it is described here first since it provides a better "retrospective" understanding of some maternal and developmental findings of the original study (which will be described further down below).

Maternal effects:

NOAEL: 80 mg/kg bw/d

Effects at LOAEL (750 mg/kg bw/d): Decreased net body weight gain, drastically increased water consumption (up to >170% of control; see below for respective amended graph from the study report), increased food consumption and clinical chemical indications for functional impairments of kidneys and liver.



Assessment of severity of maternal toxicity at highest tested dose (750 mg/kg bw/d): The maternal toxicity is considered to be very strong, even sublethal, based primarily on the kidney dysfunction and resulting dehydration, which in other repeated dose rat studies (14 week, 1 year, 2 year rat, pilot developmental tox rat) even caused mortalities at doses between 500 and 1000 mg/kg bw/d. In the same Wistar rat substrain, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25% mortality (as determined in the pilot developmental toxicity, reported also in M-067839-01-1).

Developmental effects:

NOAEL: 80 mg/kg bw/d

Effects at LOAEL (750 mg/kg bw/d): Marginal increase of fetal supernumerary rudimentary (comma-shaped) ribs (variation).

In order to investigate the specificity of microphthalmia formation observed in the original rat developmental toxicity study ([redacted], 1997, M-012279-01-1), the present study used a different Wistar rat substrain for which the available historical control database reveals a virtually zero background incidence of microphthalmia; thus, a non-specific enhancement of microphthalmia secondary to maternal toxicity could not be elicited in this strain. Since the strain was nevertheless sensitive to a direct, specific oculo-teratogenic effect (as shown with the positive control substance All Trans-Retinoic Acid (M-517045-01-1)) it is well suited to decisively investigate the specificity of microphthalmia formation caused by JAU 6476. In order to establish an objective measure for the ocular size, rather than the more subjective standard guideline observation according to the Wilson freehand slicing technique, fresh fetal eyes were extracted, weighed and morphometrically investigated. In this new rat developmental toxicity study JAU 6476 caused no specific malformations or abnormalities up to the maternally maximum tolerated dose, including microphthalmia and anophthalmia.

Classification proposal:

The highest tested dose (750 mg/kg bw/d) caused very strong, even sublethal, maternal toxicity, based primarily on the kidney dysfunction and resulting dehydration (in a pilot study using the same Wistar rat substrain, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25% mortality).

The only developmental effect at 750 mg/kg bw/d (a marginally increased incidence of the variation supernumerary rudimentary (comma-shaped) ribs is assessed as a secondary non-specific consequence of the disrupted maternal water homeostasis at the same dose.

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, this constellation of maternal and developmental effects does not warrant any reproductive toxicity classification. The classification criteria for a Category 2 classification (see 3.7.2.2. of the ECHA Guidance) are not met: *"... the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects."*

Developmental toxicity study in rats (gavage), 0, 80, 500 and 1000 mg/kg bw/d, [redacted], 1997 (M-012279-01-1)

Maternal effects:

NOAEL: 80 mg/kg bw/d

Effects at LOAEL (500 mg/kg bw/d): Decreased body weight gain, increased water consumption (126% of control) and urination.

Additional effects at highest tested dose (1000 mg/kg bw/d): Transient body weight loss, drastically increased water consumption (165% of control).

Assessment of severity of maternal toxicity at highest tested dose (1000 mg/kg bw/d): The maternal toxicity is considered to be very strong, even sublethal, based primarily on the kidney dysfunction and resulting dehydration, which in other repeated dose rat studies (14 week, 1 year, 2 year rat, pilot developmental tox rat) even caused mortalities at doses between 500 and 1000 mg/kg bw/d. In another Wistar rat substrain, dehydration of pregnant dams at 1000 mg/kg bw/d caused 25% mortality or, at 750 mg/kg bw/d, could not be fully compensated even by a drastically increased (up to >170% of control) water consumption (as determined in the pilot developmental toxicity study or the new main developmental toxicity study (see M-067839-01-1)).

Based on a comparison of water intake at 1000 mg/kg bw/d in the present study (165% of control) and at 750 mg/kg bw/d in a different rat substrain in the special developmental toxicity study (up to >170% of control) and considering that in the same rat strain used in the special developmental toxicity study 1000 mg/kg bw/d caused 25% mortality of pregnant dams due to dehydration, it can be concluded:

- The severity of maternal toxicity at the respective highest tested doses (1000 mg/kg bw/d in the first study (M-012279-01-1), 750 mg/kg bw/d in the special study (M-067839-01-1)) is comparable.
- The degree of maternal toxicity at the respective highest tested doses of both studies is very strong, even sublethal.

Developmental effects:

NOAEL: 500 mg/kg bw/d

In the low- and mid dose groups, slightly increased incidences for microphthalmia and rudimentary supernumerary ribs were observed. For the following reasons, these findings are assessed to be biological variations and unrelated to JAU 6476:

- Microphthalmia: The incidences are within the range of historical controls and show no dose response (see CA 5.6).
- Rudimentary supernumerary ribs: At the PRAPeR 04 Meeting (September 2006), the experts defined a "combined" NOAEL of 20 mg JAU 6476/kg bw/d for the formation of rudimentary 14th ribs in rats, considering the results of both the original and the new special developmental toxicity studies in rats. Thus, the experts did not accept the NOAEL of 500 mg/kg bw/d, and even not of 80 mg/kg bw/d, for rudimentary supernumerary ribs in the present study. As outlined in this dossier (see CA 5.6), the incidences at 80 and 500 mg/kg bw/d are within the range of historical controls. Furthermore, a new benchmark dose analysis (see M-531958-01-1) supports that the NOAEL for rudimentary supernumerary ribs in the present study should be conservatively set at 80 mg/kg bw/d (as meanwhile also done by PMRA Canada, US EPA, UK HSE as ECB-Rapporteur, FAO/WHO and EFSA PPR Panel (for references see CA 5.6)). In addition, in the special developmental toxicity study in rats (M-067839-01-1) a clear NOAEL for this finding was established at 80 mg/kg bw/d and only a marginally increased incidence of rudimentary supernumerary ribs was observed at the highest tested dose of 750 mg/kg bw/d. As outlined above, 750 mg/kg bw/d in the special study caused a comparably marked maternal toxicity as 1000 mg/kg bw/d in the present study. Therefore it is plausible to conclude that the treatment-related increase of rudimentary supernumerary ribs at the present study at 1000 mg/kg bw/d is also only a marginal effect (secondary to maternal toxicity) and, thus, the mid dose of 500 mg/kg bw/d is a respective NOAEL.

Effects at LOAEL (1000 mg/kg bw/d) retarded fetal development (lower fetal weights, incomplete ossification), together with increased incidences of microphthalmia, rudimentary supernumerary ribs, engorged placentas, renal pelvis dilatation. All observed developmental effects are assessed as a secondary non-specific consequence of the very strong (sublethal) maternal toxicity at this dose. Specifically in the case of microphthalmia, this assessment is substantiated as follows:

- Microphthalmia is a common spontaneous malformation in the rat strain of the present study and is concluded to be increased at 1000 mg/kg bw/d as a secondary non-specific consequence of the very strong (sublethal) maternal toxicity. A positive correlation between the degree of maternal toxicity and the occurrence of microphthalmia in the present study at 1000 mg/kg bw/d was demonstrated by grouping the maternal toxicity results separately for those dams that produced pups with microphthalmia and for those that did not have any pups with microphthalmia (see table in CA 5.6 and M-285563-01-1).
- For the same rat strain, a further example for an unspecific enhancement of microphthalmia secondary to disturbed maternal health (bradypnea, hypoxia) is described by [redacted] and [redacted], 2003 (M-075915-01-1).
- The results from the new special rat developmental toxicity study (M-067839-01-1), involving highly sophisticated measures for ocular size and weight, exclude any specific oculo-teratogenic potential of JAU 6476 (see above).
- Also in humans, there is strong evidence for an unspecific mechanism (maternal hyperthermia) being a cause of fetal microphthalmia ([redacted] et al., 1998; M-394322-01-1).

Classification proposal: Maternal effects started at 500 mg/kg bw/d; at 1000 mg/kg bw/d, maternal toxicity was very strong, even sublethal, based primarily on kidney dysfunction and resulting dehydration.

Developmental effects (mainly fetal retardation (lower fetal weights, incomplete ossification) and increased incidences of rudimentary supernumerary ribs and of the (in this rat strain) common malformation microphthalmia) occurred only at the highest tested dose of 1000 mg/kg bw/d, and are assessed as a secondary non-specific consequence of the disrupted maternal water homeostasis at the same dose.

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, this constellation of maternal and reproductive / offspring effects does not warrant any reproductive toxicity classification. Specifically, the Guidance states: *"Based on pragmatic observation, maternal toxicity may, depending on severity, influence development via non-specific secondary mechanisms, producing effects such as depressed foetal weight, retarded ossification, and possibly resorptions and* ***certain malformations in some strains of certain species****."*

The classification criteria for a Category 2 classification (see 3.7.2.2. of the ECHA Guidance) are not met: *"... the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects."*

Developmental toxicity study in rabbits (gavage), 0, 10, 30, 80 and 350 mg/kg bw/d, [redacted], 1998 (M-012237-01-1)

Maternal effects:	NOAEL: 80 mg/kg bw/d
	Effects at LOAEL (350 mg/kg bw/d): mortality, bodyweight loss, decreased body weight gain, decreased food consumption
	Assessment of severity of maternal toxicity at highest tested dose (350 mg/kg bw/d): The maternal toxicity is considered to be very strong, including mortality.
Developmental effects:	NOAEL: 80 mg/kg bw/d
	Effects at LOAEL (350 mg/kg bw/d): Abortions, total litter losses, decreased fetal weights, retarded ossification. All observed developmental effects are assessed as a secondary non-specific consequence of the very strong (sublethal) maternal toxicity at this dose. There was no evidence of a teratogenic effect.
Classification proposal:	Maternal toxicity occurred at 350 mg/kg bw/d and was very strong, including mortality.
	Developmental effects occurred also at 350 mg/kg bw/d (abortions, total litter losses, decreased fetal weights, retarded ossification) and are assessed as a secondary non-specific consequence of the very strong maternal toxicity at this dose. There was no evidence of a teratogenic effect.
	According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, this constellation of maternal and developmental effects does not warrant any reproductive toxicity classification. The classification criteria for a Category 2 classification (see 3.7.2.2. of the ECHA Guidance) are not met: *"... the adverse effect on reproduction is considered not to be a secondary non-specific consequence of the other toxic effects."*

SPECIFIC TARGET ORGAN TOXICITY – SINGLE EXPOSURE (STOT-SE)

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, a classification in **STOT-SE**

Category 2 is not applicable, if non lethal significant and/or severe toxic effects on target tissues/organs are not seen in acute toxicity studies up to the following guidance values:

Oral rat	2000 mg/kg bw
Dermal rat or rabbit	2000 mg/kg bw
Inhalation rat, dust / mist / fume	5 mg/l/4h

Furthermore, the ECHA Guidance specifies criteria that trigger a classification for **STOT-SE Category 3**. These criteria are generally independent from the aforementioned guidance values and include transient target organ effects, focusing on overt narcotic effects and respiratory tract irritation (respiratory tract irritation covers two different effects: 'sensory irritation' and 'local cytotoxic effects'). Specifically, the following examples for findings from single and repeated inhalation toxicity studies are mentioned as possible triggers for a STOT-SE Category 3 classification: clinical signs of toxicity (dyspnoea, rhinitis etc) and histopathology (e.g. hyperemia, edema, minimal inflammation, thickened mucous layer) which are reversible.

The relevant acute toxicity studies conducted with JAU 6476 (a repeated inhalation study is not available) provide the following LOAELs and toxicological effects at the respective LOAELs :

Study	LOAEL Toxicological effects at LOAEL (Reference)
Acute oral rat	6200 mg/kg bw (highest tested dose) Decreased motility and diarrhea. (M-012312-01-1)
Acute dermal rat	2000 mg/kg bw (highest tested dose) Partial reddening of the treated skin. (M-009688-01-1)
Acute inhalation rat	4.99 mg/l/4h (highest tested dose) Piloerection, absence of grooming, bradypnea, laboured breathing, serous nasal discharge, red encrustation around the muzzle/nostrils, reduced mobility on the day of exposure. All clinical signs had resolved within 3 days of exposure. Mean rectal temperatures immediately after exposure were decreased. Slight but transient weight loss during the first three post-exposure days. At necropsy, there were no treatment-related gross lesions in any of the test animals. The change in breathing rate and decreased body temperature were attributed to a non-specific response to sensory irritation from exposure to dust. (M-008846-01-1)
Acute oral neurotoxicity rat	750 mg/kg bw (highest tested dose) Partially-formed stools and perianal stain, decreases in motor and locomotor activity. (M-023861-01-1)

A comparison of these LOAELs and toxicological effects with the aforementioned classification criteria reveals that a STOT-SE Category 2 classification is not applicable.

Regarding a possible STOT-SE Category 3 classification for "overt narcotic effects", the observed toxicological findings do not indicate respective effects; the reduced mobility (acute inhalation) and decreased motor and locomotor activities (acute neurotoxicity) are seen as mild expressions of a generally affected well-being and not as a neuro-pharmaco-toxicological narcotic effect. Therefore, a respective STOT-SE Category 3 classification is not applicable.

Regarding a possible STOT-SE Category 3 classification for respiratory tract irritation (sensory irritation or local cytotoxic effects) the observed laboured breathing, serous nasal discharge and red encrustation around the muzzle/nostrils (all reversible within 3 days of exposure) could indicate respiratory tract irritation. However, at necropsy none of the aforementioned histopathological trigger findings were observed. The change in breathing rate and decreased body temperature are attributed to a non-specific response to sensory irritation from exposure to dust, and, thus, not to a specific irritative potential of JAU 6476. Altogether, the observed findings are not seen as convincing evidence for a clear and specific respiratory tract irritation due to JAU 6476 exposure and should therefore not trigger a STOT-SE Category 3 classification.

SPECIFIC TARGET ORGAN TOXICITY - REPEATED EXPOSURE (STOT-RE)

According to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, a classification in STOT-RE Category 2 is not applicable, if significant toxic effects observed in 28-day, 90-day or 12-month repeated-dose studies conducted in experimental animals are not seen up to the following guidance values:

Route of exposure	28-day	90-day	12-month	>12-month
Oral rat	300 mg/kg bw/d	100 mg/kg bw/d	25 mg/kg bw/d	ngvp
Demal rat	600 mg/kg bw/d	200 mg/kg bw/d	ngvp	ngvp

ngvp no guidance value provided

The repeated-dose studies conducted with JAU 6476 provide the following LOAELs:

Route of exposure	28-day (Reference)	90-day (Reference)	12-month (Reference)	>12-month (Reference)
Oral rat	952 mg/kg bw/d (highest tested dose) (M-012338-01-1)	500 mg/kg bw/d (highest tested dose) (M-011757-01-1)	750 mg/kg bw/d (highest tested dose) (M-030441-01-1)	24-month 50 mg/kg bw/d (M-084962-01-1)
		500 mg/kg bw/d (M-053225-01-1)		
Oral mouse	---	100 mg/kg bw/d* (M-012244-01-1)	---	18-month 70 mg/kg bw/d (M-085068-01-1)
Oral dog	---	100 mg/kg bw/d** (M-035825-01-1)	40 mg/kg bw/d (M-035967-01-1)	---
Demal rat	> 1000 mg/kg bw/d (highest tested dose) (M-044301-01-1)	---	---	---

--- not conducted

* The findings observed at this LOAEL consist of changes indicative for liver hypertrophy in response to enzyme induction,which, according to point 3.9.2.5.4. of the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015, are adaptive (compensatory) changes for which no classification would be appropriate.

** The findings observed at this LOAEL consist of histopathological changes in the kidneys (minimal to moderate acute and chronic inflammation, debris) of male and female dogs. Since kidney changes were also observed in rats and mice, they are considered to be relevant for human health. Therefore, these findings may be appropriate to trigger a STOT-RE Category 2 classification according to the ECHA Guidance to Regulation (EC) No 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures, Version 4.1, June 2015.

Based on these data and considerations it is concluded that no classification for STOT-RE is applicable, except for a possible STOT-RE Category 2 classification due to the histopathological changes in the kidneys of male and female dogs in the 90-day study. But this would be a conservative approach since these effects occurred just at the upper end of the respective range of guidance values for a 90-day study (100 mg/kg bw/d) and since similar effects observed in the 12-month dog study do not trigger a respective STOT-RE Category 2 classification since the respective upper end of the range of guidance values (25 mg/kg bw/d) is exceeded by the respective LOAEL (40 mg/kg bw/d) in the 12-month dog study.