Appendix I

Additional information report

For a Substance under Harmonised Classification and Labelling Process

Substance Name: Epoxiconazole

EC Number: 406-850-2

CAS Number: 133855-98-8

Additional information report submitter:

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Date: **15 June 2012**

Explanatory remarks:

BASF has generated new toxicological data in the process of clarifying the endocrine disruption potential of epoxiconazole according to requirements of the Annex I Inclusion Directive 2008/107/EC. BASF has been requested to submit all data available by 1 March 2012 to the EU Commission, for the purpose of data evaluation and consideration for appropriate classification and labeling of epoxiconazole by the Risk Assessment Committee (RAC) of ECHA. The requested data submission is triggered by the outcome of a REACH Committee decision not to adopt the recent RAC opinion for re-classification of epoxiconazole[1] from Repr. Cat. 2 to Repr. Cat. 1B. Instead it was agreed to ask RAC for a new opinion on the basis of all data that is available at this time.

The RAC had justified their previous opinion from March 2010 on the basis of two main adverse effects in rat studies that were considered as critical for re-classification of epoxiconazole:

1) published rat data from 2007/2008 showing **post-implantation loss and resorptions** in the reported absence of maternal toxicity
2) increased incidence of **malformations as cleft palate in rats** based on study data already evaluated by ECB in 1997 / 2002-2003 / 2007-2008 and that formed the basis for the current harmonized classification and labeling entry of epoxiconazole in the CLP Regulation 1272/2008.

During the RAC discussions, BASF had pointed out that studies with epoxiconazole are ongoing and planned to further investigate the endocrine disrupting potential (in compliance with the legal requirement to conduct such investigations as published in Commission Directive 2008/107/EC), and that results would be expected to be also of high relevance for the classification and labeling discussions. However, these data were not taken into account for the current RAC opinion for procedural reasons.

The new study data provides useful and relevant information for the choice of the appropriate reproduction toxicity classification of epoxiconazole. Robust study summaries of these investigations are included in this Additional Report. A conclusion on the relevance of the new information for developmental toxicity classification of epoxiconazole is also provided, including a clarification of the added value of the new information in comparison to already considered information in the RAC Opinion from March 2010.

[1] RAC opinion and RAC Background Document published on 17 March 2010 can be downloaded from the ECHA Website:
http://echa.europa.eu/web/guest/opinions-of-the-committee-for-risk-assessment-on-proposals-for-harmonised-classification-and-labelling/-/substance/590/search/%20/del/20/col/OPINIONDATERAC/type/desc/pre/3/view

CONTENTS

1 IDENTITY OF THE SUBSTANCE

Table 1: Substance identity

Substance name:	Epoxiconazole
EC number:	406-850-2
CAS number:	133855-98-8

2 TOXICITY FOR REPRODUCTION

- DEVELOPMENTAL TOXICITY

Note: All studies were performed with Epoxiconazole technical (CAS-No. 133855-98-8; EC-Number 406-850-2) batches that were in compliance to the authorized technical specification. On the basis of available knowledgs, batch impurities do not affect the classification.

2.1 ANIMAL DATA

2.1.1 Prenatal developmental toxicity study in rats I

STUDY REFERENCE

Report: Schneider S., Strauss V., Fabian E., van Ravenzwaay B. 2010a

BAS 480 F (Epoxiconazole) Modified Prenatal Developmental Toxicity Study in Wistar Rats Oral Administration (Gavage)

BASF DocID 2010/1062087 (unpublished report)

Date of report: 10-May-2010

Report: Schneider S., Rey Moreno M. 2011

Amendment No. 1 to the report: BAS 480 F (Epoxiconazole) Modified Prenatal Developmental Toxicity Study in Wistar Rats Oral Administration (Gavage)

BASF DocID 2011/1229835

Date of report: 09-Sep-2011

Guidelines: (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.31 No. L 142; OECD 414; OPPTS 870.3700

GLP: Yes
(laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz)

Note: The amendment to the study report includes the results from pathological and histopathological examinations of the study

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	8563
Purity:	97.0%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 30-Jun-2010.
2. Vehicle controls:	a) Corn oil b) Carboxymethylcellulose (CMC; 1% aqueous solution)
3. Test animals:	
Species:	Rat
Strain:	Wistar [Crl:WI (Han)]
Sex:	Female
Age:	Sexually mature, virgin rats of about 10-12 weeks of age
Weight at dosing (GD 7):	221.6 ± 11.5 g (188.6 to 259.7)
Source:	Charles River Lab., Germany
Acclimatization period:	at least 7 days
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type M III Makrolon cages (Becker & Co, Castrop-Rauxel, Germany), floor area about 800 cm^2 with Lignocel dustfree bedding (SSNIFF, Soest, Germany)
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work: 04-Feb-2010 to 20-Jul-2011
(in-life dates: 11-Feb-2010 (Start of treatment at gestation day (GD) 7) to 05-Mar-2010 (Sacrifice of the 7th cohort)

2. Animal assignment and treatment:

One day after supply to the test facility, untreated animals were paired at the test facility from about 15:30 until 7:30. When sperm were microscopically detected in the vaginal smear, the females were considered being impregnated and transferred into the study. This day was referred to as gestation day 0 (GD 0), the following day as GD 1. Epoxiconazole was administered to groups of 35 sperm-positive female Wistar rats as a suspension in corn oil or as a suspension in 1% carboxymethylcellulose suspension in highly deionized water (1% CMC) at dose levels of 0, 23 and 50 mg/kg bw/d. Twelve of the females in each group were treated once daily from GD 7-18. The other 23 presumably pregnant females received treatment from GD 7-21. A standard dose volume of 2 mL/kg bw was used for all corn oil groups and a standard dose volume of 10 mL/kg bw was used for all 1% CMC groups. The control groups, consisting of 35 females (12 females GD 7-18, 23 females GD 7-21) were dosed with the vehicles (corn oil or 1% CMC) in parallel. At terminal sacrifice on GD 18, seven to ten females had implantation sites. At terminal sacrifice on GD 21, seventeen to twenty-one females per group had implantation sites.

Table 2/1 Test groups and doses

Test group	Dose (mg/kg bw/d)	Concentration (g/100 mL)	Volume (mL/kg bw)	Treatment period	No. of animals (mated)
0	0 (corn oil)	0	2	GD 7-18	12
				GD 7-21	23
1	23	1.15	2	GD 7-18	12
				GD 7-21	23
2	50	2.5	2	GD 7-18	12
				GD 7-21	23
3	0 (1% CMC)	0	10	GD 7-18	12
				GD 7-21	23
4	23	0.23	10	GD 7-18	12
				GD 7-21	23
5	50	0.5	10	GD 7-18	12
				GD 7-21	23

3. Test substance preparation and analysis:

Prior to study initiation the aqueous and oily test substance preparations were shown to be stable over a 7-day period. Thus, test-substance preparations were performed at the beginning of the administration period and thereafter at maximum intervals of 7 days.

Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in corn oil or 1% CMC using a high-speed homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals.

Test-article concentration analyses were performed twice at the beginning and towards the end of the study. The homogeneity of the dose suspensions was verified at the beginning of the study by taking 3 samples from the top, middle and bottom of the beaker for the low and high dose levels (23 and 50 mg/kg bw/d) while a magnetic stirrer was running. The results of these analyses are given in the table below.

Table 2/2 Analysis of preparations for homogeneity and test-item content

Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± SD
Corn oil	10.02.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		1.15	1.081 1.135 1.115	94.0 98.7 97.0	96.6 ± 2.4
		2.5	2.315 2.374 2.436	92.6 95.0 97.4	95.0 ± 2.4
	02.03.2010 [Concentration control analyses]	0 1.15 2.5	n.d. 1.101 2.352	--- 95.7 94.1	
1% CMC	16.02.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		0.23	0.213 0.224 0.222	92.8 97.3 96.6	95.6 ± 2.5
		0.5	0.492 0.512 0.504	98.5 102.3 100.8	100.5 ± 1.9
	17.02.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		0.23	0.227 0.228 0.230	98.7 98.9 100.1	99.3 ± 0.8
		0.5	0.490 0.493 0.505	98.1 98.6 101.0	99.2 ± 1.6
	02.03.2010 [Concentration control analyses]	0 0.23 0.5	n.d. 0.225 0.464	--- 97.8 92.7	

Relative standard deviations of maximum 2.5% indicated the homogenous distribution of epoxiconazole in the dosing suspensions. The actual nominal test-item concentrations were in the range of 92.6 to 102.3% of the target nominal concentrations and thus in the acceptable range.

4. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption [a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of post-implantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal proportions

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

Statistics for pathology and clinical pathology	
Parameter	**Statistical test**
Hematology, clinical chemistry, organ weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
Mean grade of the degeneration of the placental labyrinth and trophospongium per dam	Pair wise comparison of groups 1 and 2 (GD 21) with the control group 0 was performed using the WILCOXON test (one-sided) for the hypothesis of equal medians.
Difference in degeneration of placentas belonging to live fetuses and late resorptions per dam	For groups 1 and 2 (GD 21) the WILCOXON signed-rank test (one-sided) was performed

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

2. Body weight and food consumption

All animals were weighed on GD 0, 1, 4, and daily on GD 7-21. The body weight change of the animals was calculated from these results.

In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 18 or GD 21 minus weight of the unopened uterus minus body weight on GD 7).

Food consumption was determined on GD 1, 4, 7, 10, 13, 15, 18 and 21.

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on GD 18 or GD 21 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Hematology and clinical chemistry

Blood was drawn in the morning from non-fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.

The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	White blood cell count (WBC)	✓	Platelet count (PLT)
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)		
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells (differential)		

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
		✓	Cholesterol	✓	Alkaline phosphatase (ALP)
		✓	Creatinine	✓	γ-glutamyl transferase (γ-GT)
		✓	Globulin (by calculation)		
		✓	Glucose		*Hormones:*
		✓	Protein (total)	✓	Progesterone (PROG)
		✓	Triglycerides	✓	Androstenedione (ANROS)
		✓	Urea	✓	Estradiol (E2)
				✓	Testosterone (TESTO)

4. Sacrifice

On GD 18 or GD 21, the dams were sacrificed after blood sampling in randomized order by cervical dislocation (after isoflurane anesthesia) and the fetuses were removed from the uterus. Dams were subsequently assessed by gross pathology with special attention to the placentas. The following organs from all dams sacrificed on schedule were weighed and fixed in neutral buffered 4% formaldehyde solution:

- Adrenal glands
- Liver (one slice each of the lobus dexter medialis and of the lobus sinister lateralis were fixed in Carnoy's solution and embedded in paraplast)
- Ovaries

Additionally, from all dams sacrificed on schedule, the …:

- Uterus including placental sites and decidua

… was fixed in neutral buffered 4% formaldehyde solution, and after a 24-48h fixation period, transferred to 70% alcohol solution for asservation.

The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Photograph of the unopened uterus with all contents in situ (CD with all photographs in the raw data archive)
- Number of corpora lutea
- Number and distribution of implantation sites classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to SALEWSKI in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue)
    c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]: } \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]: } \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]: } \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

5. Histopathology of placentas

The histopathological examination of rat placentas followed a stepwise procedure. In a first step, a representative number of placentas was histopathalogically evaluated from each treatment group in order to get an impression of the scope of changes between control and treatment groups as well as of the influence of treatment duration (GD 7–18 vs. GD 7–21) and of the vehicle used (corn oil vs. 1% CMC). For this purpose from each treatment and control group, up to 4 placentas from each dam were examined (i.e., two placentas with live fetus plus 1-2 placentas with late resorption).

Based on the outcome of this histopathological screening, only few histopathological differences were noted between the corn oil and 1% CMC vehicles or the GD 7–18 and GD 7–GD 21 exposure windows. Therefore, in a second step the groups of rats administered corn oil or epoxiconazole/corn oil (Groups 0-2) and sacrificed on GD 21 were selected for a complete histopathological evaluation, which comprised the examination of every placenta from each rat of these groups.

The histopathological examination of the placenta comprised an assessment of changes seen in the labyrinth, trophospongium, decidua and in fetal and maternal blood vessels. Degenerative changes of the labyrinth and of the trophospongium were graded according to their severity in Grade 1 (minimal), Grade 2 (slight), Grade 3 (moderate), Grade 4 (severe), and Grade 5 (massive). From grade 2 onwards, the degenerative changes in labyrinth and trophospongium were frequently associated with an increasing size of the placentas.

SEVERITY GRADES: DEGENERATION OF THE LABYRINTH	
Grade 1 (minimal)	Up to 10% of the labyrinth tissue affected. Small cystic spaces filled with blood and/or fibrin below the chorionic plate.
Grade 2 (slight)	10-30% of the labyrinth tissue affected. Small cystic spaces filled with blood and/or fibrin below the chorionic plate. Minimal, multifocal deposits along fetal vessels in the vicinity of the chorionic plate
Grade 3 (moderate)	30-50% of the labyrinth tissue affected. Larger cystic spaces filled with blood and/or fibrin or empty, extending to the basal and central areas. Slightly changed architecture of remaining labyrinth (overall slight dilation of sinus spaces with thinning of interhemal membranes, degenerative signs in the trilayered trophoblast with karyolysis, cytoplasmic vacuolation and eosinophilic cytoplasmic change). Increased number of fetal vessels with basophilic deposits extending from the chorionic plate to the basal labyrinth. Dilation of uteroplacental artery and distortion of the wall (folding)
Grade 4 (severe)	Cystic spaces in 50-70% of the labyrinth tissue. Remaining labyrinth tissue at the base of the labyrinth shows same features as in Grade 3. Basophilic granular and laminar deposits along fetal vessels may be increased compared with grade 3. The uteroplacental artery is dilated and displaced from the central location.
Grade 5 (massive)	In more than 70% of the labyrinth, cystic spaces replace the normal labyrinth structure. The labyrinthine tissue is reduced to cysts built by filiform and irregular remnants of interhemal membranes. The number of fetal vessels with basophilic deposits reaches the maximum severity.

SEVERITY GRADES: DEGENERATION OF THE TROPHOSPONGIUM	
Grade 1 (minimal)	The trophospongium is minimally thickened, otherwise normal architecture and differentiated cell structure.
Grade 2 (slight)	Up to 2-fold thickening of the trophospongium, with areas of disorganisation. Additionally slight congestion of venous spaces, visible eosinophilic fibrinoid deposits among the cells.
Grade 3 (moderate)	2- to 3-fold thickening of the trophospongium, increased severity of features described for grade 2. Additionally multifocal necrotic areas (eosinophilia with loss of cellular detail, mainly in the central area of the trophospongium.
Grade 4 (severe)	Same features as above with more extensive necrosis (central and peripheral areas). Increased congestion of venous spaces.
Grade 5 (massive)	Only a small rim of apparently normal tissue is observed, otherwise necrotic tissue. The thickness of trophospongium may vary.

6. Examination of fetuses

When the uterus was opened the viability of the fetuses and the condition of placentae, umbilical cords, fetal membranes, and fluids were carefully examined in situ.

After dissection from the uterus each fetus was sexed and external tissues and all orifices were examined macroscopically.

The GD 21 fetuses were additionally weighed.

The sex was determined by observing the distance between the anus and the base of the genital tubercle.

Thereafter, the fetuses were sacrificed by subcutaneous injection of pentobarbital (Narcoren®; dose: 0.1 mL per fetus) and discarded.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al (1997) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al (Chahoud et al 1999; Solecki et al 2001 and 2003):

Malformation	A permanent structural change that is likely to adversely affect the survival or health
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Note: Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on day 18 or 21 p.c. were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

For the above reasons the following females were excluded from the above-mentioned calculations:

Epoxiconazole dose	Treatment from GD 7–18		Comments
	Vehicle: corn oil	Vehicle: 1% CMC	
0 mg/kg bw/d	002; 003	210; 211	Not pregnant
23 mg/kg bw/d	036; 041; 043; 044	245; 246	Not pregnant
50 mg/kg bw/d	075; 077; 079	272; 275; 276; 277; 279	Not pregnant

Epoxiconazole dose	Treatment from GD 7–21		Comments
	Vehicle: corn oil	Vehicle: 1% CMC	
0 mg/kg bw/d	015; 018; 026	214; 216; 218; 222; 225; 229	Not pregnant
23 mg/kg bw/d	053; 063; 064; 069	252; 253; 258; 262; 264; 265; 267	Not pregnant
50 mg/kg bw/d	089; 102	284; 288; 299; 302	Not pregnant

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

There were no test-substance-related mortalities in any of the groups.

2. Clinical signs of toxicity [see Table 2/3]

In dams treated from GD 7–18, transient salivation was observed from GD 16 onwards in 4 dams shortly after treatment with 50 mg/kg bw/d epoxiconazole in corn oil. No clinical signs of toxicity were noted at 23 mg/kg bw/d or at 50 mg/kg bw/d in 1% CMC.

In dams treated from GD 7–21, transient salivation was observed in most females of the high-dose corn oil group. At 50 mg/kg bw/day, piloerection and vaginal hemorrhage were noted; high-dose treatment using the corn oil suspension resulted in slightly higher incidences than with the 1% CMC suspension. In addition, 1 dam was found with pale mucous membranes on GD 21, which may be due to anemia. At 23 mg/kg bw/d, piloerection was noted in 4 dams each from the corn oil and the 1% CMC groups. It is noteworthy that (with exception of transient salivation resulting from repeated administration of epoxiconazole in corn oil) the clinical signs in treatment group dams all became evident after GD 18.

Table 2/3 Clinical findings

	Epoxiconazole (mg/kg bw/d)		
	0	**23**	**50**
Treatment: GD 7–18			
Corn oil	(10x pregnant)	(8x pregnant)	(9x pregnant) 4x transient salivation (GD 16–18)
1% CMC	(10x pregnant)	(10x pregnant)	(7x pregnant)
Treatment: GD 7–21			
Corn oil	(20x pregnant)	(19x pregnant) 4x piloerection (GD 19–21)	(21x pregnant) 20x transient salivation (GD 10–21) 13x vaginal hemorrhage (GD 19–21) 6x piloerection (GD 19–21)
1% CMC	(17x pregnant)	(16x pregnant) 4x piloerection (GD 19–21)	(19x pregnant) 11x vaginal hemorrhage (GD 18–21) 9x piloerection (GD 19–21) 1x pale mucous membranes (GD 21)

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption [see Table 2/4 and Figure 2/1]
In dams treated from **GD 7–18**, the mean food consumption of high-dose (50 mg/kg bw/d) dams was reduced throughout the entire treatment period, the reduction was statistically significant from GD 13-18 (up to -24.1% with corn oil and up to -19.2% with 1% CMC as vehicle). At 23 mg/kg bw/d, mean food intake was significantly reduced (up to -20.4% from GD 7-18 with corn oil and up to -14.2% from GD 10-18 with 1% CMC as vehicle). In the corn-oil group administered 23 mg/kg bw/d, slight but statistically significant reductions in food consumption were noted already before the start of treatment (see Figure 2/1), which is of course not treatment-related; the extent of the reduction in food intake increased especially towards the end of pregnancy indicating a treatment-related effect from GD 15-18 at 23 mg/kg bw/d.

In dams treated from **GD 7–21**, the mean food consumption of high-dose dams was reduced throughout the entire treatment period, the reduction was statistically significant from GD 7-13 and GD 15-21 (up to -29.6% with corn oil and up to -29.4% with 1% CMC as vehicle). At 23 mg/kg bw/d, mean food intake was also significantly reduced from GD 15-21 (up to -19.1% with corn oil and up to -21.5% with 1% CMC as vehicle).

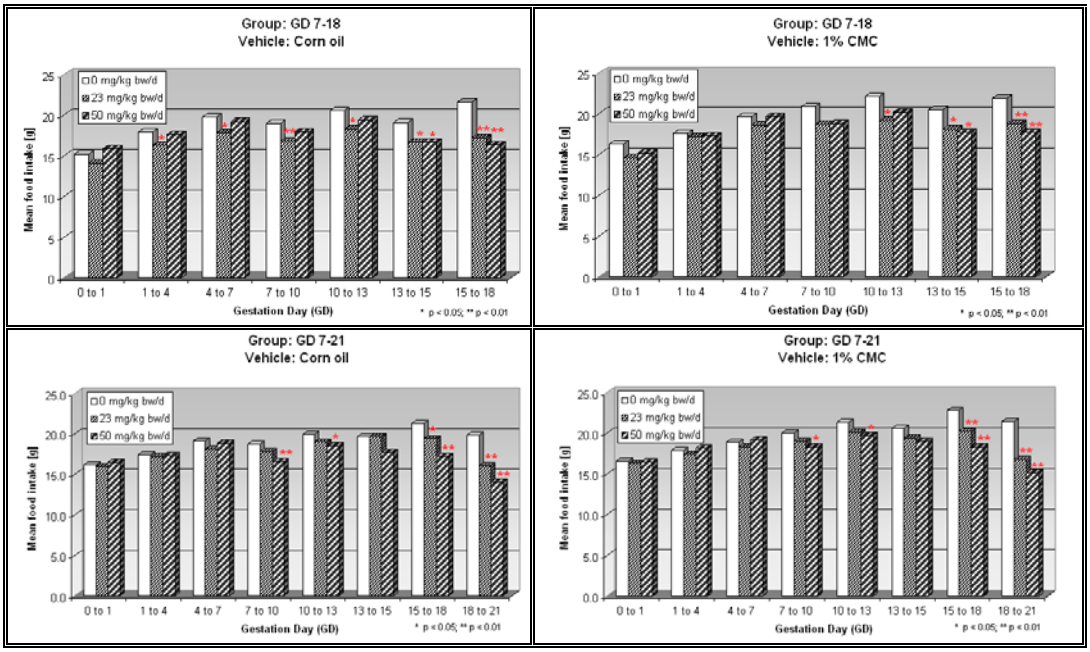
Overall, it appears that food consumption was especially affected in-high dose group dams during late pregnancy, especially from GD 15-21. The choice of vehicle (corn oil or 1% CMC) did not have a relevant impact on food consumption regardless of treatment group.

Table 2/4 Food consumption

Parameter: mean food intake (g/animal)	Epoxiconazole (mg/kg bw/d)					
	Corn oil			1% CMC		
	0	23	50	0	23	50
Groups with treatment: GD 7–18						
GD 7 -10	19.0	**16.8****	17.9	20.9	18.7	18.8
Δ%		**-11.6**	-5.8		-10.5	-10.0
GD 10 -13	20.6	**18.3***	19.4	22.1	**19.2***	20.2
Δ%		**-11.2**	-5.8		**-13.1**	-8.6
GD 13 -15	19.1	**16.7***	**16.7***	20.5	**18.1***	**17.7***
Δ%		**-12.6**	**-12.6**		**-11.7**	**-13.7**
GD 15 -18	21.6	**17.2****	**16.4****	21.9	**18.8****	**17.7****
Δ%		**-20.4**	**-24.1**		**-14.2**	**-19.2**
Groups with treatment: GD 7–21						
GD 7 -10	18.7	17.8	**16.6****	20.0	19.0	**18.3***
Δ%		-11.0	**-17.0**		-5.0	**-8.5**
GD 10 -13	20.0	19.0	**18.5****	21.3	20.1	**19.7***
Δ%		-5.0	**-7.5**		-5.6	**-7.5**
GD 13 -15	19.7	19.7	17.7	20.6	19.4	19.0
Δ%		0.0	-10.2		-5.8	-7.8
GD 15 -18	21.3	**19.4***	**17.2****	22.8	20.2**	**18.3****
Δ%		**-8.9**	**-19.2**		-11.4	**-19.7**
GD 18 - 21	19.9	**16.1****	**14.0****	21.4	16.8**	**15.1****
Δ%		**-19.1**	**-29.6**		-21.5	**-29.4**

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

Figure 2/1 Food consumption in pregnant rats administered epoxiconazole during GD 7-18 or GD 7-21 and using either corn oil or 1% CMC as vehicle



2. Body weight and body weight gain [see Table 2/5]
In dams treated from GD 7–18, mean body weights/body weight gain of low and high-dosed animals (23 and 50 mg/kg bw/d) were comparable to the concurrent control group. All differences observed in these groups during pretreatment and treatment period were without biological relevance and reflected the normal variation inherent in the strain of rats used in the present experiment.

In dams treated from GD 7–21 with 50 mg/kg bw/d in corn oil, the mean body weights were statistically significantly below the control value on GD 9–13 (up to 5%) and GD 20–21 (about 6%). Body weight gain of these dams was significantly reduced during GD 7–9 (up to 65% below the concurrent control), during GD 17-18 (about 20% and during GD 19–20 (about 31%). The average decrease of body weight gain during the treatment period (GD 7–21) was about 16% at 50 mg/kg bw/d. No treatment-related effects on body weight or body weight gain were observable in dams administered 23 mg/kg bw/d epoxiconazole with corn oil used as vehicle. In dams with epoxiconazole / 1% CMC treatment, the mean body weights of high and low dose groups were comparable to the concurrent control group. Body weight gain of the high-dose group was occasionally reduced at the beginning of treatment (GD 7–8, about 70% below control) and towards the end of treatment (GD 19–20, about 39% below control). The average decrease of body weight gain during the treatment period (GD 7–21) was about 10% at 50 mg/kg bw/d.

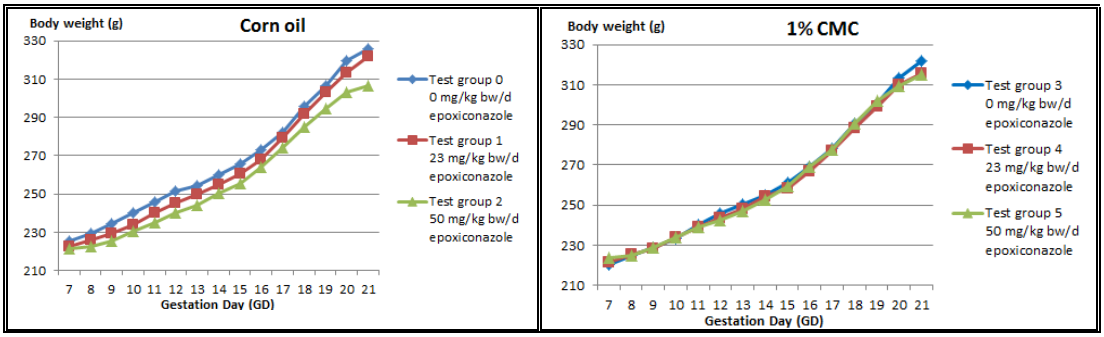
Table 2/5 Bodyweight change

Parameter: bw change (g/animal)	Epoxiconazole (mg/kg bw/d)					
	Corn oil			1% CMC		
	0	23	50	0	23	50
Groups with treatment: GD 7–18						
GD 0 -7	33.7	**24.5****	30.3	30.2	27.1	29.0
Δ%		**-27.3**	-10.1		-10.3	-4.0
GD 7 -18	61.7	51.5	66.6	64.1	63.2	56.8
Δ%		-16.5	7.9		-1.4	-11.4
GD 0 -18	95.4	76.0	96.9	94.3	90.3	85.8
Δ%		-20.3	1.6		-4.2	-9.0
Groups with treatment: GD 7–21						
GD 0 -7	29.5	**25.1***	27.8	29.1	28.2	30.1
Δ%		**-14.9**	-5.8		-3.1	3.4
GD 7 -21	100.7	99.8	**85.0***	101.3	94.5	91.2
Δ%		-0.9	**-15.6**		-6.7	-10.0
GD 0 -21	130.1	124.9	**112.8***	130.4	122.7	121.3
Δ%		-4.0	**-13.3**		-5.9	-7.0

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

Overall, it appears that body weight gain of dams was reduced by treatment especially during late pregnancy. No clear effect of vehicle could be made out. The observed reduction of body weight gain is the result of maternal and not of fetal toxicity as can be seen by evaluation of the corrected maternal body weight gain and weights of unopened uteri (see section D below).

Figure 2.1/2 Body weight development in pregnant rats administered epoxiconazole during GD 7-21 and using either corn oil or 1% CMC as vehicle



D. NECROPSY OBSERVATIONS

1. Corrected (net) body weight gain [see Table 2/6]
In dams treated with 50 mg/kg bw/day from **GD 7–18** the average carcass weight was significantly lower than the control weight (about 7% when given with corn oil and about 8% with 1% CMC). The corrected body weight gain (terminal body weight on GD 18 minus weight of the unopened uterus minus body weight on GD 7) was significantly reduced (about 38% with corn oil and about 42% with 1% CMC as vehicle). At the low dose level of 23 mg/kg bw/d the average carcass weight was significantly lower than the control weight after treatment from GD 7–18 (about 10% when given with corn oil and about 6% with 1% CMC). The corrected body weight gain of low dose group dams was significantly reduced as well (about 35% with corn oil and about 28% with 1% CMC as vehicle).

In dams treated with 50 mg/kg bw/day from **GD 7–21** the average carcass weight was significantly lower than the control weight (about 8% when given with corn oil and about 6% with 1% CMC). The corrected body weight gain (terminal body weight on GD 21 minus weight of the unopened uterus minus body weight on GD 7) was significantly reduced (about 60% with corn oil and about 64% with 1% CMC as vehicle). At the low dose level of 23 mg/kg bw/d the average carcass weight was significantly lower than the control weight after treatment from GD 7–21 (about 5% when given with corn oil and about 4% with 1% CMC). The corrected body weight gain of low dose group dams was significantly reduced as well (about 35% with corn oil and about 45% with 1% CMC as vehicle).

Table 2/6 Mean gravid uterus weights and net body weight change of pregnant rats administered epoxiconazole

	Epoxiconazole (mg/kg bw/d)					
	Corn oil			1% CMC		
	0	23	50	0	23	50
Groups with treatment: GD 7–18; sacrifice GD 18						
Gravid uterus (g)	25.0	27.6	44.0**	30.6	38.9	37.4
Δ%		+10	+76		+27	+22
Carcass (g)	262.8	235.4**	243.9**	257.2	241.5*	235.9*
Δ%		-10	-7		-6	-8
Net weight change from GD 7 (g)	36.7	23.9**	22.6**	33.5	24.2**	19.4**
Δ%		-35	-38		-28	-42
Groups with treatment: GD 7–21; sacrifice GD 21						
Gravid uterus (g)	75.4	83.3	75.0	75.0	80.0	81.8
Δ%		+10	-1		+7	+9
Carcass (g)	250.7	238.8	231.3**	246.8	235.8	233.0
Δ%		-5	-8		-4	-6
Net weight change from GD 7 (g)	25.3	16.5	10.0**	26.3	14.5	9.4**
Δ%		-35	-60		-45	-64

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

Overall, a clear reduction of the net (corrected) body weight gain was observed at both dose levels and at both sacrifice time points, the extent of maternal toxicity being greater when treatment continued until GD 21, providing evidence that maternal toxicity increased towards the end of pregnancy.

2. Hematology [see Table 2/7]
Treatment of dams with 50 mg/kg bw/d in corn oil from **GD 7–18** resulted in a significant decrease of red blood cell parameters (cell count, hemoglobin, and hematocrit) with concomitant increase in reticulocytes. The same parameters were affected also at 23 mg/kg bw/d but the changes did not reach statistical significance. In rats that were administered epoxiconazole in 1% CMC, the effects on red blood cell parameters were generally less pronounced. Statistically significant changes were confined to the low dose group, i.e., decreases in hemoglobin and hematocrit, the values were within the historical control ranges (hemoglobin: 6.6 – 7.5 mM; hematocrit: 0.302 – 0.323 L/L) and therefore were not regarded as adverse effects. In addition, and apparently not influenced by the vehicle administered, treatment with epoxiconazole resulted in a dose-dependent and statistically significant decrease in platelet counts at both 23 and 50 mg/kg bw/d.

In rats administered epoxiconazole from **GD 7-21** at 23 or 50 mg/kg bw/d, red blood cell (RBC) counts, hemoglobin and hematocrit values were significantly decreased, whereas relative reticulocyte counts were increased. Furthermore, platelet counts were decreased in dams treated with 23 and 50 mg/kg bw/d. The changes were more pronounced in groups that were administered epoxiconazole in corn oil. Total white blood cell counts were dose-dependently increased; the

differential blood cell analysis revealed that the increase was mainly due to an increase of lymphocytes.

Table 2/7 Hematology

Parameter		Epoxiconazole (mg/kg bw/d) in vehicle …					
		Corn oil			1% CMC		
		0	23	50	0	23	50
Treatment: GD 7-18							
White blood cells	[giga/L]	4.65	4.83	4.98	4.79	4.86	5.32
	(% ctrl)		104%	107%		101%	111%
Red blood cells	[tera/L]	6.52	6.25	**5.61****	6.24	5.89	6.11
	(% ctrl)		96%	**86%**		94%	98%
Hemoglobin	[mmol/L]	7.7	7.3	**6.6****	7.4	**7.0****	7.1
	(% ctrl)		95%	**86%**		**95%**	96%
Hematocrit	[L/L]	0.342	0.321	**0.291****	0.327	**0.309****	0.318
	(% ctrl)		94%	**85%**		**94%**	97%
Reticulocytes	[%]	3.6	4.4	**5.6****	3.7	4.5	4.1
	(% ctrl)		122%	**156%**		122%	111%
Platelets	[giga/L]	1081	**892****	**726****	1070	**867***	**725****
	(% ctrl)		**83%**	**67%**		**81%**	**68%**
Treatment: GD 7-21							
White blood cells	[giga/L]	4.06	**4.89****	**5.76***	4.25	**5.04***	**5.89****
	(% ctrl)		**120%**	**142%**		**119%**	**139%**
Red blood cells	[tera/L]	5.98	**5.54****	**4.65****	5.98	**5.47****	**4.37****
	(% ctrl)		**93%**	**78%**		**91%**	**73%**
Hemoglobin	[mmol/L]	7.0	**6.6****	**5.6****	6.8	**6.3****	**5.0****
	(% ctrl)		**94%**	**80%**		**93%**	**74%**
Hematocrit	[L/L]	0.314	**0.286****	**0.244****	0.306	**0.283****	**0.229****
	(% ctrl)		**91%**	**78%**		**92%**	**75%**
Reticulocytes	[%]	2.3	**3.3***	**10.2****	2	**3.8***	**8.8****
	(% ctrl)		**143%**	**443%**		**190%**	**440%**
Platelets	[giga/L]	1041	**824****	**650****	1020	**777****	**615****
	(% ctrl)		**79%**	**62%**		**76%**	**60%**

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis and Wilcoxon, two-sided)

3. Clinical chemistry [see Table 2/8]

In blood analyses performed on **GD 18**, the only clinical chemistry change seen in all dose groups was a decrease in globulin concentrations. The other statistically significant findings were confined to the 50 mg/kg bw/d / corn oil dose group: decreases in total protein, albumin, bilirubin and calcium and an increase in glucose.

On GD 21, the clinical chemistry analyses showed a slight increase of aspartate aminotransferase (AST) activity in rats from all dose groups indicating that liver cell membranes were affected by treatment. Inorganic phosphate concentrations

were also increased over controls in all groups. Total protein, globulin and albumin concentrations were reduced dose-dependently in all treatment groups (not statistically significant in Group 4). Triglycerides and cholesterol were consistently increased at 50 mg/kg bw/d; both of these parameters were also increased at 23 mg/kg bw/d when corn oil was used as vehicle. Triglycerides but not cholesterol was increased upon treatment with 23 mg/kg bw/d epoxiconazole in 1% CMC.. In treatment groups with corn oil administration, bilirubin levels were decreased and glucose concentrations significantly increased by treatment.

Table 2/8 Clinical chemistry (selected parameters)

Dose group			0	1	2	3	4	5
Vehicle			Corn oil			1% CMC		
Dose level (mg/kg bw/d)			0	23	50	0	23	50
AST	[µkat/L]	GD 18	1.45	1.47	1.62	1.31	1.30	1.57
		GD 21	1.51	**1.83****	**2.36****	1.53	**1.86****	**2.57****
Total bilirubin	[µM]	GD 18	1.37	1.18	**0.76***	1.75	**1.23***	1.72
		GD 21	1.10	**0.49****	**0.38****	1.46	1.01	1.04
Total protein	[g/L]	GD 18	63.60	59.11	**50.06****	61.06	59.04	58.52
		GD 21	58.54	**51.69****	**46.41****	57.66	**50.88****	**44.63****
Albumin	[g/L]	GD 18	38.59	36.09	**31.80****	36.59	33.28	36.65
		GD 21	33.20	**29.96****	**28.35****	32.45	29.94	**27.02****
Globulins	[g/L]	GD 18	25.00	**23.02***	**18.27****	24.47	**22.75***	**21.86***
		GD 21	25.34	**21.73****	**18.07****	25.22	**20.94****	**17.61****
Calcium	[mM]	GD 18	2.58	2.51	**2.44****	2.55	2.52	2.61
		GD 21	2.42	2.42	2.42	2.44	2.44	2.40
Glucose	[mM]	GD 18	4.76	5.21	**5.28***	5.70	5.61	5.90
		GD 21	4.41	**4.84****	**5.35****	5.15	4.97	5.32
Inorg. Phosph.	[mM]	GD 18	1.89	1.61	1.82	1.82	1.82	2.04
		GD 21	1.35	**1.59****	**1.82****	1.49	**1.83****	**1.96****
Triglycerides	[mM]	GD 18	4.05	4.15	4.70	3.39	3.99	3.15
		GD 21	2.47	**5.23****	**8.11****	1.34	**4.11****	**5.51****
Cholesterol	[mM]	GD 18	1.71	1.96	1.86	1.70	1.82	1.68
		GD 21	1.96	**2.30****	**2.79****	2.02	2.10	**2.43***

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis and Wilcoxon, two-sided)

A likely explanation for the observed reduction of total serum bilirubin is a substance-related increase in the bilirubin conjugation via enzyme induction, resulting in increased bilirubin excretion. No patho-physiological correlate can be found for the decreased bilirubin levels and therefore this mechanism is regarded as an adaptive and not an adverse effect

The increased glucose values (50 mg/kg bw/d at GD 18 and 23 and 50 mg/kg bw/d at GD 21) may reflect a higher stress of these animals. Further evidence for induction of stress is the observed increase in total white blood cell counts.

The significance of the apparently treatment-related increase of inorganic phosphate in all dosed rats at GD 21 is unclear.

The direct comparison of the absolute control values revealed differences in glucose, bilirubin and triglyceride concentrations, suggesting that the choice of the vehicle itself had an influence on liver metabolism.

Overall, indications of altered liver cell metabolism and first signs of liver damage were observed in treatment group dams of both dose levels. Effects were frequently more pronounced if corn oil was used as vehicle. More and/or stronger effects were seen on GD 21 compared to GD 18.

4. Hormone changes [see Table 2/9]
Hormone analyses **of GD 18** blood samples from corn oil groups showed decreased progesterone and markedly decreased estradiol values at both dose levels (progesterone increase not statistically significant at 23 mg/kg bw/d); in the 1% CMC groups progesterone concentrations were decreased only at 50 mg/kg bw/d, while marked reductions in estradiol concentration were noted at both dose levels. Androstenedione and testosterone levels were higher in the dams of the 50 mg/kg bw/d / corn oil dose group (not statistically significant for testosterone). Androstenedione was also increased in the 1% CMC groups (statistically significant at 23 mg/kg bw/d). Testosterone concentrations were not significantly changed by treatment.

Table 2/9 Hormones

		Dose group	0	1	2	3	4	5
		Vehicle	Corn oil			1% CMC		
		Dose level (mg/kg bw/d)	0	23	50	0	23	50
Progesterone	[nM]	GD 18	405.08	**271.52****	299.76	377.09	347.54	**277.19****
		GD 21	174.69	208.62	245.78	157.80	195.55	206.05
Estradiol	[pM]	GD 18	17.28	**5.59***	**1.21****	26.40	**4.86****	**4.03****
		GD 21	33.46	**0.47****	**0.55****	39.13	**0.56****	**0.55****
Androstenedione	[nM]	GD 18	5.68	6.74	**8.19***	5.94	**8.31****	7.29
		GD 21	5.77	7.29	6.46	4.70	6.70	6.25
Testosterone	[nM]	GD 18	1.11	1.13	1.65	1.06	1.39	1.20
		GD 21	1.22	1.42	1.04	1.09	1.15	1.03

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis and Wilcoxon, two-sided)

The hormone levels in **GD 21** blood samples from control rats showed the changes expected to occur in rat pregnancy shortly before the onset of parturition: compared to GD 18 values the control group estradiol concentrations at GD 21 more than doubled, and progesterone levels on GD 21 were reduced to about half of the progesterone concentrations determined on GD 18. In treatment groups, the maternal progesterone levels were slightly above control group values but the differences did not attain statistical significance. The estradiol concentrations on GD 21 however almost approached zero and were thus further reduced compared to the already low estradiol values measured in GD 18 samples. Slightly increased

androstenedione concentrations were observed at both 23 and 50 mg/kg bw/d (not statistically significant), while testosterone concentrations were comparable to control group values.

4. Gross necropsy observations

No gross necropsy changes were noted in dams at Caesarian section.

5. Organ weights [see Table 2/10]

Table 2/10 Organ weight

Dose group		0	1	2	3	4	5
Vehicle		Corn oil			1% CMC		
Dose level (mg/kg bw/d)		0	23	50	0	23	50
Terminal body wt [g]	GD 18	256	**232** [91%]	**241*** [94%]	252	**239*** [95%]	**237*** [94%]
	GD 21	248	**237*** [96%]	**232**** [94%]	242	232.97 [96%]	235.32 [97%]
Abs. liver wt [g]	GD 18	10.36	9.185 [89%]	10.60 [102%]	10.21	9.67 [95%]	9.33 [91%]
	GD 21	9.40	10.01 [107%]	**10.88**** [116%]	8.90	9.26 [104%]	**10.54**** [118%]
Rel. liver wt [%]	GD 18	4.02	3.95 [98%]	4.40 [110%]	4.03	4.03 [100%]	3.93 [97%]
	GD 21	3.77	**4.20**** [111%]	**4.69**** [124%]	3.65	3.95 [108%]	**4.49**** [123%]
Abs. Adrenal gland wt [mg]	GD 18	75.25	74.83 [99%]	**84.17*** [112%]	84.67	82.25 [97%]	80.67 [95%]
	GD 21	72.04	**78.44*** [109%]	**79.74*** [111%]	74.17	80.44 [108%]	**86.00**** [116%]
Rel. Adrenal gland wt [%]	GD 18	0.029	0.032 [110%]	**0.035**** [119%]	0.034	0.034 [102%]	0.034 [101%]
	GD 21	0.029	**0.033**** [114%]	**0.034**** [118%]	0.031	**0.035*** [113%]	**0.037**** [121%]
Abs. ovary wt [mg]	GD 18	101.17	100.58 [99%]	111.92 [111%]	110.75	102.42 [92%]	111.33 [101%]
	GD 21	113.17	111.83 [99%]	110.17 [97%]	111.39	109.48 [98%]	116.04 [104%]
Rel. ovary wt [%]	GD 18	0.040	0.043 [110%]	**0.047**** [118%]	0.044	0.043 [98%]	0.047 [107%]
	GD 21	0.046	0.047 [103%]	0.048 [104%]	0.046	0.047 [102%]	0.049 [107%]

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis H and Wilcoxon, two-sided)

Liver weights were significantly increased in dams killed on GD21 at 50 mg/kg bw/d with relative weight increases exceeding 20%. Slight liver weight increases attained statistical significance also in the low-dose group administered epoxiconazole with corn oil [+11%]. No treatment-related liver weight changes were noted in dams killed on GD 18.

Adrenal weights were slightly increased in GD 18 dams at 50 mg/kg bw/d [+12%, corn-oil group only] and dose-dependently increased in GD 21 dams at both 23 and 50 mg/kg bw/d [up to +21%].

No clear effects of treatment were noted with regard to ovary weights.

E. CESAREAN SECTION DATA [see Table 2/11]

The pregnancy rate of all groups was sufficient to allow meaningful evaluation at both termination time points. When compared with the control group, the post-implantation loss was increased in dams receiving epoxiconazole at 50 mg/kg bw/d. The post-implantation loss resulted from an increased incidence of late fetal resorptions, while early fetal resorptions were not increased by treatment. This increase in late fetal resorptions was statistically significant at both time points and with both vehicles and considered to be related to treatment.

The sex distribution of the fetuses in all treated groups (23 and 50 mg/kg bw/d) was comparable to the control group. Observable differences were without biological relevance.

The mean fetal weights (determined only on GD 21) did not show any biologically relevant differences between the test substance-treated groups and the control. The observable differences between the groups reflect the usual fluctuation for this parameter.

Table 2/11 Summary of reproduction toxicity data

Treatment duration:	GD 7–18						GD 7–21					
Group:	0	1	2	3	4	5	0	1	2	3	4	5
Vehicle:	Corn oil			1% CMC			Corn oil			1% CMC		
Epoxiconazole [mg/kg bw/d]:	0	23	50	0	23	50	0	23	50	0	23	50
Females mated	12	12	12	12	12	12	23	23	23	23	23	23
Dams pregnant	10	8	9	10	10	7	20	19	21	17	16	19
Dams with viable fetuses	10	7	9	10	9	6	20	19	20	17	16	18
Dams with all resorptions	0	1	0	0	1	1	0	0	1	0	0	1
Corpora lutea	13.0	11.5	13.9	13.6	12.8	12.6	14.1	13.8	13.1	13.9	14.2	14.5
Implantation sites	8.9	8.6	12.9	11.2	11.8	10.0	12.3	11.8	11.9	12.2	11.9	13.4
Pre-implantation loss	32.1	31.2	7.2	17.9	12.0	24.1	13.1	14.0	9.4	12.8	17.0	7.2
Post-implantation loss	9.8	27.5	39.7*	10.0	16.9	34.8*	8.0	9.1	42.1**	6.2	10.9	32.3**
Resorptions, total	0.9	1.1	4.9**	1.3	1.0	2.4	0.8	1.1	4.9**	0.8	1.3	4.4**
Resorptions, early	0.9	1.0	0.3	1.3	0.7	0.3	0.7	0.4	0.7	0.8	0.7	0.6
Resorptions, late	0.0	0.1	4.6***	0.0	0.3	2.1**	0.1	0.6	4.1**	0.1	0.6	3.8**
Dead fetuses	0	0	0	0	0	0	0	0	0	0	0	0
Live fetuses	8.0	8.6	8.0	9.9	12.0	8.8	11.5	10.8	7.4**	11.4	10.6	9.5
Fetal weight [g]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4.7	5.0	4.7	4.9	5.0	4.6

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, two-sided)

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. External examination [see Table 2/12 and Table 2/13]

Fetal external malformations

External malformations were seen in all dose groups including controls. In fetuses from dams that were treated from **GD 7-18**, anarsarca was observed in one fetus in the high-dose / corn oil group and in two fetuses from one litter of the high-dose / 1% CMC group. Anarsaca is present in the historical control data and is often associated with maternal stress. This finding was not observed in fetuses from any animal treated from GD 7–21. Single fetuses were observed with open eye (control or low dose) or short tail (control).

Among the groups administered the test substance from **GD 7–21**, one fetus from the low dose / 1% CMC showed agnathia. In addition, malrotated limb was diagnosed in 1 fetus from the high-dose group with corn oil, in 1 fetus from the low-dose group with 1% CMC and in 2 fetuses from 1 high-dose litter with 1% CMC. Since malrotated limbs are a common finding in this rat strain and dose-response relationship is missing, these findings were considered to be spontaneous in nature and without a relation to dosing. The overall incidences of external malformations as listed in Table 2/13 did not demonstrate a dose-response relationship.

Table 2/12 Individual fetal external malformations

	GD 7-18						GD 7-21					
	Corn oil			1% CMC			Corn oil			1% CMC		
Dose [mg/kg bw/d]	0	23	50	0	23	50	0	23	50	0	23	50
Agnathia											1	
Anarsarca			1			3 / 2[$]						
Malrotated limb									1		1	2 / 1[$$]
Open eye	1				1							
Short tail	1											

[$] 3 fetuses from 2 litters; [$$] 2 fetuses from 1 litter

Table 2/13 Total external malformations

Parameter		Epoxiconazole (mg/kg bw/d) in vehicle …					
		Corn oil			1% CMC		
		0	23	50	0	23	50
Treatment: GD 7-18							
Litter	N	10	7	9	10	9	6
Fetuses	N	80	60	72	99	108	53
Fetal incidence	N	2	0	1	0	1	3
Litter incidence	N	2	0	1	0	1	2
Affected fetuses / litter	Mean%	2.8	0.0	0.9	0.0	0.9	4.9
Treatment: GD 7-21							
Litter	N	20	19	20	17	16	18
Fetuses	N	230	205	148	193	170	171
Fetal incidence	N	0	0	1	0	2	2
Litter incidence	N	0	0	1	0	2	1
Affected fetuses / litter	Mean%	0.0	0.0	0.4	0.0	1.3	1.1

Fetal external variations

External variations were not observed in this study.

Fetal external unclassified observations [see Table 2/14]

In fetuses from rats treated during **GD 7–18**, blood coagulum around placenta was recorded for single fetuses of test groups 1, 2, 3 and 5 (23 and 50 mg/kg bw/d). This is a common finding in this rat strain and a relation to dosing is not present [see Table 2/14] if normal biological variation is taken into account. Therefore, a test substance-induced effect is not assumed.

In fetuses from rats treated during **GD 7–21,** blood coagulum around placenta was recorded in fetuses from single litters. In addition, fetuses from the high-dose groups were found with necrobiotic placenta. The increase in the mean percentage of affected fetuses per litter was statistically significant. Since the placenta is a potential target of the test compound, thorough histopathological examinations of these organs were conducted.

Table 2/14 Total external unclassified observations

Test group		0	1	2	3	4	5
Vehicle		Corn oil			1% CMC		
Epoxiconazole [mg/kg bw/d]		0	23	50	0	23	50
Treatment: GD 7-18							
Litter	N	10	7	9	10	9	6
Fetuses	N	80	60	72	99	108	53
Blood coagulum around placenta							
Fetal incidence	N	0	1	1	2	0	2
Litter incidence	N	0	1	1	1	0	2
Affected fetuses / litter	Mean%	0.0	1.3	1.0	5.0	0.0	5.4
Total unclassified observations							
Fetal incidence	N	0	1	1	2	0	2
Litter incidence	N	0	1	1	1	0	2
Affected fetuses / litter	Mean%	0.0	1.3	1.0	5.0	0.0	5.4
Treatment: GD 7-21							
Litter	N	20	19	20	17	16	18
Fetuses	N	230	205	148	193	170	171
Blood coagulum around placenta							
Fetal incidence	N	0	1	4	0	0	2
Litter incidence	N	0	1	1	0	0	1
Affected fetuses / litter	Mean%	0.0	0.6	2.2	0.0	0.0	1.1
Placenta necrobiotic							
Fetal incidence	N	0	0	8	0	0	9
Litter incidence	N	0	0	3	0	0	3
Affected fetuses / litter	Mean%	0.0	0.0	6.4*	0.0	0.0	5.6*
Total unclassified observations							
Fetal incidence	N	0	1	12	0	0	11
Litter incidence	N	0	1	4	0	0	3
Affected fetuses / litter	Mean%	0.0	0.6	8.6*	0.0	0.0	6.7*

* $p \leq 0.05$ (Wilcoxon-test, one-sided))

G. HISTOPATHOLOGY OF THE PLACENTA

COMPARISON OF CONTROL PLACENTAS SAMPLED GD 18 AND GD 21

Labyrinth: no relevant differences between GD 18 and GD 21

Trophospongium: At GD 21, glycogen trophoblast cells were reduced in number compared to GD 18, or glycogen trophoblast cells showed liquefactive change. The reduction in glycogen trophoblast cells resulted in a slight reduction in trophospongium thickness

Decidua: On GD 21 apparent reduction of metrial and glycogen cells. Lacunes of liquefied clusters of glycogen trophoblast cells located around the uteroplacental area in the stromal decidua were frequently observed (not seen on GD 18).

FINDINGS IN PLACENTAS FROM TREATMENT GROUPS SAMPLED ON GD 18 AND GD 21

Histopathology revealed a treatment-related and dose-dependent degeneration overall in placentas of treated dams on GD 18 and GD 21. No relevant differences were noted between the corn oil and the 1% CMC test groups. Placental degeneration was characterized by cystic dilation of maternal sinuses and rupture of interhemal membrane in the labyrinth with concomitant changes in the trophospongium, such as thickening, congestion and necrosis. These changes were observed in placentas with live fetuses as well as in placentas with late resorptions. The main difference lies in the severity of the degeneration: placentas with live fetuses exhibited a slight to moderate degeneration, while placentas with late resorptions displayed a severe to massive degeneration.

On GD 18 dose-dependent degenerative changes were noted, increasing from slight at 23 mg/kg bw/d to moderate/severe at 50 mg/kg bw/d. This finding was associated with a dose-dependent increase of degeneration in placentas with live fetus (from slight at 23 mg/kg bw/d to moderate at 50 mg/kg bw/d) and with an increase in the number of placentas with late resorption at 50 mg/kg bw/d.

On GD 21, all placentas displayed a dose-dependent increase in degenerative changes from moderate at 23 mg/kg bw/d to moderate/severe at 50 mg/kg bw/d. Other than on GD 18, degeneration in placentas with live fetuses did not increase dose-dependently and was moderate at 23 and 50 mg/kg bw/d. Therefore, the overall increase in the severity of degenerative findings in placentas on GD 21 was attributed to the increased number of placentas with late resorptions at 50 mg/kg bw/d.

III. SUMMARY AND CONCLUSIONS

The aim of this modified prenatal developmental toxicity study in rats was to …

a) verify results of investigatons reported in publications by Taxvig et al (2007, 2008)
b) clarify the extent of maternal toxicity under the study conditions
c) examine hormone changes (progesterone, estradiol, testosterone and androstenedione)
d) study the impact of vehicle and of treatment duration / sacrifice timepoint on fetal findings
e) investigate the relationship between fetal toxicity and potential effects on the placenta

Epoxiconazole was administered as a suspension in corn oil or as a suspension in 1% Carboxymethylcellulose suspension in highly deionized water (1% CMC) to 35 paired female Wistar rats per group by gavage at doses of 23 and 50 mg/kg body weight or with the vehicle only. Twelve of the females in each group were administered the test compound from GD 7 through GD 18. The other groups each consisting of twenty-three mated females were treated from GD 7 through 21.

Maternal toxicity examinations comprised regular recording of food consumption, body weight and health status, blood sampling for clinical chemistry, hematological and hormone investigations on the day of sacrifice, determination of corrected body weight gain, and weights of liver, adrenal gland and ovaries and gross necroscopy. Following weight determination of the unopened uterus a photograph of the uterus and its contents was taken. For each dam, corpora lutea were counted and number and distribution of implantation sites (differentiated by resorptions, live and dead fetuses) were determined. The fetuses were removed from the uterus, sexed and investigated for external findings. The uterus with placental sites and decidua were processed to allow for histopathological examination of the placentas.

The treatment-related increase of late fetal resorptions observed in this study at the dose of 50 mg/kg bw/d was consistent with the data reported by Taxvig et al in 2007. However, in-depth clinical and clinical pathological examinations, including determination of serum levels of major sexual hormones, showed that these resorptions occurred in the presence of distinct maternal toxicity. Maternal toxicity was dose-dependently observed at 23 and 50 mg/kg bw/d, comprising significant reductions in food consumption, corrected body weight gain, approx. 22 - 27% reduction of red blood cells, 4-5 fold increase of reticulocytes at 50 mg/kg bw/d (indicating a clear anemia). Maternal serum estradiol levels were markedly decreased. In the 23 mg/kg bw/d dose group, serum estradiol levels were very low but still appreciable on GD 18 and were almost completely suppressed on GD 21, whereas in the 50 mg/kg bw/d dose group an almost complete suppression of serum estradiol was noted both on GD 18 and 21. Although some clinical pathology alterations appeared to be more pronounced in the corn oil groups than in the carboxymethylcellulose groups, no essential difference between vehicles was noted when all effects were assessed at large. No treatment-related increase of craniofacial malformations (i.e., cleft palates) was observed in this study at any dose level. No substance-related fetal effects were noted at 23 mg/kg bw/d.

Histopathological examination of the placentas revealed treatment-related degenerative findings in the labyrinth and trophospongium of placentas in almost all treated animals, independently of receiving corn oil or 1% CMC as vehicle. The degenerative changes were dose-dependent and associated with an enlargement of the placentas. Both, placentas with live fetuses and with late resorptions were affected, with the highest degree of severity in placentas with late resorptions. At 50 mg/kg bw/d on GD 18 or GD 21, a relevant increase in the number of late resorptions was observed. Degenerative changes in the labyrinth led to a loss of feto/maternal blood compartmentalization, which appeared to be critical if more than 70% of the labyrinth was affected (massive degeneration), being associated with an increased number of fetal deaths at 50 mg/kg bw/d.
The results of the study indicate that the late fetal resorptions are not caused by an independent adverse effect of the test substance on the development of the fetus, but because mothers are unable to nourish their progeny properly ultimately resulting from the loss of placental function. In this experiment, two conditions had to concur to cause late fetal resorptions: a) degenerative damage to the placenta affecting at least 70% of its area and b) the fetus must have developed to a certain size and its demand of nutrients exceeded the supply that the mother

could provide through the damaged placenta. A relationship of the placenta degeneration to the marked estradiol deficiency seen in this study is highly likely, and was therefore further investigated in a separate mechanistic study.

STUDY RELEVANCE

The study is relevant for assessing the appropriate classification and labeling of epoxiconazole. The current RAC opinion for classification of epoxiconazole for developmental toxicity in Category1B is based on two findings from rat studies, (1) an increased incidence of late fetal resorptions without reported maternal toxicity (Taxvig et al 2007, 2008) and (2) a high incidence of cleft palates observed at an excessive dose level in a range-finding study in the presence of marked maternal toxicity plus isolated occurrences of cleft palate in single fetuses at lower dose levels from other studies for which a treatment-relationship was not excluded (BASF unpublished studies). This study provides relevant new information for both issues of concern. No cases of cleft palate were observed in the study up to the highest dose level tested (50 mg/kg bw/d). With regard to late fetal resorptions, it is important to note that in the publications by Taxvig et al., the reported information did not allow to adequately evaluate the extent of maternal toxicity. In addition questions remained regarding the reliability of the results reported by as a result of several shortcomings (e.g. low animal group size used, poor reporting of experimental design, of material and methods and of results, inconsistent findings in hormone investigations, unclear impact of corn oil used as vehicle for dose administration).

The new study data clarifies these issues, provides important information on the extent of maternal toxicity and gives a first indication on the potential mechanism for the occurrence of late fetal resorptions. The study was performed under GLP conditions using an experimental design with sufficient animal group sizes that are adequate for drawing meaningful conclusions on the extent of maternal and fetal toxicity under the study conditions.

While the study confirms the statistically significantly increased occurrence of late fetal resorptions when administered to pregnant rats at a dose level of 50 mg/kg bw/d as reported by Taxvig et al., the new study data clearly demonstrates that these late fetal resorptions occurred in the presence of significant maternal toxicity (clear reduction of corrected body weight, signs of anemia, marked reduction of maternal estradiol plasma levels and significant damage of the placenta). The RAC opinion on classification of epoxiconazole from 2010, which supported a Cat. 1B (CLP) classification and a Repr. Cat. 2 / R61 classification (Dangerous Substance Directive), relied on information that claimed the absence of significant maternal toxicity at dose levels causing late fetal resorptions. Therefore, a classification of epoxiconazole in Cat. 2 (CLP) and Repr. Cat. 3 / R63 (DSD) for the finding of late fetal resorptions may be considered based on these study results. However, by taking into account all available data in an overall weight-of-evidence approach, no classification is required for this rat finding (see chapters 3 and 4).

2.1.2 Prenatal developmental toxicity study in rats II

STUDY REFERENCE

Report:	Schneider S., Strauss V., Fabian E., van Ravenzwaay B. 2010b BAS 480 F (Epoxiconazole) Modified Prenatal Developmental Toxicity Study in Wistar Rats Oral Administration (Gavage) - Subcutaneous Co-administration of Estradiol cyclopentylpropionate BASF DocID 2010/1062088 Date of report: 10-May-2010
Report:	Schneider S., Rey Moreno M., Fabian E. 2011 Amendment No. 1 to the report: BAS 480 F (Epoxiconazole) Modified Prenatal Developmental Toxicity Study in Wistar Rats Oral Administration (Gavage) - Subcutaneous Co-administration of estradiol cyclopentylpropionate BASF DocID 2011/1229836 Date of report: 09-Sep-2011
Report:	Stinchcombe S., Schneider S, Fegert I., Fussel K.C., Rey Moreno M.C,. Strauss V., Gröters S., Fabian E., Richter M., Piggot G., van Ravenzwaay B (2012) Effects of estrogen co-administration on epoxiconazole toxicity (Publication in preparation)
Report:	Rey Moreno M.C., Schneider S, Stinchcombe S., Fegert I., Fussel K.C., Strauss V., Gröters S., Fabian E., Richter M., Piggot G., van Ravenzwaay B (2012) Epoxiconazole-induced degeneration in rat placenta and the effects of estradiol supplementation (Publication in preparation)
Guidelines:	There are no guidelines for this mechanistic study. Reference was made to: (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.31 No. L 142; OECD 414; OPPTS 870.3700
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz)

Note: The amendment to the study report includes the results from pathological and histopathological examinations of the study

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1a. Test Material	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	8563
Purity:	97.0%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 30-Jun-2010.
1b. Test Material	Estradiol cyclopentylpropionate (ECP)
Description:	Solid, white with a yellow cast
Lot/Batch #:	049H1244
Purity:	99.8%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 04-Feb-2013.
2. Vehicle control:	Corn oil
3. Test animals:	
Species:	Rat
Strain:	Wistar [Crl:WI (Han)]
Sex:	Female
Age:	Sexually mature, virgin rats aged 8-10 and 10-12 weeks
Weight at dosing (GD 7):	218.8 ± 13.5 g (195.8 to 257.4)
Source:	Charles River Lab., Germany
Acclimatization period:	at least 1 day before mating and at least 7 days before start of test substance administration
Diet:	Kliba maintenance diet for mouse/rats "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type M III Makrolon cages (Becker & Co, Castrop-Rauxel, Germany), floor area about 800 cm² with

	Lignocel dustfree bedding (SSNIFF, Soest, Germany) and wooden gnawing blocks (Abedd Lab. and Vet. Service GmbH, Vienna) offered for enrichment
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work: 12-Feb-2010 to 20-Jul-2011 (in-life dates: 19-Feb-2010 (Start of treatment at gestation day (GD) 7) to 10-Mar-2010 (Sacrifice of the 4th cohort)

2. Animal assignment and treatment:

After an acclimatization period of at least one day, untreated animals were paired at the test facility from about 15:30 until 7:30 in the morning of the following day. When sperm were microscopically detected in the vaginal smear, the females were considered being impregnated and transferred into the study. This day was referred to as gestation day 0 (GD 0), the following day as GD 1. Epoxiconazole in corn oil suspension or corn oil only were administered once daily to groups of 18 sperm-positive females animals by oral gavage from GD 7 to 21. The volume administered each day was 2 mL/kg body weight. The calculation of the administration volume was based on the most recent individual body weight. The estradiol cyclopentylpropionate (ECP) formulations were administered at dose levels of 0 (corn oil), 0.5 or 1 µg/animal by subcutaneous injection, once a day from GD 7 to 21 in parallel to the epoxiconazole treatment. The volume administered each day was 0.2 mL/animal. At terminal sacrifice on GD 21, eleven to seventeen females per group had implantation sites.

Table 2/15 Test groups and doses

Test group	Dose epoxiconazole (mg/kg bw/d)	Concentration epoxiconazole (mg/100 mL)	Volume (mL / kg bw)	Dose ECP (µg/animal/ d)	Volume (mL / animal)	No. of mated animals	No. of pregnant animals
0	0 (corn oil)	0	2	0 (corn oil)	0.2	18	11
1	0 (corn oil)	0	2	0.5	0.2	18	15
2	0 (corn oil)	0	2	1	0.2	18	17
3	50	2500	2	0 (corn oil)	0.2	18	14
4	50	2500	2	0.5	0.2	18	16
5	50	2500	2	1	0.2	18	16

3. Test substance preparation and analysis:

Dose formulations of epoxiconazole in corn oil were prepared at the beginning of the administration period and thereafter at intervals of 7 days, which took into account the period of established stability. Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in corn oil using a high-speed homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals. The administered ECP dose formulations were prepared by dilution of an ECP / corn oil stock solution.

Epoxiconazole concentration analyses were performed twice at the beginning and towards the end of the study. The homogeneity of the 50 mg/kg bw/d dose suspension was verified at the beginning of the study by taking 3 samples from the top, middle and bottom of the beaker while a magnetic stirrer was running. The results of these analyses are given in the table below. Also ECP concentration control analyses were performed twice during the study period. Since ECP was completely miscible with corn oil, the solutions were considered to be homogenous without further analysis.

Relative standard deviations of maximum 2.1% indicated the homogenous distribution of epoxiconazole in the dosing suspensions. The actual nominal test-item concentrations were in the range of 97.4 to 101.4% of the target nominal concentrations and thus in the acceptable range.

Analysis of preparations for homogeneity and content of epoxiconazole					
Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± SD
Corn oil	18.02.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		2.5	2.465	98.6	99.1 ± 2.1
			2.435	97.4	
			2.536	101.4	
	04.03.2010 [Concentration control analyses]	0	n.d.	---	
		2.5	2.444	97.8	

Analysis of preparations for content of estradiol cyclopentylpropionate				
Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]	% of nominal concentration
Corn oil	18.02.2010	0.5	0.4428	88.6
		1.0	1.040	104.0
	10.03.2010	0.5	0.5528	104.6
		1.0	0.9408	94.1

4. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption [a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of post-implantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal proportions

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

Statistics for pathology and clinical pathology	
Parameter	**Statistical test**
Hematology, clinical chemistry	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
Organ weight parameters	Pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
Mean grade of the degeneration of the placental labyrinth and trophospongium per dam	Pair wise comparison of groups 3,4 and 5 with the control group was performed using the WILCOXON test (one-sided) for the hypothesis of equal medians.
Difference in degeneration of placentas belonging to live fetuses and late resorptions per dam	For groups 3 and 4 the WILCOXON signed-rank test (one-sided) was performed

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

2. Body weight and food consumption

All animals were weighed on GD 0, 1, 4, and daily on GD 7-21. The body weight change of the animals was calculated from these results.

In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 21 minus weight of the unopened uterus minus body weight on GD 7).

Food consumption was determined on GD 1, 4, 7, 10, 13, 15, 18 and 21.

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on GD 21 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Hematology and clinical chemistry

On the morning of sacrifice on GD 21, blood was drawn from non-fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.

The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	White blood cell count (WBC)	✓	Platelet count (PLT)
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)		
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells (differential)		

Clinical chemistry:					
	Electrolytes		*Metabolites and Proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
		✓	Cholesterol	✓	Alkaline phosphatase (ALP)
		✓	Creatinine	✓	γ-glutamyl transferase (γ-GT)
		✓	Globulin (by calculation)		
		✓	Glucose		*Hormones:*
		✓	Protein (total)	✓	Progesterone (PROG)
		✓	Triglycerides	✓	Androstenedione (ANROS)
		✓	Urea	✓	Estradiol (E2)
				✓	Testosterone (TESTO)

4. Sacrifice

On GD 21, the dams were sacrificed after blood sampling in randomized order by cervical dislocation (after isoflurane anesthesia) and the fetuses were removed from the uterus. Dams were subsequently assessed by gross pathology with special attention to the placentas. The following organs from all dams sacrificed on schedule were weighed and fixed in neutral buffered 4% formaldehyde solution:

- Adrenal glands
- Liver (one slice each of the lobus dexter medialis and of the lobus sinister lateralis were fixed in Carnoy's solution and embedded in paraplast)
- Ovaries

Additionally, from all dams sacrificed on schedule, the …:

- Uterus including placental sites and decidua

… was fixed in neutral buffered 4% formaldehyde solution, and after a 24-48h fixation period, transferred to 70% alcohol solution for asservation.

The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Photograph of the unopened uterus with all contents in situ (CD with all photographs in the raw data archive)
- Number of corpora lutea

- Number and distribution of implantation sites classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to SALEWSKI in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue)
    c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]: } \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]: } \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]: } \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

5. Histopathology of placentas

All placentas from animals of control group 0 and of epoxiconazole groups 3, 4 and 5 (with 0, 0.5 or 1 µg ECP co-treatment, respectively) were histopathologically examined. In case of the control groups 1 and 2 (with ECP treatment: 0.5 or 1 µg ECP/rat), two placentas per dam were subjected to histopathological examination. Beside of H&E staining, sections from single animals were specially stained for detection of glycogen, calcium/phosphate carbonate, for collagen and elastic fibers or for ferric iron (Fe3+). The degree of placental degeneration was assessed according to the grading system described previously.

6. Examination of fetuses

When the uterus was opened the viability of the fetuses and the condition of placentae, umbilical cords, fetal membranes, and fluids were carefully examined in situ. After dissection from the uterus each fetus was sexed and external tissues and all orifices were examined macroscopically. The fetuses were weighed. The sex was determined by observing the distance between the anus and the base of the genital tubercle. Thereafter, the fetuses were sacrificed by subcutaneous injection of pentobarbital (Narcoren®; dose: 0.1 mL per fetus) and discarded.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al. (1997) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al. (Chahoud et al. 1999; Solecki et al. 2001 and 2003):

Malformation	A permanent structural change that is likely to adversely affect the survival or health
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Note: Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on day 21 p.c. were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

For the above reasons the following females were excluded from the above-mentioned calculations:

Epoxiconazole dose	0 µg ECP/rat	0.5 µg ECP/rat	1.0 µg ECP/rat	Comments
0 mg/kg bw/d	004, 005, 006, 007, 009, 010, 017	026, 032, 034	046	Not pregnant
50 mg/kg bw/d	067, 069, 071, 072	073, 078	097, 102	Not pregnant
	057			Sacrificed moribund

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

There were no test-substance-related mortalities in any of the groups. One female from test group 3 (No. 057) was killed in a moribund state on GD 9; no association with the test substance was assumed for the health condition of this animal.

2. Clinical signs of toxicity [see Table 2/3]

Clinical findings were exclusively observed in the epoxiconazole-treated (50 mg/kg bw/d) groups 3, 4 and 5, independent of the ECP co-treatment. No clinical findings were seen in the groups not exposed to epoxiconazole but administered with ECP only (groups 1 and 2, 0.5 and 1 µg/animal/d ECP).

Vaginal hemorrhage was noted in all epoxiconazole-treated animals, namely in four animals of dose group 5 (50 mg/kg bw/d + 1 µg/animal/d ECP), six animals of dose group 4 (50 mg/kg bw/d + 0.5 µg/animal/d ECP) and eight animals of dose group 3 (50 mg/kg bw/d + no ECP). The symptom was observed towards the end of pregnancy, i.e. from GD 17 onwards. Piloerection was noted in four animals of dose group 5, in one animal of dose group 4 and in four animals of dose group 3, immediately before term. All animals of dose group 5 and twelve animals of dose group 4 and 3 showed transient salivation after treatment. Salivation occurred from GD 9 onwards and persisted in the respective females for a few minutes immediately after each administration.

Table 2/16 Clinical findings

	Epoxiconazole (mg/kg bw/d)	
	0 (corn oil)	**50**
0 µg ECP/animal	GROUP 0 (11x pregnant) 0x transient salivation 0x vaginal hemorrhage 0x piloerection	GROUP 3 (14x pregnant) 12x transient salivation (GD 9–21) 8x vaginal hemorrhage (GD 18–21) 4x piloerection (GD 20–21) 1x poor general state (GD 8-9)*
0.5 µg ECP/animal	GROUP 1 (15x pregnant) 0x transient salivation 0x vaginal hemorrhage 0x piloerection	GROUP 4 (16x pregnant) 12x transient salivation (GD 9–21) 6x vaginal hemorrhage (GD 17–21) 1x piloerection (GD 21)
1.0 µg ECP/animal	GROUP 2 (17x pregnant) 0x transient salivation 0x vaginal hemorrhage 0x piloerection	GROUP 5 (16x pregnant) 18x transient salivation (GD 10–21) 4x vaginal hemorrhage (GD 20–21) 4x piloerection (GD 20–21)

* Group 3 rat no. 057 was sacrificed moribund on GD 9 after showing poor general state, piloerection, nasal red crust formation, accelerated respiration.

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption [see Table 2/4]

The mean food consumption of the epoxiconazole-treated dams (50 mg/kg bw/d) was reduced during major parts of the treatment period, the decrease becoming statistically significant on GD 15 –21 in test group 3 (no ECP, up to 32% below the concurrent control) and on GD 18 – 21 in test groups 4 an 5 (0.5 and 1 µg/animal/d ECP, about 22 - 23% below the concurrent control). No changes of food consumption were seen in the groups not exposed to epoxiconazole but administered with ECP only (groups 1 and 2).

Table 2/17 Food consumption

Parameter: mean food intake (g/animal)	0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
GD 7 -10	18.5	20.0	19.5	17.6	17.5	18.0
Δ%		+8.1	+5.4	-4.9	-5.4	-2.7
GD 10 -13	20.6	21.0	20.8	19.3	19.2	19.7
Δ%		+1.9	+1.0	-6.3	-6.8	4.4
GD 13 -15	20.3	21.1	21.1	19.4	20.0	20.4
Δ%		+3.9	+3.9	-4.4	-1.5	0.5
GD 15 -18	21.3	21.4	21.7	**18.3****	20.3	20.7
Δ%		+0.5	+1.9	**-14.1**	-4.7	-2.8
GD 18 - 21	20.9	20.4	21.2	**14.2****	**16.4***	**16.1***
Δ%		-2.4	+1.4	**-32.1**	**-21.5**	**-23.0**

* p < 0.05; ** p < 0.01 (Dunnett-Test, two-sided)

2. Body weight and body weight gain [see Table 2/5 and Figure 2/3]
Mean body weights/body weight gain of epoxiconazole-exposed animals (50 mg/kg bw/d) with ECP co-treatment (groups 4 and 5, 0.5 and 1 µg/animal/d ECP) were comparable to the concurrent control group, as were body weights/body weight gain in the groups administered with ECP only (groups 1 and 2). All differences observed in these groups during the treatment period were without biological relevance and reflected the normal variation inherent in the strain of rats used in the present experiment.

Animals of dose group 3, treated with 50 mg/kg bw/d epoxiconazole alone, had decreased body weights on GD 21 (about 9% below the concurrent control value) and the body weight gain was statistically significantly reduced on GD 8–9 (about 82% below control) and on GD 19–21 (60–130% below control). The average decrease of body weight gain during the treatment period (GD 7 – 21) was 29% in this dose group.

Figure 2/3 Body weight change

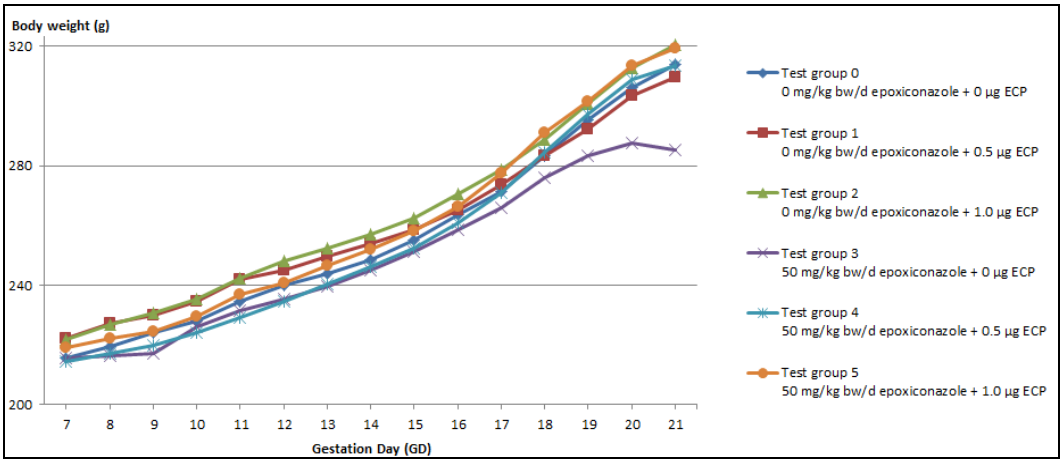


Table 2/18 Bodyweight change

Parameter: bw change (g/animal)	0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
GD 8 - 9	4.5	2.9	3.8	**0.8***	2.4	2.3
Δ%		-36	-16	**-82**	-47	-49
GD 19 - 20	11.0	11.1	12.0	**4.4***	11.8	11.9
Δ%		+1	+9	**-60**	+7	+8
GD 20 - 21	7.7	6.2	7.9	**-2.3****	4.4	5.8
Δ%		-19	+3	**-130**	-43	-25
GD 0 -7	27.3	33.1*	29.8	33.7*	29.8	31.2
Δ%		+21	+9	+23	+9	+14
GD 7 -21	98.5	87.3	98.7	**69.6***	99.0	100.1
Δ%		-11	0	**-29**	+1	+2
GD 0 -21	125.8	120.4	128.5	103.5	128.8	131.3
Δ%		-4	2	-18	+2	+4

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

Overall, it appears that body weight gain of dams was reduced by treatment especially during late pregnancy. No clear effect of vehicle could be made out. The observed reduction of body weight gain is the result of maternal and not of fetal toxicity as can be seen by evaluation of the corrected maternal body weight gain and weights of unopened uteri (see section D below).

D. NECROPSY OBSERVATIONS

1. Corrected (net) body weight gain [Table 2/19]

Mean carcass weights and also gravid uterus weights in all test groups were comparable to control. The corrected (net) body weight gain (terminal body weight on GD 21 minus weight of the unopened uterus minus body weight on GD 7) was decreased in the epoxiconazole-treated dams (50 mg/kg bw/d), independent of the ECP co-treatment. The reduction was about 46% below control in test group 5 (1 µg/animal/d ECP), about 42% below control in test group 4 (0.5 µg/animal/d ECP) and about 58% below control in test group 3 (no ECP). No changes of corrected (net) body weight gain were seen in the groups not exposed to epoxiconazole but administered with ECP only (groups 1 and 2, 0.5 and 1 µg/animal/d ECP).

Table 2/19 Mean gravid uterus weights and net body weight change of pregnant rats administered epoxiconazole

Parameter: bw change (g/animal)	0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
Gravid uterus (g)	73.3	61.5	70.0	58.9	84.2	86.5
Δ%		-16	-5	-20	15	+18
Carcass (g)	240.8	248.2	250.7	226.4	229.3	232.9
Δ%		+3	+4	-6	-5	-3
Net weight change from GD 7 (g)	25.3	25.8	28.7	10.7	14.8	13.6
Δ%		+2	+13	-58%	-42%	-46%

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

2. Hematology (see Table 2/20)

In rats administered epoxiconazole without ECP, red blood cell (RBC) counts, hemoglobin and hematocrit values were significantly decreased, whereas relative reticulocyte counts were increased. In rats additionally receiving ECP, the decreases in red blood cell parameters were less pronounced but still statistically significant. However, the reticulocyte counts were no longer increased in groups 4 and 5. Total white blood cell counts were significantly increased in dams administered epoxiconazole without ECP; this increase was mainly due to increased lymphocyte counts. Platelet counts were significantly decreased in dams treated with 50 mg/kg bw/d epoxiconazole; with ECP, the decrease was less severe but still significant.

Table 2/20 Hematology (GD 21)

Parameter		0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
White blood cells	[giga/L]	4.38	4.37	4.53	**6.20***	5.42	5.05
	(Δ% ctrl)		-0.2	+3.4	**+41.6**	+23.7	+15.3
Red blood cells	[tera/L]	5.90	5.86	5.96	**4.94***	**5.03****	**5.27****
	(Δ% ctrl)		-0.7	+1.0	**-16.3**	**-14.7**	**-10.7**
Hemoglobin	[mmol/L]	6.9	6.9	7.0	**5.9***	**6.0****	**6.3***
	(Δ% ctrl)		0.0	+1.4	**-14.5**	**-13.0**	**-8.7**
Hematocrit	[L/L]	0.306	0.306	0.309	**0.259***	**0.259****	**0.268****
	(Δ% ctrl)		0.0	+1.0	**-15.4**	**-15.4**	**-12.4**
Reticulocytes	[%]	1.9	2.0	2.2	**8.3****	3.2	1.7
	(Δ% ctrl)		+5.3	+15.8	**+337**	+68.4	-10.5
Platelets	[giga/L]	1110	1008	1054	**631****	**802****	**802****
	(Δ% ctrl)		-9.2	-5.0	-43.2	-27.7	-27.7

* $p < 0.05$; ** $p<0.01$ (Wilcoxon-test, two-sided)

No changes of hematology parameters were measured in the groups not exposed to epoxiconazole, regardless of ECP supplementation.

3. Clinical chemistry [see Table 2/8]
Aspartate aminotransferase (AST) activity in rats was higher in the epoxiconazole-treated groups (statistically significant in groups 3 and 4); a slightly decreased activity of alanine aminotransferase in group 3 rats was considered incidental because the observed value was still within the historical control range.

In dams of test group 3 (50 mg/kg bw/d epoxiconazole) the inorganic phosphate, glucose, triglyceride and cholesterol values were higher compared to controls. In rats with additional ECP administration (test group 4: + 0.5 µg/animal/d ECP; test group 5: +1.0 µg/animal/d ECP) the extent of the increased phosphate, glucose and triglyceride concentrations became smaller, but remained statistically significant (except for inorganic phosphate in group 5 dams). Cholesterol values did not seem affected by ECP co-treatment.

The following clinical chemistry parameter levels were decreased in rats of test group 3 (50 mg/kg bw/d epoxiconazole) compared to controls: total bilirubin, total protein, albumin (not statistically significantly) and globulins. The values were not changed when rats were co-administered with ECP.

Table 2/21 Clinical chemistry (GD 21, selected parameters)

Parameter		0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
ALT	[µkat/L]	0.90	0.83	0.83	**0.76****	0.87	0.87
AST	[µkat/L]	1.62	1.46	1.45	**2.09****	**1.83***	1.77
Total bilirubin	[µM]	1.12	1.38	1.19	**0.58****	**0.54****	**0.53****
Total protein	[g/L]	57.23	57.92	58.67	**47.96***	**46.65****	**48.14****
Albumin	[g/L]	32.37	32.97	33.53	29.53	**27.94****	**27.96****
Globulins	[g/L]	24.86	24.95	25.14	**18.43****	**18.72****	**20.19****
Glucose	[mM]	4.46	4.79	4.93	**5.34***	**5.16***	**4.91***
Inorg. phosphate	[mM]	1.46	1.50	1.41	**1.82****	**1.72****	1.69
Triglycerides	[mM]	1.72	1.70	1.93	**5.90****	**4.96****	**4.93****
Cholesterol	[mM]	1.93	1.93	1.95	**2.50***	2.19	**2.46***

* $p < 0.05$; ** $p<0.01$ (Wilcoxon-test, two-sided)

No clinical findings were seen in the groups not exposed to epoxiconazole but administered ECP only (groups 1 and 2, 0.5 and 1 µg/animal/d ECP).

Clinical chemistry changes in epoxiconazole-treated rats focused on an altered liver cell metabolism.

Regardless if rats were treated with epoxiconazole only or additionally with ECP the total protein, albumin and globulin as well as total bilirubin levels were decreased, but the cholesterol and triglyceride levels were increased. The cell membranes of some liver cells seemed to be affected especially in epoxiconazole-only treated rats because of elevated serum aspartate aminotransferase (AST) activities. AST is located in the cytoplasm of liver cells, but additionally in cells of some other tissues. The co-administration of ECP seemed to reduce the AST activity increases. Lower serum bilirubin levels were judged to be due to a higher conjugation rate in the liver followed by a higher excretion of bilirubin via bile and kidneys. No patho-physiological correlate can be found to decreased bilirubin levels and therefore this mechanism was regarded as an adaptive and not an adverse effect. A definite reason for the higher glucose and inorganic phosphate levels in all epoxiconazole-treated rats (test groups 3, 4 and 5) cannot be found, but an effect due to the epoxiconazole-treatment cannot be excluded.

4. Hormone changes [see Table 2/22]
Hormone measurements revealed that ECP doses of 0.5 and 1 µg/animal/d administered to rats of groups 1 and 2 did not affect maternal serum estradiol concentrations as measured on GD 21. The ECP molecule does not cross react with the antibodies of the specific estradiol ELISA which was used in this study and was therefore not detectable in the serum samples. The cleavage of the ECP molecule to estradiol and a possible feedback resulting in down-regulation of the endogenous estradiol synthesis in ECP-treated rats may explain the comparable serum estradiol concentrations of control group 0 and ECP-treated groups 1 and 2. In dams treated with epoxiconazole-only, no serum estradiol levels were detected anymore proving the aromatase inhibiting character of epoxiconazole. In rats administered both epoxiconazole and ECP (test groups 4 and 5) increasing numbers of individuals with low levels of serum estradiol were identified dependent on the ECP dose. This is assessed to be related to ECP administration and its subsequent cleavage to estradiol. Androstenedione levels were higher in rats receiving both epoxiconazole and ECP compared to controls and the epoxiconazole only dose group (test group 3). During pregnancy, androstenedione is mainly synthetized in the placenta of rats and is converted to estradiol in the ovaries by the aromatase enzyme (Jackson and Albrecht, 1985; Gibori et al., 1988). The aromatase is inhibited by the administered epoxiconazole in test groups 3, 4 and 5. By co-administration of ECP serum androstenedione levels were increased in test groups 4 and 5, maybe via functioning feedback mechanisms and/or due to reduced placental damage (see section G below). Higher progesterone levels were seen in rats of both epoxiconazole-exposed ECP co-treated groups (test groups 4 and 5) compared to controls and the epoxiconazole-only dose group (test group 3).

Table 2/22 Hormones (GD 21)

Parameter	0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
Estradiol [pM]	41.0	36.8	38.9	**0.0** (-100%)**	**0.2** (-100%)**	**2.2** (-95%)**
Progesterone [nM]	132.2	**218.5***	173.1	161.7	**242.8** (+84%)**	**252.0* (+91%)**
Androstenedione [nM]	5.1	5.2	**4.5* (-12%)**	4.6	**6.6* (+28%)**	**6.7* (+30%)**
Testosterone [nM]	1.2	1.1	1.0	0.98	1.1	1.2

* p < 0.05; ** p<0.01 (Wilcoxon, two-sided)

4. Gross necropsy observations

Animal No. 57 that had been sacrificed in a moribund condition revealed macroscopically an effusion in the thoracic cavity and grey-yellow deposition in the lungs. These findings are often seen in animals as a result of gavage errors. Enlarged adrenals were seen in two dams of treatment group 5 (50 mg/kg bw/d epoxiconazole + 1 µg/d ECP). All other gross lesions observed in the test animals occurred singularly and were considered to be incidental and not related to treatment.

Findings related to embryo-fetal toxicity are summarized in section E.

5. Organ weights [see Table 2/23]

Table 2/23 Organ weight

Parameter	0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
Terminal body wt [g]	231	242 [105%]	**248** [108%]**	226 [98%]	229 [99%]	231 [100%]
Abs. liver wt [g]	8.341	9.254 [111%]	**9.963* [119%]**	**10.051** [121%]**	**11.402** [137%]**	**11.256** [135%]**
Rel. liver wt [%]	3.587	3.792 [106%]	**4.002* [112%]**	**4.452** [124%]**	**4.968** [139%]**	**4.863** [136%]**
Abs. adrenal gland wt [g]	0.074	0.074 [101%]	0.074 [100%]	**0.086* [116%]**	**0.087** [118%]**	**0.093* [127%]**
Rel. adrenal gland wt [%]	0.032	0.031 [96%]	0.030 [93%]	**0.038* [120%]**	**0.038** [119%]**	**0.041* [129%]**
Abs. ovary wt [mg]	101.56	105.17 [104%]	102.22 [101%]	106.18 [105%]	103.00 [101%]	96.89 [95%]
Rel. ovary wt [%]	0.044	0.043 [98%]	0.041 [94%]	0.047 [108%]	0.045 [102%]	0.042 [96%]

* p < 0.05; ** p<0.01 (Wilcoxon-test, two-sided)

Terminal body weights appeared to be slightly increased by ECP-alone treatment. Liver weights were also increased by both ECP and epoxiconazole alone, but increases were the highest when administered in combination. Adrenal gland weights were increased by epoxiconazole and further increased by combined epoxiconazole / high-dose-ECP treatment. No clear effects of treatment were noted with regard to ovary weights.

E. CESAREAN SECTION DATA [see Table 2/24]

No significant differences between control and treated animals were observed for the conception rate, mean number of corpora lutea, implantation sites and pre-implantation loss.

Table 2/24 Summary of reproduction toxicity data

Parameter	0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
Females mated	18	18	18	18	18	18
Dams pregnant	11	15	17	14	16	16
Female mortality	0	0	0	1	0	0
Dams with all resorptions	0	2	0	3	0	0
Dams with viable fetuses	11	13	17	10	16	16
Corpora lutea (mean)	13.8	13.0	13.9	12.8	14.1	13.8
Implantation sites (mean)	11.9	10.4	11.5	10.6	12.5	13.1
Pre-implantation loss [mean %]	[14.9]	[24.4]	[17.9]	[22.1]	[12.7]	[5.7]
Post-implantation loss [mean %]	[6.3]	[19.2]	[5.5]	**[51.6**]**	12.8	[9.4]
Resorptions, total (mean)	0.7	0.9	0.6	**4.6****	1.7	1.1
Resorptions, early (mean) [mean %]	0.6 [5.6]	0.9 [19.2]	0.6 [5.5]	1.7 [21.5]	0.8 [6.3]	0.9 [6.8]
Resorptions, late (mean) [mean %]	0.1 [0.6]	0.0 [0.0]	0.0 [0.0]	**2.9**** **[30.1**]**	0.9 [6.5]	0.3 [2.6]
Dead fetuses	0	0	0	0	0	0
Live fetuses / litter (mean) [mean%]	11.2 [93.7%]	11.0 [93.2%]	10.9 [94.5%]	**7.8** **[62.9%**]**	10.8 [87.2%]	11.9 [90.6%]
Fetal weight [g] (mean)	4.9	4.7	4.8	4.8	4.9	4.8

* $p < 0.05$; ** $p<0.01$ (Dunnett-test, two-sided)

In test group 3 (50 mg/kg bw/d epoxiconazole without ECP co-treatment) the mean number of late resorptions was statistically significantly increased (2.9** or 30.1%** [p≤0.01] vs. 0.1 or 0.6% in control). This resulted in a significantly increased post-implantation loss (51.6%** vs. 6.3% in control) and, hence, a significantly decreased percentage of live fetuses.

In groups that were administered epoxiconazole in combination with ECP (groups 4 and 5), no statistically significant increases in resorption rate or post-implantation loss were observed. In the ECP-only treated animals (groups 1 and 2) the resorption rate / post-implantation loss was also not statistically different from vehicle control group animals.

No dead fetuses were noted in any group.

The sex distribution of the fetuses in all treated groups was comparable to the control group. Observable differences were without biological relevance.

The mean fetal weights did not show any biologically relevant differences between the test substance-treated groups and the control. The observable differences between the groups reflect the usual fluctuation for this parameter.

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. External examination [see Table 2/25]

Fetal external malformations

External malformations were recorded for one fetus in test group 2 (malrotated limb); for two fetuses in test group 3 (one with short snout, one with malrotated limb) and for one fetus in test group 4 (malrotated limb). The overall incidences did not demonstrate a relationship to any treatment or co-treatment.

Table 2/25 Fetal external malformations

		0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
Short snout	Fetal incidence*	0 / 123	0 / 143	0 / 185	1 / 78	0 / 173	0 / 191
	Litter incidence*	0 / 11	0 / 13	0 / 17	1 / 10	0 / 16	0 / 16
Malrotated limb	Fetal incidence*	0 / 123	0 / 143	1 / 185	1 / 78	1 / 173	0 / 191
	Litter incidence*	0 / 11	0 / 13	1 / 17	1 / 10	1 / 16	0 / 16
Total	Fetal incidence*	0 / 123	0 / 143	1 / 185	2 / 78	1 / 173	0 / 191
	Litter incidence*	0 / 11	0 / 13	1 / 17	2 / 10	1 / 16	0 / 16

* No. of fetuses (litters) with findings / no. fetuses (litters) examined

Fetal external variations
External variations were not observed in this study.

Fetal external unclassified observations
Unclassified external observations, such as blood coagulum around placenta and placentae fused, were recorded for single fetuses of test groups 2 - 5. A relation to dosing is not present if normal biological variation is taken into account. Therefore, a relation to any treatment or co-treatment is not assumed. However, since the placenta is a potential target of epoxiconazole, thorough histopathological examinations were conducted and are summarized in section G.

Table 2/26 Total external unclassified observations

		0 mg epoxiconazole/kg bw/d			50 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	0.5 µg ECP	1.0 µg ECP	0 µg ECP	0.5 µg ECP	1.0 µg ECP
Blood coagulum around placenta	Fetal incidence[1]	0 / 123	0 / 143	1 / 185	3 / 78	2 / 173	2 / 191
	Litter incidence[1]	0 / 11	0 / 13	1 / 17	2 / 10	2 / 16	2 / 16
Placenta fused	Fetal incidence[1]	0 / 123	0 / 143	0 / 185	0 / 78	1 / 173	0 / 191
	Litter incidence[1]	0 / 11	0 / 13	0 / 17	0 / 10	1 / 16	0 / 16
Total	Fetal incidence[1]	0 / 123	0 / 143	1 / 185	3 / 78	3 / 173	2 / 191
	Litter incidence[1]	0 / 11	0 / 13	1 / 17	2 / 10	3 / 16	2 / 16

[1] No. of fetuses (litters) with findings / no. fetuses (litters) examined

G. HISTOPATHOLOGY OF THE PLACENTA

FINDINGS IN CONTROL GROUP RATS WITH ECP TREATMENT (Gr. 1, 2)
Placentas of test group 1 (0.5 µg/animal/d ECP) and 2 (1 µg/animal/d ECP) showed changes comparable to the control placentas of test group 0 and were therefore regarded as not affected by treatment.

FINDINGS IN EPOXICONAZOLE GROUP RATS WITHOUT AND WITH ECP-TREATMENT (Gr. 3, 4, 5)
Treatment-related degenerative findings were observed in the placentas of test groups 3 (50 mg/kg bw/d epoxiconazole), 4 (50 mg/kg bw/d epoxiconazole + 0.5 µg/animal/d ECP) and 5 (50 mg/kg bw/d epoxiconazole + 1 µg/animal/d ECP). Placental degeneration was characterized by cystic dilation of maternal sinuses and rupture of interhemal membrane in the labyrinth with concomitant changes in the trophospongium, such as thickening, congestion and necrosis. Degenerative changes were associated with enlargement of the placentas. The same pattern of alteration was observed in placentas with live fetuses as in placentas with late resorptions. However, placentas with live fetuses exhibited a slight to moderate degeneration, while placentas with late resorptions displayed a severe to massive degeneration.

Table 2/27 Placenta histopathology

Gr.	EPX (mg/kg bw/d)	ECP (µg/d)	Examined placentas[a]			Mean severity grade of placental degeneration					
						Labyrinth			Trophospongium		
			All	with late resorpt.	with live fetus	All	with late resorpt.	with live fetus	All	with late resorpt.	with live fetus
0	0	0	122	1	121	0.0	2.0	0.0	0.0	2.0	0.0
3	50	0	108	29	79	3.7**	4.7	3.3	3.8**	4.1	3.7
4	50	0.5	186	12	174	2.9**	4.9	2.8	3.3**	4.0	3.2
5	50	1.0	195	4	191	1.8**	3.5	1.8	2.2**	3.5	2.2

EPX: Epoxiconazole; ECP: Estradiol cyclopentylpropionate
[a] placentas with live fetuses or with late resorptions were considered
[b] Severity grades: 1 (minimal), 2 (slight), 3 (moderate), 4 (severe), 5 (massive)
Statistical analysis: ** p <= 0.01

This difference in the extent of degeneration of "late-resorption-placentas" compared to "live-fetus-placentas" was found to be statistically significant (severity grades: 4.0* - 4.9* vs. 2.8 - 3.7), based on separate statistical evaluations performed for groups 3 and 4.

When evaluating all placentas without differentiating those with live fetus or late resorption, histopathology revealed severe degenerative changes in the placentas of dams of test group 3 (50 mg/kg bw/d epoxiconazole) associated with a high number of late resorptions: 29 out of 108 total examined placentas, in 8 of 12 dams. By co-administration of estradiol the degenerative changes decreased dose-dependently, to moderate in test groups 4 (50 mg/kg bw/d epoxiconazole + 0.5 µg/animal/d ECP), and to slight in test group 5 (50 mg/kg bw/d epoxiconazole + 1 µg/animal/d ECP).

Associated with this, an ECP-dose-dependent decrease in the degeneration of placentas with live fetus and a concomitant regression in the number of fetal resorptions was noted in test group 4 (12 out of 186 total examined placentas, in 5 of 16 dams) and test group 5 (4 out of 195 examined placentas, in 2 of 16 dams).

Data from this study and a previous study on epoxiconazole (see chapter 2.1.1, Schneider et al. 2010a, DocID 2010/1062087) suggest that severe to massive degenerative changes in the labyrinth led to a loss of feto-maternal blood compartmentalization, which appeared to be critical if more than 70% of labyrinth was affected (massive degeneration), being associated with an increased incidence of fetal deaths.

III. SUMMARY AND CONCLUSIONS

The aim of this mechanistic prenatal developmental toxicity study in rats was to:

a) investigate whether the observed fetal toxicity and placental damage in rats is causally related to previously observed pronounced reduction of maternal serum estradiol
b) study whether estradiol co-supplementation impacts placental histology and extent of late fetal resorptions caused by epoxiconazole treatment

Epoxiconazole suspended in corn oil was administered by oral gavage to three groups of 18 paired female Wistar rats each at a dose level of 50 mg/kg bw/d once

daily from GD 7 through GD 18. Two of these three groups additionally received daily subcutaneous injections of either 0.5 of 1 µg/animal estradiol cyclopentylpropionate (ECP). Three control groups were dosed with the vehicle (corn oil), two of which additionally received 0.5 of 1 µg/animal ECP by s.c. injection from GD 7-21. Dose volumes were 2 mL/kg bw for epoxiconazole in corn oil suspension and 0.2 mL/animal for ECP also in corn oil. At sacrifice on GD 21, eleven to seventeen females per group had implantation sites.

Maternal toxicity examinations comprised regular recording of food consumption, body weight and health status, blood sampling for clinical chemistry, hematological and hormone investigations on the day of sacrifice, determination of corrected body weight gain, and weights of liver, adrenal gland and ovaries and gross necroscopy. Following weight determination of the unopened uterus a photograph of the uterus and its contents was taken. For each dam, corpora lutea were counted and number and distribution of implantation sites (differentiated by resorptions, live and dead fetuses) were determined. The fetuses were removed from the uterus, sexed and investigated for external findings and subsequently discarded without any other examinations. The uterus with placental sites and decidua were processed to allow for histopathological examination of the placentas.

In epoxiconazole treated dams, vaginal hemorrhage and piloerection were seen irrespective of ECP co-treatment. Food consumption was decreased during major parts of the treatment period especially during late pregnancy (from GD 15-21 without ECP, from GD 18-21 with ECP). Corrected body weight gain was significantly decreased especially in the group with ECP supplementation. Anemia was indicated by reductions in red blood cell count, hemoglobin and hematocrit and increased reticulocytes. Platelets were decreased and lymphocytes were increased; neutrophil counts were decreased. Impaired liver function was indicated by decreased protein levels; aspartate aminotransferases (ASAT), inorganic phosphate, glucose, cholesterol and triglyceride values. Estradiol values were substantially reduced: In fetuses from dams administered epoxiconazole alone, a significantly increased number of late resorptions and total post implantation loss was observed, in conjunction with significantly decreased percentage of live fetuses compared to controls. When epoxiconazole was administered in combination with ECP-treatment, no treatment-related adverse fetal effects were seen.

Histopathology data of this study clearly demonstrate that supplementation of estradiol reduces the severity of degenerative placental changes caused by epoxiconazole below the threshold of a functional loss. This recovery of placental morphology and function is beneficial to fetal survival.

In conclusion, there is evidence that fetal deaths are not caused by an independent adverse effect of epoxiconazole on the development of the fetus, but by maternal toxicity, which pertubates the ability of the mother to maintain pregnancy with all fetuses. Estradiol deficiency and the associated loss of placental function is the crucial endpoint of maternal toxicity caused by epoxiconazole in rats under the study conditions. Estradiol supplementation restored placental function although the ECP co-treatment seemed not to have a distinct effect on serum estradiol

levels themselves, and serum estradiol was still quite low in the ECP co-treated rats.

Data from this epoxiconazole study confirms findings from a preceding study indicating a correlation between massive estradiol reduction (almost down to the limit of detection for serum estradiol), degenerative damage to the placenta and, subsequently, fetal mortality. This and the previous experiment suggest that two conditions had to concur to cause late resorptions:

1) degenerative damage to the placenta affecting at least 70% of its normal morphology and function, and
2) advanced fetal development to the extent that the increased nutritional demand of the fetus exceeded the maternal supply that the mother could provide through the damaged placenta.

Histopathology data of this study clearly demonstrate that supplementation of estradiol reduces the severity of degenerative placental changes caused by epoxiconazole below the threshold of a functional loss. This recovery of placental morphology and function is beneficial to fetal survival.

In conclusion, there is evidence that fetal deaths are not caused by an independent adverse effect of epoxiconazole on the development of the fetus, but by maternal toxicity, which pertubates the ability of the mother to maintain pregnancy with all fetuses. Estradiol deficiency and the associated loss of placental function is the crucial endpoint of maternal toxicity caused by epoxiconazole in rats under the study conditions. Estradiol supplementation restored placental function although the ECP co-treatment seemed not to have a distinct effect on serum estradiol levels themselves, and serum estradiol was still quite low in the ECP co-treated rats.

STUDY RELEVANCE

The study is relevant for assessing the appropriate classification and labeling of epoxiconazole. The current RAC opinion for classification of epoxiconazole for developmental toxicity in Category1B is based on two findings from rat studies, (1) an increased incidence of late fetal resorptions without reported maternal toxicity (Taxvig et al. 2007, 2008) and (2) a high incidence of cleft palates observed at an excessive dose level in a range-finding study in the presence of marked maternal toxicity plus isolated occurrences of cleft palate in single fetuses at lower dose levels from other studies for which a treatment-relationship was not excluded (BASF unpublished studies). This study provides relevant new information for both issues of concern. No cases of cleft palate were observed in the study up to the highest dose level tested (50 mg/kg bw/d). With regard to late fetal resorptions, this new BASF study clearly shows that, following exposure of pregnant females to 50 mg/kg bw/d during GD 7-21, late fetal resorptions occur in the presence of distinct maternal toxicity (significantly decreased corrected body weight gain, anemia, marked decrease of maternal plasma estradiol levels, severe placental damage); this observation is in contrast to conclusions previously drawn by RAC regarding the association of late fetal resorptions and maternal toxicity on the basis of information provided in the publications by Taxvig et al. (2007,

2008). The study also reveals for the first time that epoxiconazole-mediated inhibition of maternal ovarian aromatase, placental damage and late fetal resorptions are causally linked, providing highly relevant information for the underlying mode of action of epoxiconazole and also for the assessment of the human relevance of the observed late fetal resorptions in rat studies. Already in 1999, specialist experts from the Scientific Committee on Plants (SCP) had concluded that the rat may not be the appropriate species for assessment of reproduction toxicity of aromatase inhibitors, due to fundamental differences in the hormonal regulation of murid rodent and human pregnancy. The SCP has recommended the guinea pig as relevant alternative to study the developmental toxicity of aromatase inhibitors for the purpose of human hazard and risk assessment. In this regard, one very important aspect is that in the murid rodent, estradiol during pregnancy is exclusively supplied by the maternal rat ovary, while no estradiol is produced in the rat placenta. This is in striking contrast to humans and guinea pigs, where the placenta is the almost exclusive source of estradiol during late pregnancy. In previous investigations already evaluated by RAC [Wuttke 1995, 2001, summarized in RAC Background Document (2010)], the in-vitro comparison of the aromatase inhibiting potential of epoxiconazole in rat and in human granulosa cells showed that human granulosa cells are considerably less sensitive to aromatase inhibition by epoxiconazole, at least by a factor of 10 to 100. A positive control substance, vorozole also showed the same type of species-specific effect In this context, the results of this new study provide clear evidence to support the conclusion that the observed late fetal resorptions resulting from treatment of pregnant rats are not only secondary to maternal toxicity but also of limited relevance to humans.

The current RAC opinion in support of a Repr. 1B (CLP) classification and of a Repr. Cat. 2 / R61 classification (DSD) for epoxiconazole relied on information that claimed the absence of significant maternal toxicity at dose levels causing late fetal resorptions in rats, via an (unknown) mechanism for which human relevance could not be excluded. With the new study results clearly indicating an indirect effect on the rat fetus via maternal toxicity, plus reliable evidence linking late fetal resorptions in rats to maternal aromatase inhibition, a Repr. 1B classification based on increased late fetal resorptions is not considered to be justified. By taking into account all available data in an overall weight-of-evidence approach, no classification is required for this rat finding (see chapters 3 and 4).

2.1.3 Maternal toxicity (dose range finding) study in guinea pigs

STUDY REFERENCE

Report:	Schneider S., Strauss V., Rey Moreno M.C., Becker M., van Ravenzwaay B. 2011a BAS 480 F (Epoxiconazole) Modified Maternal Toxicity Study in Guinea Pigs Oral Administration (Gavage) BASF DocID 2011/1229837 Date of report: 13-Sep-2011
Guidelines:	There are no guidelines for this range-finding study in guinea pigs. Reference was made to Commission Reg. 440/2008 (30 May 2008), OECD TG 414 (22 January 2001) and OPPTS 870.3700 (EPA, August 1998)
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz)

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	8563
Purity:	97.0%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 30-Jun-2010.
2. Vehicle controls:	Carboxymethylcellulose (CMC; 1% aqueous solution)
3. Test animals:	
Species:	Guinea pig
Strain:	Dunkin-Hartley [Crl:HA]
Sex:	Female
Age:	Time-mated, supplied on GD 4
Weight at arrival (GD 4):	551 to 1215 g
Source:	Charles River Lab., Germany
Acclimatization period:	at least 2 days

Diet:	Kliba maintenance diet for rabbits & guinea pigs “GLP” meal, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type H-temp (PSU) cages (Tecniplast, Germany), floor area about 2065 cm² with dust free wooden bedding
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work: 08-Mar-2010 to 04-Aug-2011
(in-life dates: 10-Mar-2010 [Start of treatment at gestation day (GD) 6] to 17-Jun-2010 [Sacrifice of the 5th cohort on GD 63])

2. Animal assignment and treatment:

Upon arrival of time-mated female guinea pigs on GD 4, the animals were randomly allocated to the different test groups and placed to the cages. Starting on GD 6, groups of 19-20 animals received a daily dose of epoxiconazole in 1% aqueous carboxymethylcellulose suspension (1% CMC) by oral gavage at dose levels of 5, 15, 50, and 180 mg/kg bw/d until GD 63. A concurrent control group received the vehicle only; all groups were administered the same volume of 10 mL/kg bw. All animals from group 4 (180 mg/kg bw/d) were sacrificed prematurely by decapitation under anesthesia for humane reasons. By the time of sacrifice, the dose of 180 mg/kg was lethal for 7 out of 20 animals in this group and caused further distinct signs of intoxication in the remaining animals, such as clinical observations (abortion, poor general state), distinctly reduced food consumption and loss of body weight.

Table 2/28 Test groups and doses

Test group	Dose (mg/kg bw/d)	Concentration (mg/100 mL)	Volume (mL/kg bw)	Treatment period	No. of mated animals
0	0 (1% CMC)	0	10	GD 6–63	20
1	5	50	10	GD 6–63	20
2	15	150	10	GD 6–63	19
3	50	500	10	GD 6–63	20
4	180	1800	10	GD 6–63	20

3. Test substance preparation and analysis:

Prior to study initiation the aqueous test substance preparations were shown to be stable over a 7-day period. Thus, test-substance preparations were performed at the beginning of the administration period and thereafter at maximum intervals of 7 days.

Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in 1% CMC using a high-speed homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals.

Test-article concentration analyses were performed twice at the beginning and towards the end of the study. The homogeneity of the dose suspensions was verified at the beginning of the study by taking 3 samples from the top, middle and bottom of the beaker for the low and high dose levels (5 and 180 mg/kg bw/d) while a magnetic stirrer was running. The results of these analyses are given in the table below.

Relative standard deviations of maximum 7% indicated the homogenous distribution of epoxiconazole in the dosing suspensions. The actual nominal test-item concentrations were in the range of 94.0 to 100.6% of the target nominal concentrations and thus in the acceptable range.

Analysis of preparations for homogeneity and test-item content

Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± SD
1% CMC	09.03.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		0.05	0.046	92.5	100.6 ± 7.0
			0.052	104.4	
			0.052	104.4	
		0.15	0.142	94.9	94.9
		0.50	0.472	94.3	94.3
		1.80	1.700	94.9	96.8 ± 2.3
			1.780	94.3	
			1.746	94.6	
	15.06.2010 [Concentration control analyses]	0.05	0.05	100.0	100.0
		0.15	0.14	93.3	93.3
		0.50	0.47	94.0	94.0

4. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption [a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of post-implantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal proportions

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

Statistics for pathology and clinical pathology	
Parameter	**Statistical test**
Hematology, clinical chemistry, hormones: DHEAS, Estriol, E2 and 17-OHP; organ weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
Hormones (except DHEAS, Estriol, E2 and 17-OHP)	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the MANN-WHITNEY-U test (two-sided) for the hypothesis of equal medians.

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

2. Body weight and food consumption

All animals were weighed on GD 4, 6, 9, 13, 16, 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62 and 63. The body weight change of the animals was calculated from these results.

In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 63 minus weight of the unopened uterus minus body weight on GD 6).

Food consumption was determined for the time periods GD 4-6, 6-13, 13-20, 20-27, 27-34, 34-41, 41-48, 48-55, 55-62 and 62-63.

Only pregnant sows were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant sows with scheduled sacrifice on GD 63 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Hematology and clinical chemistry

Blood sampling was performed during mid-pregnancy on GD 42 for hormone measurements and on the day of sacrifice (GD 63) for hematological, clinical chemistry and hormone analyses. For this purpose, blood was drawn in the morning from non-fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence. Blood sampling was performed also on GD 42 to determine hormone levels during mid-pregnancy

The following hematological and clinical chemistry parameters were determined for all surviving test group animals:

Hematology (GD 63):					
	Red blood cells		*White blood cells*		*Clotting potential*
✓	Erythrocyte count (RBC)	✓	White blood cell count (WBC)	✓	Platelet count (PLT)
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)		
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells (differential)		

Clinical chemistry (GD 63):					
	Electrolytes		*Metabolites and proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
		✓	Cholesterol	✓	Alkaline phosphatase (ALP)
		✓	Creatinine	✓	γ-glutamyl transferase (γ-GT)
		✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

Hormones (GD 42 and GD 63):			
✓	11-desoxycorticosterone	✓	Corticosterone
✓	11-desoxycortisol	✓	Cortisol
✓	Progesterone	✓	17-OH-Progesterone (17OHP)
✓	Androstenedione	✓	Testosterone (TESTO)
✓	Estriol (ESTRIOL)	✓	Estradiol (E2)
✓	18-hydroxycorticosterone	✓	Dehydroepiandrosterone sulfate (DHEAS)

4. Sacrifice

On GD 63, the sows were sacrificed after blood sampling in randomized order by decapitation (after isoflurane anesthesia). The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Photograph of the unopened uterus with all contents in situ (CD with all photographs in the raw data archive)
- Number of corpora lutea
- Number and distribution of implantation sites classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to SALEWSKI in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue visible
    c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]: } \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]: } \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]: } \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

Dams were subsequently assessed by gross pathology. The following organs from all dams sacrificed on schedule were weighed and fixed in neutral buffered 4% formaldehyde solution:

- Adrenal glands
- Liver
- Ovaries

Additionally, from all dams sacrificed on schedule, the …:

- Placentas (detached from the uterus wall) and
- Uteri

… were fixed in neutral buffered 4% formaldehyde solution. After a 24-48h fixation period, the placentas were transferred to 70% ethanol solution for asservation.

5. Histopathology of placentas

The placentas of one animal per test groups 0-3 were processed histotechnically and examined by light microscopy. For this purpose the placentas were trimmed by a transverse cut in the center (parallel to the umbilical cord) in order to obtain a section visualizing the different placental zones including the subplacenta.

The placentas were stained with Hematoxylin-eosin (H&E). Special stains, such as PAS (Periodic-Acid-Schiff), H-MG (Hart stain combined with Masson’s Goldner Trichrome stain) and Kossa’s stain were performed on individual placentas of each test group

6. Examination of fetuses

When the uterus was opened the viability of the fetuses and the condition of placentae, umbilical cords, fetal membranes, and fluids were carefully examined in situ. After dissection from the uterus each fetus was sexed and external tissues and all orifices were examined macroscopically. The fetuses and the corresponding placentas were weighed. Thereafter, the fetuses were sacrificed by subcutaneous injection of pentobarbital (Narcoren®; dose: 0.5 mL per fetus) and discarded.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al. (1997) and its updated version by Makris et al. (2009) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al. (Chahoud et al. 1999; Solecki et al. 2001 and 2003):

Malformation	A permanent structural change that is likely to adversely affect the survival or health
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Note: Only pregnant sows were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on gestation day 63 p.c. were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

For the above reasons the following females were excluded from the above-mentioned calculations:

Epoxiconazole dose (mg/kg bw/d)				
0	5	15	50	180
9, 11, 13	24, 31, 33, 34, 36	53, 54	64, 73, 75, 78	Dose group terminated; not evaluated

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

At the top dose group of 180 mg/kg bw/d, 7 out of 20 animals died during treatment, which was considered to be a substance-related finding. At 15 mg/kg bw/d, one sow (No. 42) was found dead on GD 47, and one animal at 15 mg/kg bw/d (No. 54) and one control group guinea pig (No. 16) was sacrificed in a moribund condition; these deaths were considered to be incidental. Cases of early delivery or absorption occurred in all groups including control (4, 1, 2, and 2 sows at 0, 5, 15, and 50 mg/kg bw/d, respectively); these sows were sacrificed without further investigation; the deaths were thus not related to substance intake.

2. Clinical signs of toxicity

Sows of the 180 mg/kg bw/d group that died during the course of the treatment period showed clinical signs of general toxicity (abortion, poor general state, distinctly reduced food consumption and loss of body weight). In combination with mortality, the effects observed at 180 mg/kg bw/d were considered to represent exaggerated toxicity that prevented a meaningful assessment of potential developmental toxicity effects in a definitive study in guinea pigs. Therefore the data obtained in this group until unscheduled termination was not reported. There were no treatment related clinical effects in guinea pigs of other dose groups (up to 50 mg/kg bw/d).

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption

There were no test substance-related changes of food consumption during the whole study in guinea pigs exposed to epoxiconazole dose levels of up to 50 mg/kg bw/d.

2. Body weight and body weight gain
Body weight and body weight gain of pregnant guinea pigs was not influenced by epoxiconazole administration in groups treated with up to 50 mg/kg bw/d.

D. NECROPSY OBSERVATIONS

1. Corrected (net) body weight gain
The average carcass weight and the corrected body weight gain of treated females were not influenced by the treatment.

2. Hematology (Table 2/29)
At the end of pregnancy in guinea pigs administered 5 or 15 mg/kg bw/d absolute eosinophil counts, in individuals given 15 mg/kg bw/d relative eosinophil counts, and in guinea pigs treated with 50 mg/kg bw/d absolute basophil counts were lower compared to controls. In the absence of a dose-response relationship, the changes in the eosinophilic counts were regarded as incidental and not treatment-related.

Table 2/29 Hematology

	Epoxiconazole dose (mg/kg bw/d)			
	0	5	15	50
Total white blood cell count (WBC) [giga/L]	7.13 ± 3.90	6.65 ± 1.62	6.93 ± 2.32	6.32 ± 2.15
Absolute eosinophil count (EOSA) [giga/L]	0.11 ± 0.05	**0.07 ± 0.03***	**0.05 ± 0.02****	0.09 ± 0.05
Relative eosinophil count (EOS) [%]	1.6 ± 0.7	1.1 ± 0.5	**0.7 ± 0.4****	1.5 ± 1.0
Absolute basophil count (BASOA) [giga/L]	0.04 ± 0.02	0.04 ± 0.02	0.03 ± 0.01	**0.02 ± 0.01****
Relative basophil count (BASO) [%]	0.5 ± 0.2	0.6 ± 0.3	0.4 ± 0.2	**0.3 ± 0.1****

Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis + Wilcoxon test, 2-sided)

3. Clinical chemistry
No treatment-related changes of clinical-chemistry parameters were measured.

4. Hormone changes [see Table 2/30]
At **GD 42** testosterone and 11-desoxycortisol were dose-dependently increased in test groups 5, 15 and 50 mg/kg bw/d. Compared to controls, 11-desoxycorticosterone, androstenedione and corticosterone were higher in guinea pigs of test group 3 (50 mg/kg bw/d).

At **GD 63** androstenedione, testosterone and 11-desoxycortisol were increased in individuals administered 15 or 50 mg/kg bw/d. Compared to controls, 17-hydroxyprogesterone and progesterone were higher in the 50 mg/kg bw/d dose group.

Estradiol levels were not significantly affected by treatment at either time point of measurement.

Table 2/30 Hormones

Parameter		Dose group	0	1	2	3
		dose level (mg/kg bw/d)	0	5	15	50
Progesterone[b]	[nM]	GD 42	939 ± 282	1087 ± 417	1008 ± 468	920 ± 345
		GD 63	696 ± 262	784 ± 243	813 ± 292	**939 ± 326***
17-OH-progesterone[a]	[nM]	GD 42	5.13 ± 1.94	4.56 ± 1.68	4.85 ± 1.83	5.41 ± 1.78
		GD 63	7.30 ± 2.68	7.22 ± 1.89	9.23 ± 3.33	**9.89 ± 3.44***
Estradiol[a]	[pM]	GD 42	324.99 ± 77.71	320.57 ± 65.43	333.65 ± 65.21	332.68 ± 58.33
		GD 63	159.83 ± 16.11	172.50 ± 43.71	153.83 ± 21.28	152.65 ± 17.46
Androstenedione[b]	[nM]	GD 42	16.1 ± 9.6	22.5 ± 10.1	24.0 ± 17.4	**34.9 ± 21.5*****
		GD 63	6.7 ± 4.6	12.8 ± 4.7	**18.0 ± 9.4****	**19.0 ± 8.2****
Testosterone[b]	[nM]	GD 42	0.73 ± 0.24	**0.88 ± 0.23***	**1.01 ± 0.31****	**1.34 ± 0.34*****
		GD 63	0.79 ± 0.29	0.90 ± 0.22	**1.16 ± 0.4***	**1.30 ± 0.41****
11-deoxycortisol[b]	[nM]	GD 42	21.3 ± 8.4	**38.9 ± 23.7***	44.0 ± 31.9	**112.6 ± 97.1*****
		GD 63	32.0 ± 22.7	39.0 ± 15.1	**94.7 ± 123***	**92.3 ± 81.7****
Cortisol[b]	[nM]	GD 42	3549 ± 1057	3605 ± 1272	3229 ± 1062	3702 ± 996
			7102 ± 2415	7576 ± 1719	7460 ± 1731	6727 ± 1978
11-deoxycorticosterone[b]		GD 42	151 ± 80	162 ± 67	158 ± 83	**213 ± 85***
	[nM]	GD 63	241 ± 98	219 ± 89	217 ± 53	259 ± 123
Corticosterone[b]	[nM]	GD 42	32.5 ± 9.9	42.1 ± 20.6	42.8 ± 23.7	**63.6 ± 49.7****
		GD 63	67.1 ± 48.0	78.2 ± 37.0	91.3 ± 55.3	75.7 ± 51.7
18-OH-corticosterone[b]		GD 42	9.2 ± 6.1	**16.9 ± 10.1***	11.8 ± 8.3	20.7 ± 19.0*
	[nM]	GD 63	12.7 ± 9.4	15.4 ± 7.9	22.1 ± 13.4*	20.6 ± 17.2

* p≤0.05; ** p≤0.01; ***p≤0.001 (Kruskal-Wallis followed by either Wilcoxon[a], or Mann-Whitney-U-test[b,] 2-sided)

4. Gross necropsy observations

All gross lesions observed in the test animals occurred singularly and were considered to be incidental and not related to treatment.

5. Organ weights [see Table 2/31]

None of the mean absolute and relative weight parameters in the treated groups showed differences when compared with the control group and were considered within the normal range.

Table 2/31 Organ weight

Parameter	Dose group	0	1	2	3
	Dose level (mg/kg bw/d)	0	5	15	50
Terminal body wt	[g]	864.927	814.484 [94%]	837.273 [97%]	830.956 [96%]
Abs. liver wt	[g]	23.986	23.046 [96%]	25.02 [104%]	24.152 [101%]
Rel. liver wt	[%]	2.769	2.828 [102%]	2.973 [107%]	2.906 [105%]
Abs. adrenal gland wt	[mg]	702.933	633.947 [90%]	666.867 [95%]	703.389 [100%]
Rel. adrenal gland wt	[%]	0.081	0.079 [97%]	0.081 [100%]	0.085 [105%]
Abs. ovary wt	[mg]	228.533	272.474 [119%]	202.400 [89%]	217.944 [95%]
Rel. ovary wt	[%]	0.026	0.032 [124%]	0.024 [92%]	0.026 [99%]

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis H and Wilcoxon-test, 2-sided)

E. CESAREAN SECTION DATA [see Table 2/32]

The conception rate reached 85% in the control group, 75% in test group 1 (5 mg/kg bw/d), 89% in test group 2 (15 mg/kg bw/d) and 80% in test group 3 (50 mg/kg bw/d). A sufficient number of dams were available for the purpose of the study.

No test substance-related and/or biologically relevant differences with regard to conception rate, mean number of corpora lutea, implantation sites, pre- and post-implantation loss and resorptions (total, early and late) were observed.

No dead fetuses were noted at any dose level.

The sex distribution of the fetuses in all treated groups was comparable to the control group. Observable differences were without biological relevance.

The mean fetal weights and also the mean placental weights did not show any biologically relevant differences between the test substance-treated groups and the control. The observable differences between the groups reflect the usual fluctuation for this parameter.

Table 2/32 Summary of reproduction toxicity data

Dose group	0	1	2	3
Dose level (mg/kg bw/d)	0	5	15	50
Females mated	20	20	19	20
Dams pregnant	17	15	17	16
Dams mortality	2	0	1	1
Females with abortions / premature births	4	1	2	2
Dams with viable fetuses	12	14	14	14
Corpora lutea (mean)	5.9	6.8	6.4	6.1
Implantation sites (mean)	4.9	5.3	4.6	4.6
Pre-implantation loss (mean %)	20.3	20.2	22.6	22.6
Post-implantation loss (mean %)	18.4	15.5	4.8	5.3
Resorptions, total (mean)	1.0	0.8	0.2	0.4
Resorptions, early (mean)	0.6	0.6	0.1	0.1
Resorptions, late (mean)	0.4	0.2	0.1	0.2
Dead fetuses	0	0	0	0
Live fetuses / litter (mean) [mean%]	3.9 [81.6%]	4.5 [84.5%]	4.4 [95.2%]	4.2 [94.7%]
Sex distribution (% live males)	46.8	61.9	53.2	50.8
Fetal weight [g] (mean)	85	79	83	78
Placental weight [g] (mean)	4.8	4.3	4.3	4.6

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, two-sided)

F. EXTERNAL EXAMINATION OF FETUSES

1. External examination

Fetal external malformations [see Table 2/33 and Table 2/34]
External malformations were recorded for one fetus in each dose group (5, 15 and 50 mg/kg bw/d), Table 2/33. The overall incidences as listed in Table 2/34 as well as the individual findings did not show a dose-response relationship, nor a specific pattern of abnormalities. Therefore, they were not regarded as treatment-related findings.

Table 2/33 Individual external malformations

Dose grp	Epoxiconazole dose	Dam No.-Fetus No. (Sex)	Malformations
1	5 mg/kg bw/d	32-01 (M)	Meningocele, small eye, supernumerary digit, supernumerary claw
2	15 mg/kg bw/d	58-04 (F)	Umbilical hernia
3	50 mg/kg bw/d	68-05 (M)	Umbilical hernia

Table 2/34 Total external malformations

Parameter	Dose group	0	1	2	3
	Dose level (mg/kg bw/d)	0	5	15	50
Litter	N	12	14	14	14
Fetuses	N	46	63	62	59
Fetal incidence	N	0 (0.0%)	1 (1.6%)	1 (1.6%)	1 (1.7%)
Litter incidence	N	0 (0.0%)	1 (7.1%)	1 (7.1%)	1 (7.1%)
Affected fetuses/litter	Mean%	0.0	3.6	1.8	1.8

Fetal external variations
External variations were not observed in this study.

Fetal external unclassified observations [see Table 2/35]
Fused placentas were recorded for single fetuses of the control and test group 1 (5 mg/kg bw/d). This appears to be a common finding and a relation to dosing is not present. Therefore, a test substance-induced effect is not assumed. However, since the placenta is a potential target of epoxiconazole, thorough histopathological examinations were conducted and are summarized in section G.

Table 2/35 Total external unclassified observations (fused placenta)

Parameter	Dose group	0	1	2	3
Dose level (mg/kg bw/d)		0	5	15	50
Litter	N	12	14	14	14
Fetuses	N	46	63	62	59
Fetal incidence	N	1 (2.2%)	1 (1.6%)	0 (0.0%)	0 (0.0%)
Litter incidence	N	1 (8.3%)	1 (7.1%)	0 (0.0%)	0 (0.0%)
Affected fetuses/litter	Mean%	1.7	1.0	0.0	0.0

G. HISTOPATHOLOGY OF THE PLACENTA

All findings noted in the placentas were either single observations, or were biologically equally distributed between control and treated animals. All of them were considered to be incidental and/or spontaneous in origin. Special stains, such as PAS (Periodic-Acid-Schiff), H-MG (Hart stain combined with Masson's Goldner Trichrome stain) and Kossa's stain did not reveal special pathological findings.

III. SUMMARY AND CONCLUSIONS

In the range-finding study for selection of dose levels for a prenatal developmental toxicity study in guinea pigs, the highest test dose level of 180 mg/kg bw/d caused exaggerated toxicity resulting in the death of one third of the treated animals until mid-pregnancy (7 out of 20 guinea pigs died). Thus, this dose was out of the question as a suitable dose for a definitive prenatal developmental toxicity study and the remaining animals of this group were sacrificed prematurely, for humane reasons.

At the other dose levels, top dose now being 50 mg/kg bw/d, neither significant signs of systemic toxicity nor effects on reproduction or offspring were detected. Maternal effects were confined to slight hematological changes and to alterations of certain hormone levels.

With regard to hematological findings, lower absolute basophil counts were observed in guinea pigs treated with 50 mg/kg bw/d. A reduction of basophilic leukocytes are known to occur as a consequence of increased corticoid hormone levels (Saavedra-Delgado, 1980), which were also observed at this dose level. The decreased basophil count was not accompanied by any other hematological change, the finding was therefore regarded as treatment-related but not adverse.

Blood sampling for hormone investigations were carried out during mid-pregnancy on GD 42 and towards the end of pregnancy on GD 63. On GD 42 higher testosterone levels in all dosed guinea pigs and increased androstenedione levels at 50 mg/kg bw/d were observed, which may be related to aromatase inhibition Furthermore, an increase of the mineralocorticoids 11-

desoxycorticosterone, corticosterone and 18-hydroxycorticosterone occurred in guinea pigs treated with 50 mg/kg bw/d as well as an increase of the glucocorticoid 11-desoxycortisol in all dosed animals; this finding may be a result of a higher steroid hormone production of the adrenals.

At the end of pregnancy on GD 63, testosterone and androstenedione were still higher in guinea pigs of test groups 2 and 3 (15 and 50 mg/kg bw/d); in addition at 50 mg/kg bw/d, higher progesterone and 17-hydroxyprogesterone levels were noted. At this time point mineralocorticoids were not significantly increased anymore, but there were still higher 11-desoxycortisol levels in guinea pigs of test groups 2 and 3 (15 and 50 mg/kg bw/d) compared to controls.

The administration of the test substance to female guinea pigs from GD 6 through GD 63 at dose levels of 5, 15 and 50 mg/kg bw/d led to no treatment-related effects with regard to gross pathology, organ weights and histopathology of placentas. The evaluation of the reproductive parameters revealed no test substance-related effects on conception rate, mean number of corpora lutea, implantation sites, pre- and post-implantation loss and resorptions (total, early and late). No dead fetuses were noted at any dose level. No influence of the test compound on sex distribution of the fetuses and fetal body weights was noted. Examination of the fetuses revealed incidental fetal external malformations in individual fetuses, in all test groups. A consistent pattern and a dose-response relationship were missing. Thus, there was no evidence of an adverse effect of epoxiconazole on external fetal morphology at the tested dose levels up to 50 mg/kg bw/d. In view of the effects seen at 50 and 180 mg/kg bw/day, the top dose level of 90 mg/kg bw/day was selected for the definitive prenatal developmental toxicity study in guinea pigs.

STUDY RELEVANCE

This maternal toxicity study in guinea pigs, in which epoxiconazole was administered to pregnant guinea pigs from gestation day (GD) 6-63, is considered as supplemental information. It provides the justification of the dose selection for both the definitive prenatal developmental toxicity and pre/postnatal developmental toxicity studies in guinea pigs. The study gives relevant information regarding maternal toxicity in guinea pigs as well as effects on guinea pig fetuses, which is useful for interpretation of findings reported in the subsequent investigations with guinea pigs, especially in light of the scarceness of historical control information from developmental toxicity studies in guinea pigs. In this study placental damage or late fetal resorptions were not observable in guinea pigs at a dose level of 50 mg/kg bw/d, a dose level which caused severe placental damage and increased post-implantation loss in rats after treatment from GD 7-21.

2.1.4 Prenatal developmental toxicity study in guinea pigs

STUDY REFERENCE

Report:	Schneider S., Strauss V., Rey Moreno M.C., Becker M., van Ravenzwaay B. 2011b BAS 480 F (Epoxiconazole) Modified Prenatal Developmental Toxicity Study in Guinea Pigs Oral Administration (Gavage) BASF DocID 2011/1229838 Date of report: 14-Sep-2011
Report:	Schneider S., Strauss V., van Ravenzwaay B. 2012 Amendment No. 1 to Report: BAS 480 F (Epoxiconazole) Modified Prenatal Developmental Toxicity Study in Guinea Pigs Oral Administration (Gavage) BASF DocID 2012/… Date of report: in preparation Note: The amendment to the original report includes corrected individual, mean and median values for the adrenal steroid hormone 18-hydroxycorticosterone; the original report had by mistake reported the "norm relative areas" (NRA) solely for 18-hydroxycorticosterone instead of the absolute values. The corrected values for mean and standard deviation are given in this robust study summary.
Guidelines:	Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.31 No. L 142; OECD 414 (2001); OPPTS 870.3700 (1998)
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz)

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.

2. Vehicle controls:	Carboxymethylcellulose (CMC; 1% aqueous solution)
3. Test animals:	
Species:	Guinea pig
Strain:	Dunkin-Hartley [Crl:HA]
Sex:	Female
Age:	Time-mated, supplied on GD 4
Weight at arrival (GD 4):	719 to 1162 g
Source:	Charles River Lab., Germany
Acclimatization period:	at least 2 days
Diet:	Kliba maintenance diet for rabbits & guinea pigs "GLP" meal, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type H-temp (PSU) cages (Tecniplast, Germany), floor area about 2065 cm² with dust free wooden bedding
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work:	30-Aug-2010 to 05-Aug-2011 (in-life dates: 1-Sep-2010 (Start of treatment of 1st cohort at gestation day (GD) 6) to 25-Nov-2010 (Sacrifice of the 5th cohort on GD 63)

2. Animal assignment and treatment:

Upon arrival of time-mated female guinea pigs on GD 4, the animals were randomly allocated to the different test groups and placed to the cages. Starting on GD 6, groups of 30 animals received a daily dose of epoxiconazole in 1% aqueous carboxymethylcellulose suspension (1% CMC) by oral gavage at dose levels of 15, 50, and 90 mg/kg bw/d until GD 63. A concurrent control group received the vehicle only; all groups were administered the same volume of 10 mL/kg bw.

Table 2/36 Test groups and doses

Test group	Dose (mg/kg bw/d)	Concentration (mg/100 mL)	Volume (mL/kg bw)	Treatment period	No. of animals (mated)	Animal code nos.
0	0 (1% CMC)	0	10	GD 6–63	30	001-030
1	15	150	10	GD 6–63	30	031-060
2	50	500	10	GD 6–63	30	061-090
3	90	900	10	GD 6–63	30	091-120

3. Test substance preparation and analysis:

Prior to study initiation the aqueous test substance preparations of a comparable batch were shown to be stable over a 7-day period. Thus, test-substance preparations were performed at the beginning of the administration period and thereafter at maximum intervals of 7 days.

Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in 1% CMC using a high-speed homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals.

Test-article concentration analyses were performed twice at the beginning and towards the end of the study. The homogeneity of the dose suspensions was verified at the beginning of the study by taking 3 samples from the top, middle and bottom of the beaker for the low and high dose levels (15 and 90 mg/kg bw/d) while a magnetic stirrer was running. The results of these analyses are given in the table below.

Relative standard deviations of maximum 0.5% indicated the homogenous distribution of epoxiconazole in the dosing suspensions. The actual nominal test-item concentrations were in the range of 98.6 to 102.9% of the target nominal concentrations and thus in the acceptable range.

Analysis of preparations for homogeneity and test-item content					
Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Mean analytical concentration [g/100 mL]	% of nominal concentration	Mean ± SD
1% CMC	31.08.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		0.15	0.148	98.9	99.5 ± 0.5
			0.150	100.0	
			0.150	99.7	
		0.50	0.502	100.5	---
		0.90	0.895	99.5	99.0 ± 0.4
			0.887	98.6	
			0.890	98.8	
	11.11.2010 [Concentration control analyses]	0	n.d.	----	---
		0.15	0.153	102.0	---
		0.50	0.514	102.9	---
		0.90	0.917	101.9	---

4. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption [a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of post-implantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal proportions

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

Statistics for pathology and clinical pathology	
Parameter	**Statistical test**
Hematology, clinical chemistry, organ weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
Hormones	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the MANN-WHITNEY-U test (two-sided) for the hypothesis of equal medians.

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

2. Body weight and food consumption

All animals were weighed on GD 4, 6, 9, 13, 16, 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62 and 63. The body weight change of the animals was calculated from these results.

In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 63 minus weight of the unopened uterus minus body weight on GD 6).

Food consumption was determined for the time periods GD 4-6, 6-13, 13-20, 20-27, 27-34, 34-41, 41-48, 48-55, 55-62 and 62-63.

Only pregnant sows were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant sows with scheduled sacrifice on GD 63 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Hematology and clinical chemistry

On the day of sacrifice, blood was drawn in the morning from non-fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.

The following hematological and clinical chemistry parameters were determined for all surviving test group animals:

Hematology (GD 63):					
	Red blood cells		*White blood cells*		*Clotting potential*
✓	Erythrocyte count (RBC)	✓	White blood cell count (WBC)	✓	Platelet count (PLT)
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)		
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells (differential)		

Clinical chemistry (GD 63):					
	Electrolytes		*Metabolites and proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
		✓	Cholesterol	✓	Alkaline phosphatase (ALP)
		✓	Creatinine	✓	γ-glutamyl transferase (γ-GT)
		✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

Hormones (GD 63):			
✓	11-desoxycorticosterone	✓	Corticosterone
✓	11-desoxycortisol	✓	Cortisol
✓	Progesterone	✓	Testosterone (TESTO)
✓	Androstenedione	✓	Estradiol (E2)
✓	18-hydroxycorticosterone		

4. Sacrifice

On GD 63 morning, blood was sampled in randomized order from anesthetized sows, which were subsequently killed by decapitation. The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Photograph of the unopened uterus with all contents in situ (CD with all photographs in the raw data archive)
- Number of corpora lutea
- Number and distribution of implantation sites classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to SALEWSKI in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue visible

c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]: } \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]: } \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]: } \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

Dams were subsequently assessed by gross pathology. The following organs from all dams sacrificed on schedule were weighed and fixed in neutral buffered 4% formaldehyde solution:

- Adrenal glands
- Liver
- Ovaries

Additionally, from all sows sacrificed on schedule, the …:

- Placentas (including uterine wall)

… were fixed in neutral buffered 4% formaldehyde solution. After a 24-48h fixation period, the placentas were transferred to 70% ethanol solution for asservation.

5. Histopathology

The ovaries and placentas from all animals that were sacrificed on schedule were examined by light microscopy. For this purpose the placentas were trimmed by a transverse cut in the center (parallel to the umbilical cord) in order to obtain a section visualizing the different placental zones including the subplacenta. The ovaries and placentas were stained with Hematoxylin-eosin (H&E).

6. Examination of fetuses

When the uterus was opened the viability of the fetuses and the condition of placentae, umbilical cords, fetal membranes, and fluids were carefully examined in situ. After dissection from the uterus each fetus was sexed and external tissues and all orifices were examined macroscopically. The fetuses were weighed, the corresponding placentas processed for gross and microscopical examination. Thereafter, the fetuses were sacrificed by subcutaneous injection of pentobarbital (Narcoren®; dose: 0.5 mL per fetus).

After the fetuses had been sacrificed, the abdomen and the thorax were opened in order to examine the organs in situ before they were removed. The heart and the kidneys were sectioned in order to evaluate their internal structure. The sex of the

fetuses was determined by examination of the gonads in situ. After these examinations, the heads of approximately one half of the fetuses per litter and the heads of those fetuses which revealed severe findings already during the external examination were severed from the trunk. These heads were fixed in Bouin's solution and were, after fixation, processed and evaluated according to Wilson's method (Wilson and Warkany, 1965). About 10 transverse sections were prepared per head. After the examination these heads were discarded.

All fetuses (partly without heads) were skinned and fixed in ethyl alcohol.

After fixation for approx. 1-5 days, a number of fetuses in cohorts I / II were removed from the fixative for a few minutes. With a scalpel, a transversal incision was made into the frontal / parietal bone in the heads of the intact fetuses. The two halves of the calvarium were then cautiously bent outward and the brain was thoroughly examined. Subsequently, the fetuses were placed back into the fixative for further fixation. No cross section of the heads fixed in ethanol was conducted for cohorts III, IV; V and partly for cohort II. This investigation was not completed for all cohorts because it became clear from the assessment in cohorts I / II that the ossification of guinea pig heads of this age was very advanced and there was a high risk to sliver the frontal and other skull bones.

After fixation in ethyl alcohol the skeletons were stained according to a modified method of Kimmel and Trammell (1981). Thereafter, the stained skeleton of each fetus was examined. After the examination the stained skeletons were retained individually.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al. (1997) and its updated version by Makris et al. (2009) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al. (Chahoud et al. 1999; Solecki et al. 2001 and 2003; for detailed references see study report):

Malformation	A permanent structural change that is likely to adversely affect the survival or health
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Note: Only pregnant sows were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on gestation day 63 p.c. were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data. For the above reasons the following females were excluded from the above-mentioned calculations:

Epoxiconazole dose (mg/kg bw/d)			
0	15	50	90
5, 6, 28	35, 43, 55, 57	63, 75, 83, 84, 86	94, 101s, 108, 111, 118, 119

Thus, each test group including the controls contained a sufficient number of pregnant females to allow meaningful interpretation of the data.

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

Four guinea pigs of the control group and two animals from each treatment group aborted or delivered early, and were subsequently sacrificed. In addition, 2-4 guinea pigs per treatment groups were either found dead or were sacrificed in a moribund state during the course of the treatment period (GD 6-63). None of these mortalities were considered to be related to treatment.

Table 2/37 Mortality

Parameter / Dose group	0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)	0	15	50	90
Number of pregnant sows	27	26	25	23
… with early delivery / aborted	4	2	2	2
… died during gavaging		1		
… sacrificed moribund		1	2	2
… found dead			2	
Pregnant at terminal sacrifice	23	22	19	19

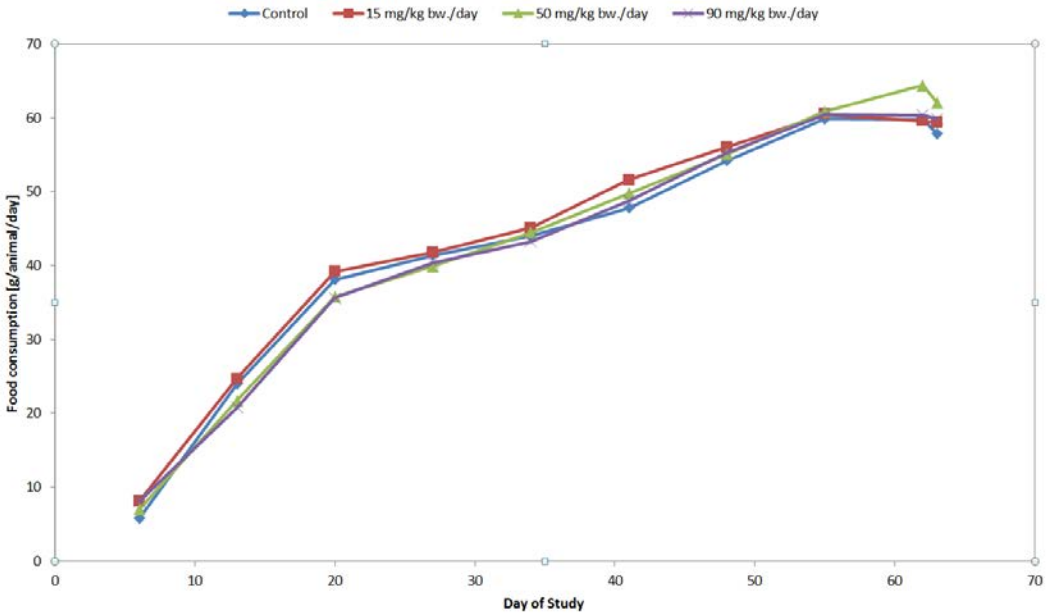
2. Clinical signs of toxicity

Reduced or no defecation was observed in some guinea pigs from both control and treatment groups; in addition, one pregnant sow of the high dose group showed reduced nutritional state, vaginal hemorrhage and piloerection. None of these findings was considered to be related to treatment.

C. BODY WEIGHT AND FOOD CONSUMPTION

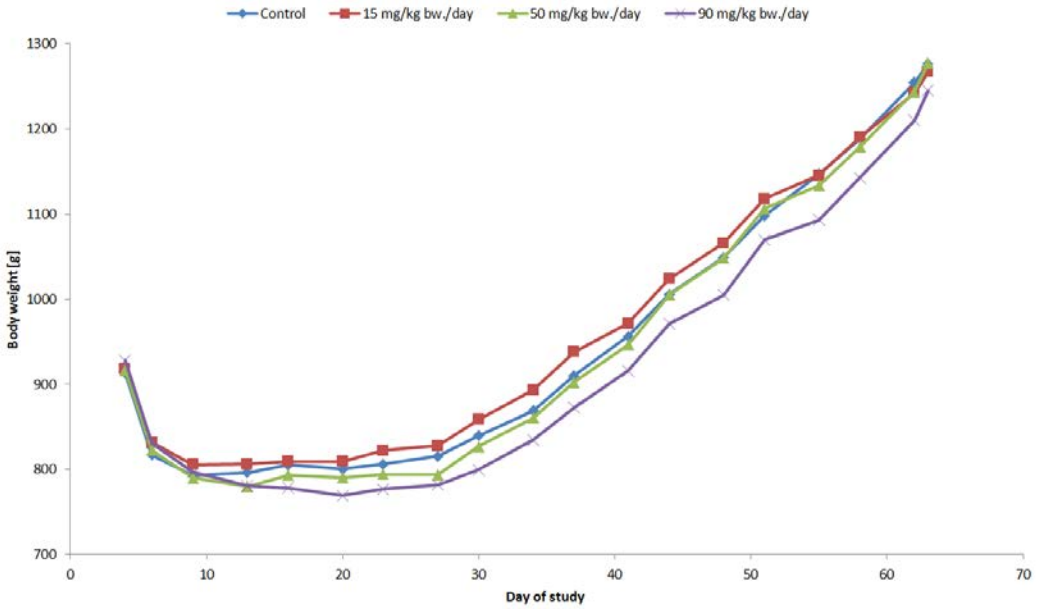
1. Food consumption (see Figure 2/4)
There were no test substance-related changes of food consumption during the whole study in guinea pigs exposed to epoxiconazole dose levels of up to 90 mg/kg bw/d.

Figure 2/4 Food consumption



2. Body weight and body weight gain (see Figure 2/5)
Body weight and body weight gain of treated pregnant guinea pigs (15, 50 and 90 mg/kg bw/d) was not significantly influenced by epoxiconazole administration during the study.

Figure 2/5 Body weight development



D. NECROPSY OBSERVATIONS

1. Corrected (net) body weight gain

The average carcass weight and the corrected body weight gain of treated females were not influenced by the treatment. Mean gravid uterus weights were also not affected by exposure to epoxiconazole.

Table 2/38 Gravid uterus wt, maternal carcass wt and maternal bw change

	Epoxiconazole dose (mg/kg bw/d)			
	0	15	50	90
N	23	22	19	19
Gravid uterus wt [g]	417.8 ± 146.70	401.2 ± 142.39	393.1 ± 91.23	392.1 ±158.31
Carcass wt [g]	858.4 ±123.20	865.5 ± 62.33	883.9 ± 113.73	852.9 ± 84.43
Net wt change from GD 6 [g]	41.0 ± 107.56	52.2 ± 64.80	58.3 ± 69.27	30.3 ± 68.16

Carcass wt = terminal body wt minus uterine wt; Net wt change from GD 6 = Carcass wt minus GD 6 bw
Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, 2-sided)

2. Hematology (Table 2/39)

At the end of pregnancy, slightly decreased values were measured for red blood cell (RBC) counts (-6.4%), hematocrit (-7.5%) and hemoglobin concentration (-5.8%) in guinea pigs administered 90 mg/kg bw/d. Mean corpuscular hemoglobin concentrations of treatment groups were marginally higher than controls, but were not dose-dependently changed and therefore this finding was considered to be incidental. Differential analysis of the white blood cell composition revealed reductions in the absolute and relative values obtained for basophilic cell counts and in the absolute large unstained cell (LUC) counts at 90 mg/kg bw/d. Lower LUC counts have no toxicological relevance.

Table 2/39 Hematology

	Epoxiconazole dose (mg/kg bw/d)			
	0	15	50	90
N	23	21	18	18
Red blood cell count [tera/L]	4.36 ± 0.40	4.35 ± 0.48	4.45 ± 0.25	**4.08 ± 0.34***
Hemoglobin [mM]	6.9 ± 0.6	6.8 ± 0.7	7.1 ± 0.4	**6.5 ± 0.5***
Hematocrit [L/L]	0.346 ± 0.028	0.340 ± 0.035	0.351 ± 0.020	**0.320 ± 0.026****
Mean corpuscular Hb conc (MCHC)	19.90 ± 0.24	**20.06 ± 0.26***	**20.16 ± 0.26****	**20.19 ± 0.32****
Platelets [giga/L]	410 ± 88	414 ± 117	470 ± 130	411 ± 113
White blood cells (WBC) [giga/L]	6.08 ± 1.46	5.57 ± 1.15	6.76 ± 1.72	5.38 ± 1.00
Abs. basophils (BASOA) [giga/L]	0.04 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	**0.02 ± 0.01****
Rel. basophils (BASO) [%]	0.6 ± 0.2	0.6 ± 0.3	0.5 ± 0.2	**0.4 ± 0.2****
Abs. large unstained (LUCA) [giga/L]	0.17 ± 0.09	0.14 ± 0.08	0.14 ± 0.05	**0.10 ± 0.05****
Rel. large unstained (LUC) [%]	2.9 ± 1.5	2.5 ± 1.5	2.1 ± 0.6	1.8 ± 0.8

Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis + Wilcoxon test, 2-sided)

3. Clinical chemistry
Lower total bilirubin and cholesterol levels were measured in pregnant guinea pigs administered 90 mg/kg bw/d epoxiconazole.

Table 2/40 Clinical chemistry

	Epoxiconazole dose (mg/kg bw/d)			
	0	15	50	90
N	23	21	19	18
Total bilirubin [µM]	0.80 ± 0.23	0.70 ± 0.26	0.70 ± 0.20	**0.56 ± 0.24****
Cholesterol [mM]	0.15 ± 0.07	0.14 ± 0.07	0.11 ± 0.07	**0.07 ± 0.04****

4. Hormone changes [see Table 2/41]
All dosed guinea pigs had higher androstenedione, testosterone as well as 11-desoxycortisol serum levels. This increase was not dose-dependent for the androstenedione levels. In addition, guinea pigs of test groups 3 (90 mg/kg bw/d) had higher 18-hydroxycorticosterone as well as corticosterone serum levels compared to controls.

Estradiol levels were not significantly affected by treatment at either time point of measurement.

Table 2/41 Hormones

Dose group		0	1	2	3
Dose level (mg/kg bw/d)		0	15	50	90
N		23	20	18	18
Progesterone	[nM]	820 ± 299	889 ± 287	876 ± 283	799 ± 242
Estradiol	[pM]	18.7 ± 57.98	3.08 ± 6.13	4.1 ± 6.45	5.32 ± 14.63
Androstenedione	[nM]	9.09 ± 3.76	**14.26 ± 7.35***	**17.05 ± 9.48****	**16.93 ± 7.63****
Testosterone	[nM]	0.63 ± 0.19	**0.89 ± 0.40***	**1.12 ± 0.34****	**1.25 ± 0.41****
Cortisol	[nM]	8300 ± 2419	7954 ± 2020	8214 ± 2647	8682 ± 2100
11-deoxycortisol	[nM]	34.90 ± 14.35	**50.39 ± 23.64***	**75.71 ± 63.03****	**126.26 ± 99.90****
11-deoxycorticosterone	[nM]	250 ± 104	220 ± 123	220 ± 100	255 ± 100
Corticosterone	[nM]	62.38 ± 36.63	62.89 ± 23.60	76.57 ± 41.00	**89.70 ± 27.80****
18-OH-corticosterone	[nM]	9.4 ± 5.3	9.7 ± 4.0	11.5 ± 0.28	**15.3 ± 6.5****

* p≤0.05; ** p≤0.01; ***p≤0.001 (Kruskal-Wallis and Mann-Whitney-U-test, 2-sided)

4. Gross necropsy observations
No test substance-related findings were observed in treated females at necroscopy.

5. Organ weights [see Table 2/42]
When compared to the control group 0 (set to 100%), the adrenal glands in pregnant females of test group 3 (90 mg/kg bw/d) showed a statistically significant increase of +10% (absolute weight) and +12% (relative weight), respectively. A treatment-related effect cannot be ruled out, however no

histopathological investigation of this organ was performed. In the liver, a statistically significant increase of the relative weight was observed in test groups 2 and 3 (50 and 90 mg/kg bw/d). However, the change was only minimal and without a clear dose-dependent relationship (+8% and +6%, at 50 and 90 mg/kg bw/d, respectively). Therefore the liver weight change was not regarded as treatment-related.

All other weight parameters did not show relevant differences when compared with the control group.

Table 2/42 Organ weight

Dose group	0	1	2	3
Dose level (mg/kg bw/d)	0	15	50	90
N	26	25	24	26
Terminal body wt [g]	854.662	858.628 [100%]	876.267 [103%]	835.189 [98%]
Abs. liver wt [g]	23.874	24.846 [104%]	26.795 [112%]	25.037 [105%]
Rel. liver wt [%]	2.827	2.894 [102%]	**3.048** ** **[108%]**	**2.991** * **[106%]**
Abs. adrenal gland wt [mg]	632.769	606.12 [96%]	665.917 [105%]	**697.962** ** **[110%]**
Rel. adrenal gland wt [%]	0.075	0.071 [94%]	0.076 [101%]	**0.084** * **[112%]**
Abs. ovary wt [mg]	266.615	269.84 [101%]	263.0 [99%]	227.462 [85%]
Rel. ovary wt [%]	0.031	0.031 [99%]	0.029 [93%]	0.027 [85%]

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis H and Wilcoxon-test, 2-sided)

6. Histopathology [see Table 2/43 and Table 2/53]
All findings noted in the ovaries and placentas were either single observations, or were biologically equally distributed between control and treated animals. All of them were considered to be incidental and/or spontaneous in origin.

The tabulated results of the histopathological examination of the placenta are summarized in Section G.

Table 2/43 Histopathological findings of the ovaries

Parameter Dose group	0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)	0	15	50	90
Ovaries exam.	26	25	24	26
… Corpora lutea present	26	25	24	26
… Cyst(s)	10	6	5	6
… Mineralization, (multi)focal	10	13	13	8
… Pigment storage,(multi)focal		1	1	

E. CESAREAN SECTION DATA [see Table 2/44]

The conception rate reached 90% in the control group, 87% in test group 1 (15 mg/kg bw/d), 83% in test group 2 (50 mg/kg bw/d) and 77% in test group 3 (90 mg/kg bw/d). A sufficient number of dams were available for the purpose of the study. No test substance-related and/or biologically relevant differences with regard to conception rate, mean number of corpora lutea, implantation sites, pre- and post-implantation loss and resorptions (total, early and late) were observed. No dead fetuses were noted at any dose level.

Table 2/44 Summary of reproduction toxicity data

Dose group	0	1	2	3
Dose level (mg/kg bw/d)	0	15	50	90
Females mated	30	30	30	30
Pregnant	27	26	25	23
Dams mortality	3	3	5	2
Females with abortions / premature births	4	2	2	2
Pregnant at terminal sacrifice	23	22	19	19
Dams with viable fetuses	23	21	19	18
Dams with all resorptions	0	1	0	1
Corpora lutea (mean)	5.7	5.6	5.7	5.8
Implantation sites (mean)	4.7	4.4	4.7	4.8
Pre-implantation loss (mean %)	18.4	21.4	16.4	16.9
Post-implantation loss (mean %)	12.1	8.8	10.7	14.3
Resorptions, total (mean)	0.5	0.4	0.5	0.7
Resorptions, early (mean)	0.3	0.4	0.3	0.7
Resorptions, late (mean)	0.1	0.0	0.2	0.1
Dead fetuses	0	0	0	0
Live fetuses / litter (mean) [mean%]	4.2 [87.9%]	4.1 [95.5%]	4.2 [89.3%]	4.3 [90.5%]
Sex distribution (% live males)	52.1	51.7	46.8	49.4
Fetal weight [g] (mean)	80 ± 13.1	81 ± 10.9	74 ± 10.1	76 ± 7.6

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, two-sided)

The sex distribution of the fetuses in all treated groups was comparable to the control group. Observable differences were without biological relevance.

The mean fetal weights did not show any biologically relevant differences between the test substance-treated groups and the control. The observable differences between the groups reflect the usual fluctuation for this parameter.

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. External examination

Fetal external malformations [see Table 2/45]
Umbilical hernia was the only external malformation which was recorded in fetuses of all dose groups including the controls (0, 15, 50 and 90 mg/kg bw/d). Individual cases and incidences are displayed in the following tables.

Table 2/45 Total external malformations (umbilical hernia)

Dose group		0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)		0	15	50	90
Litter	N	23	21	19	18
Fetuses	N	96	87	79	77
Fetal incidence	N	1 (1.0%)	1 (1.1%)	2 (2.5%)	4 (5.2%)
Litter incidence	N	1 (4.3%)	1 (4.8%)	2 (11%)	3 (17%)
Affected fetuses/litter	Mean%	0.9	0.8	2.4	4.3

Fetal external variations and unclassified observations
External variations were not observed in this study. The unclassified observation "Blood coagulum around the placenta" was recorded for one control fetus.

2. Soft tissue examination

Fetal soft tissue malformations
No soft tissue malformations were observed in any of the test groups.

Fetal soft tissue variations [see Table 2/46]
Malpositioned carotid origin was detected in the low- and mid-dose groups as well as the control group as the only type of soft tissue variation. As there is no dose-response relationship, this finding was considered to have occurred spontaneously and not to be related to treatment.

Table 2/46 Total soft tissue variations (malpositioned carotid origin)

Dose group		0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)		0	15	50	90
Litter	N	23	21	19	18
Fetuses	N	96	87	79	77
Fetal incidence	N (%)	5 (5.2%)	2 (2.3%)	1 (1.3%)	0 (0.0%)
Litter incidence	N (%)	5 (22%)	2 (9.5%)	1 (5.3)%	0 (0.0%)
Affected fetuses/litter	Mean%	5.6	1.9	1.1	0.0

Fetal soft tissue unclassified observations [see Table 2/47]
Discolored kidneys were recorded for some fetuses of test group 1 (50 mg/kg bw/d). As there is no dose-response relationship, these findings were considered to be spontaneous in nature and without a relation to treatment.

Table 2/47 Total unclassified soft tissue observations

Dose group		0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)		0	5	15	50
Litter	N	23	21	19	18
Fetuses	N	96	87	79	77
Fetal incidence	N (%)	0 (0.0%)	3 (3.4%)	0 (0.0%)	0 (0.0%)
Litter incidence	N (%)	0 (0.0%)	2 (9.5%)	0 (0.0%)	0 (0.0%)
Affected fetuses/litter	Mean%	0.0	3.3	0.0	0.0

3. Skeletal examination [see Table 2/48]
Malformations of the fetal skeletons were recorded in fetuses of all dose groups including the controls (0, 15, 50 and 90 mg/kg bw/d). Neither for the individual findings nor the overall incidences a dose-response relationship was present. Thus, these findings were considered to be spontaneous in nature and without a relation to treatment.

Variations in different skeletal structures were detected with or without effects on the corresponding cartilages. The observed skeletal variations were related to various parts of the fetal skeletons and were statistically significantly increased in the low and the high-dose groups on a fetus per litter basis.

Table 2/48 Total skeletal malformations and variations

Dose group			0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)			0	15	50	90
Litter		N	23	21	19	18
Fetuses		N	96	87	79	77
TOTAL MALFORMATIONS						
Fetal incidence		N	6 (6.3%)	7 (8.0%)	3 (3.8%)	7 (9.1%)
Litter incidence	(Fi)	N	5 (22%)	6 (29%)	3 (16%)	4 (22%)
Affected fetuses/litter	(Wi)	Mean%	6.2	6.9	4.1	11.4
TOTAL VARIATIONS						
Fetal incidence		N	43 (45%)	56 (64%)	54 (68%)	56 (73%)
Litter incidence	(Fi)	N	20 (87%)	20 (95%)	17 (89%)	18 (100%)
Affected fetuses/litter	(Wi)	Mean%	50.4	**68.1***	**66.1***	**75.5****

* $p \leq 0.05$; ** $p \leq 0.01$ (Fi: Fisher's Exact test, 1-sided; Wi: Wilcoxon test, 1-sided)

For a better overview, all individual skeletal variations with statistically significant differences between the control and the treated groups were compiled in the table below:

Table 2/49 Individual skeletal variations with statistically significant increases

Dose group			0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)			0	15	50	90
Litter		N	23	21	19	18
Fetuses		N	96	87	79	77
THORACIC CENTRUM FUSED WITH ARCH						
Fetal incidence		N	7 (7.3%)	19 (22%)	23 (29%)	25 (32%)
Litter incidence	(Fi)	N	6 (26%)	11 (52%)	**12 (63%)***	**13 (72%)****
Affected fetuses/litter	(Wi)	Mean%	6.6	**21.7***	**26.3****	**38.8****
MISSHAPEN STERNEBRA						
Fetal incidence		N	5 (5.2%)	11 (13%)	6 (7.6%)	12 (16%)
Litter incidence	(Fi)	N	4 (17%)	7 (33%)	5 (26%)	**9 (50%)***
Affected fetuses/litter	(Wi)	Mean%	6.6	11.8	7.1	**18.8****

* $p \leq 0.05$; ** $p \leq 0.01$ (Fi: Fisher's Exact test, 1-sided; Wi: Wilcoxon test, 1-sided)

Additionally, isolated cartilage findings without influence on the respective bone structures, which were designated as unclassified cartilage observations, occurred in all groups including the control. The observed unclassified cartilage findings were exclusively related to the sternum and the ribs and do not exhibit a specific pattern or a dose response. Thus, a toxicological relevance for these findings is not assumed.

Table 2/50 Total skeletal unclassified cartilage observations

Dose group			0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)			0	15	50	90
Litter		N	23	21	19	18
Fetuses		N	96	87	79	77
Fetal incidence		N	2 (2.1%)	13 (15%)	6 (7.6%)	8 (10%)
Litter incidence	(Fi)	N	1 (4.3%)	**6 (29%)***	5 (26%)	**5 (28%)***
Affected fetuses/litter	(Wi)	Mean%	1.4	**12.9***	**7.6***	**17.0***

* $p \leq 0.05$; ** $p \leq 0.01$ (Fi: Fisher's Exact test, 1-sided; Wi: Wilcoxon test, 1-sided)

SUMMARY OF ALL CLASSIFIED FETAL EXTERNAL, SOFT TISSUE AND SKELETAL OBSERVATIONS AND THEIR ASSESSMENT

Malformations

One external malformation (umbilical hernia, see Table 2/45) occurred at litter incidences of 1, 1, 2, 3 in all test groups including control (0, 15, 50, 90 mg/kg bw/d). The presence of this finding in control animals and the low general incidence indicate that a specific effect of the test substance on complete closure of abdominal wall at the umbilical ring cannot be assumed. The lack of historical data for guinea pigs makes an assessment, whether the litter incidence of 3 constitutes a treatment-related increase, difficult. However, this finding appears to be common in guinea pigs. In a recent publication [Rocca and Wehner 2009], the presence of omphalocele (which is a slightly more distinct form of umbilical hernia) in 4 Dunkin-Hartley fetuses from 3 separate litters out of 32 control litters is reported. Thus, a relationship of this finding to treatment seems to be rather unlikely.

No soft tissue malformations were noted in this study and skeletal malformations were evenly distributed about the test groups and did not form a specific pattern. Consequently, the incidence of total external, soft tissue and skeletal malformations showed no dose-response relationship (Table 2/51).

Table 2/51 Total fetal malformations (external, soft-tissue and skeletal)

Dose group		0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)		0	15	50	90
Litter	N	23	21	19	18
Fetuses	N	96	87	79	77
Fetal incidence	N (%)	7 (7.3%)	8 (9.2%)	5 (6.3%)	9 (12%)
Litter incidence	N (%)	6 (26%)	7 (33%)	4 (21)%	6 (33%)
Affected fetuses/litter	Mean%	7.1	7.7	6.5	13.4

Variations

External variations did not occur in any of the fetuses in this study. Soft tissue variations, in the form of malpositioned carotid origin (Table 2/46) occurred in the low- and mid-dose group and in the control without any relation to dosing. Skeletal variations (Table 2/48 and Table 2/49) consisted primarily of minor delays or disturbances of ossification. The majority of the skeletal variations are equally distributed among the different test groups including controls, if normal biological variation is taken into account. However, two skeletal variations (thoracic centrum fused with arch and misshapen sternebra) were noted at significantly higher incidences either in all dose groups (though not dose-related) or in the high-dose group only. Such slight retardations of the ossification process occur very frequently in rat and rabbit fetuses and they seem also to be common skeletal abnormalities in guinea pigs. Rocca and Wehner [2009] report skeletal abnormalities such as short rib, discontinuous rib, supernumerary rib, unossified

vertebra, bipartite ossification, and extra sites of ossification to be observable in 35 to 50% of litters (9.6 to 24.7% of fetuses) examined.

An increase of this spontaneously high incidence of skeletal variations is often noted in the presence of maternal toxicity or maternal stress, as has been substantiated in this study for the high-dose females displaying microcytic anemia and increased adrenal weights as well as 18-hydroxycorticosterone and corticosterone serum levels. Taking all this into consideration, the increased skeletal variations in this study and the subsequently increased total incidence of variations were regarded to be of no toxicological relevance and are not classified as adverse events.

Table 2/52 Total fetal variations

Dose group		0	1	2	3
Dose level (mg/kg bw/d)		0	15	50	90
Litter	N	23	21	19	18
Fetuses	N	96	87	79	77
Fetal incidence	N (%)	45 (47%)	56 (64%)	55 (70%)	56 (73%)
Litter incidence	N (%)	21 (91%)	20 (95%)	17 (89)%	18 (100%)
Affected fetuses/litter	Mean%	51.8	**68.1***	**67.2***	**75.5***

Unclassified observations

A spontaneous origin is also assumed for the unclassified cartilage observations (Table 2/50) which were recorded for several fetuses of all test groups (0, 15, 50, or 90 mg/kg bw/d). Distribution and type of these findings do not suggest any relation to treatment.

Thus, the oral administration of epoxiconazole to pregnant guinea pigs had no direct and specific effect on morphology of offspring at any dose level tested (15, 50 and 90 mg/kg bw/d).

G. HISTOPATHOLOGY OF THE PLACENTA [see Table 2/53]

All findings noted in the placentas were either single observations, or were biologically equally distributed between control and treated animals. All of them were considered to be incidental and/or spontaneous in origin.

When compared with the control group, the mean placenta diameter in pregnant females of test groups 1, 2 and 3 did not reveal relevant changes. Therefore, a statistical evaluation was not performed.

Table 2/53 Histopathological findings of the placenta

Dose group	0	1	2	3
Dose level (mg/kg bw/d)	0	15	50	90
PLACENTA EXAMINED / GROUP	99	87	83	78
LABYRINTH				
… Thrombosis, (multi)focal	18	22	19	19
… Necrosis, (multi)focal	4	5	7	2
… Mineralization, (multi)focal	14	15	17	9
… Degeneration, diffuse			1	1
… Hyperplasia, (multi)focal	3			1
… Autolysis / partial autolysis	2		3	
… Atrophy, lobar	2			1
… Hemorrhage, (multi)focal		1		
… Congestion	2			
INTERLOBIUM				
… Thrombosis, (multi)focal	3	8	7	3
… Necrosis, (multi)focal	5	4	5	2
… Mineralization, (multi)focal	2	2		3
… Degeneration, diffuse	1		1	1
… Autolysis / partial autolysis	2		3	
… Malformation, (multi)focal	2	1		1
… Congestion	2			
DECIDUA				
… Congestion			1	
YOLK SAC				
… Crystals	1		2	
… Metaplasia, squamous (multi)focal	1			
… Thrombosis, (multi)focal	3			
… Necrosis, (multi)focal		1		
… Autolysis / partial autolysis	2		1	1
Mean placenta diameter [mm]	28.0	28.6	27.6	28.3

III. SUMMARY AND CONCLUSIONS

In a prenatal developmental toxicity study performed according to OECD test guideline 414, epoxiconazole was administered to pregnant Dunkin-Hartley guinea pigs by stomach tube daily from implantation (GD 6) to a day shortly prior to the expected day of parturition (GD 63). Caesarean sectioning was performed on GD 63.

Up to a dose of 90 mg/kg bw/d, no test substance related effects on **clinical parameters**, such as clinical observations, food consumption, gross and corrected body weights/body weight gain were noted. The mortalities and preterm deliveries were not considered to be test substance-related.

Regarding **clinical pathology**, pregnant guinea pigs of test group 3 (90 mg/kg bw/d) exhibited a mild anemia, indicated by decreased RBC counts, hemoglobin and hematocrit values. A lower total bilirubin level in individuals of this test group as a consequence of the anemia cannot be excluded. Moreover, lower cholesterol levels were observed in guinea pigs of this test group. Similar indications of an adverse effect of the test compound on pregnant females, as this anemia, were noted in previous experiments in rats. The anemic changes were, however, more distinct in rats.

Lower basophil counts in the high-dose guinea pigs (90 mg/kg bw/d) can be correlated with increased corticoid hormone levels in these animals. Because these are the only relevant changed cell counts and they were altered secondarily to higher corticoid hormone levels [Saavedra-Delgado et al, 1980], this decrease was regarded as treatment-related but not adverse.

Higher androgen levels in guinea pigs of all dose groups may be a consequence of the aromatase inhibition in individuals gavaged with the compound. Higher 18-hydroxycorticosterone and corticosterone levels in animals of test group 3 (90 mg/kg bw/d) and increased 11-desoxycortisol levels in all dose groups may be a result of a higher steroid hormone production of the adrenals.

Regarding **pathology**, the only noteworthy finding was a statistically significant increase in the absolute and relative weight of the adrenal glands in the high-dose females (90 mg/kg bw/d). No histopathological investigation of this organ was performed, but the weight increase fits well to the increased 18-hydroxycorticosterone and corticosterone levels in these animals and thus a treatment-related effect cannot be ruled out. It is noteworthy, that other than in rats, histopathology of all guinea pig placentas revealed no adverse effect caused by epoxiconazole up to and including a dose of 90 mg/kg bw/d.

The evaluation of the **reproductive parameters** revealed no test substance-related effects on conception rate, mean number of corpora lutea, implantation sites, pre- and post-implantation loss and resorptions (total, early and late). No dead fetuses were noted at all dose levels.

No influence of the test compound on sex distribution of the fetuses and fetal body weights was noted. Examination of the fetuses revealed a slightly higher rate of

mainly skeletal variations in all dose groups. Such slight retardations of the ossification process occur very frequently in rat and rabbit fetuses and they seem also to be common skeletal abnormalities in guinea pigs as they were also experienced by Rocca and Wehner [2009]**Fehler! Textmarke nicht definiert.**. Taking this and potential consequences of maternal stress at the top dose into consideration, the increased skeletal variations in this study and the subsequently increased total incidence of variations were regarded to be of no toxicological relevance and are not classified as adverse events, although the limited historical data are a significant obstacle for a thorough interpretation of these findings in guinea pigs.

In conclusion, the high dose of 90 mg/kg bw/d caused a mild anemia and signs for a higher steroid hormone production of the adrenals, possibly related to stress in pregnant guinea pigs. It is noteworthy, that other than in rats, histopathology of all placentas revealed no adverse effect caused by epoxiconazole up to and including a dose of 90 mg/kg bw/d. Thus, the no observed adverse effect level (NOAEL) for maternal toxicity is 50 mg/kg body weight/day.

Steroid hormone level alterations were observed starting at a dose of 15 mg/kg bw/d. The increases of estradiol precursors such as testosterone and androstenedione may suggest that guinea pigs are sensitive to aromatase inhibition. In contrast to rats, histopathology of all placentas from guinea pigs revealed no adverse effect caused by epoxiconazole up to and including a dose of 90 mg/kg bw/d.

Test substance-related, specific adverse effects on fetal morphology were not observed in this study, at the tested dose levels up to 90 mg/kg bw/d.

STUDY RELEVANCE

A prenatal developmental toxicity study was conducted in guinea pigs, a species which is more similar to humans than rats in regards to hormonal regulation during pregnancy and parturition. In particular, the guinea pig shares with humans the luteal-placental shift of estradiol production which occurs during mid-pregnancy, with the placenta being the main source of estradiol during late gestation, whereas in rats the maternal ovary is the source of estradiol throughout the complete duration of pregnancy. The study is relevant for classification and labeling of epoxiconazole because it addresses the question of species-specificity of developmental toxicity and also of severe placental damage; both effects were observed in rat studies. The results of the guinea pig study clearly show that both placental damage and late fetal resorptions do not occur in guinea pigs despite epoxiconazole treatment with higher dose levels over a longer duration compared to developmental toxicity studies in rats. Hence the present study supports the conclusion that late fetal resorptions observable in rat studies are of no relevance to humans.

Moreover, there was no evidence of cleft palates in this study with guinea pigs dosed up to 90 mg/kg bw/d from GD 6-63, which was found to be half the lethal dose in the preceded maternal toxicity study. Therefore, the observed absence of

craniofacial malformations in this guinea pig study at half the lethal dose raises doubts on the human relevance of the rat findings.

For an overall relevance assessment of all new data, and for a weight-of-evidence evaluation of all epoxiconazole data relevant for developmental toxicity classification, please refer to Chapters 3 and 4.

2.1.5 Pre-postnatal reproductive toxicity study in guinea pigs

STUDY REFERENCE

Report:	Schneider S., Strauss V., Rey Moreno M.C., Becker M., van Ravenzwaay B. 2011c BAS 480 F (Epoxiconazole) Pre-Postnatal Reproductive Toxicity Study in Guinea Pigs Oral Administration (Gavage) BASF DocID 2011/1140627 Date of report: 12-Jan-2012
Guidelines:	The study was conducted with reference to the following guidelines: Commission Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B; OECD 416 (2001); OPPTS 870.3800 (1998) Note: the cited guidelines are designed for rat models, therefore necessary adaptations were made to account for guinea pig-specific reproductive biology
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz)

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.

2. Vehicle controls:	1% aqueous carboxymethylcellulose solution (1% CMC)
3. Test animals:	
Species:	Guinea pig
Strain:	Dunkin-Hartley [Crl:HA]
Sex:	Female
Age:	nulliparous young adult
Weight at mating (GD 0):	389.6 to 856.3 g
Source:	Charles River Lab., Germany
Acclimatization period:	at least 1 week before mating
Diet:	Kliba maintenance diet for rabbits & guinea pigs "GLP" meal, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type H-temp (PSU) cages (Tecniplast, Germany), floor area about 2065 cm² with dust free wooden bedding; enrichment: non-transparent plastic pipe, length 25 cm, diameter 13.5 cm
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work:	08-Feb-2011 to 05-Aug-2011 [in-life dates: 8-Feb-2011 (first pairing); start of treatment of 1st cohort at gestation day (GD) 6): 15-Feb-2011; sacrifice of last litter after weaning: 30-Jul-2011; sacrifice of last (6th) cohort of parental animals: 05-Aug-2011]

2. Animal assignment and treatment:

The animals were supplied by the breeder at least a week before they were subjected to estrous checks and pairing. Untreated stock males were used as pairing partners. Twice a day (early morning and late afternoon) the females were inspected for evidence of estrous by checking the vaginal membrane. Animals where the vaginal membrane was open were placed in the cage of a male mating partner. When copulation was observed within about 30 min (by observation of several mountings of the male) the female were left with the male for several hours (morning pairing) or overnight (afternoon pairing). When no copulation was observed, the respective female was removed from the cage of the male and

placed back in the stock until it showed evidence of estrous again. Thus, only females where copulation was actually observed were presumed pregnant.

The day of observed copulation is referred to as gestation day (GD) 0, the following day as GD 1.

Presumed pregnant females were checked for pregnancy by palpation of uterine contents during weeks 5 - 7 of pregnancy. Animals that failed this check were removed from the study (including hitherto recorded data) and were replaced by fresh animals until the number of approved pregnancies reached at least 25 in all groups. Removed animals were sacrificed and their uteri were checked for the presence of implantations. Pregnant F_0 female guinea pigs were allowed to deliver pups and to bring them up until weaning on PND 21.

The test substance suspensions were administered to the animals orally (by gavage), once a day from implantation (GD 6) through one day before necropsy (on PND 28, about 1 week after weaning on PND 21), always at approx. the same time in the morning. The animals of the control group were treated with the vehicle (1% carboxymethylcellulose suspension in highly deionized water) in the same way. The dose volume was 10 mL/kg body weight. The calculation of the volume administered was based on the most recent individual body weight.

On the day of necropsy, blood samples were obtained from all remaining surviving parental females by puncturing the retro-orbital venous plexus following isoflurane anesthesia. After the blood sampling the females were sacrificed and examined macroscopically. Thus, the guinea pigs were treated daily for a period of at least 84 days (ca. 63 days during pregnancy, plus 21 days from parturition to end of lactation, plus the time period until necroscopy).

Table 2/54 Test groups and doses

Test group	Dose (mg/kg bw/d)	Concentration (mg/100 mL)	Volume (mL/kg bw)	Treatment period	No. of animals (mated)
0	0 (1% CMC)	0	10	GD 6 – PND 28	27
1	15	150	10	GD 6 – PND 28	25
2	50	500	10	GD 6 – PND 28	27
3	90	900	10	GD 6 – PND 28	27

3. Test substance preparation and analysis:

Prior to study initiation the aqueous test substance preparations of a comparable batch were shown to be stable over a 7-day period. Thus, test-substance preparations were performed at the beginning of the administration period and thereafter at maximum intervals of 7 days.

Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in 1% CMC using a high-speed homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals.

Test-article concentration analyses were performed twice at the beginning and towards the end of the study. The homogeneity of the dose suspensions was verified at the beginning of the study by taking 3 samples from the top, middle and bottom of the beaker for the low and high dose levels (15 and 90 mg/kg bw/d) while a magnetic stirrer was running. The results of these analyses are given in the table below.

Relative standard deviations of maximum 1.7% indicated the homogenous distribution of epoxiconazole in the dosing suspensions. The actual nominal test-item concentrations were in the range of 91.9 to 105.4% of the target nominal concentrations and thus in the acceptable range.

Analysis of preparations for homogeneity and test-item content

Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Mean analytical concentration [g/100 mL]	% of nominal concentration	Mean ± SD
1% CMC	14.02.2011 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		0.15	0.143	95.4	97.4 ± 1.7
			0.147	98.2	
			0.148	98.5	
		0.50	0.494	98.8	---
		0.90	0.889	98.8	99.4 ± 1.0
			0.905	100.5	
			0.889	98.8	
	20.07.2011 [Concentration control analyses]	0.15	0.138	91.9	---
		0.50	0.500	100.0	---
		0.90	0.948	105.4	---

4. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption (parental animals) [a)], body weight, body weight change (parental animals and pups; for the pup weights the litter means were used), duration of gestation, number of pups delivered per litter, post-implantation loss, number of pups, liveborn pups, pups alive PND 4, pups alive PND 21, live birth index, viability index, lactation index	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Gestation index, females with liveborn pups, females with stillborn pups, females with all stillborn pups, pups stillborn, pups died, pups cannibalized, pups sacrificed moribund, number of litters with affected pups at necroscopy	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (one-sided) for the hypothesis of equal means

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

Statistics for pathology and clinical pathology	
Parameter	**Statistical test**
Hematology, clinical chemistry, estradiol, organ weight parameters	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.
Hormones except estradiol	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the MANN-WHITNEY-U test (two-sided) for the hypothesis of equal medians.

C. METHODS

1. Clinical observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily.

The parturition and lactation behavior of the dams was generally evaluated in the mornings in combination with the daily clinical inspection of the dams. Only particular findings (e.g. disability to deliver) were documented on an individual dam basis. On weekdays the parturition behavior of the dams was inspected in the afternoons in addition to the evaluations in the mornings. The day of parturition was considered the 24-hour period from about 15.00 h of one day until about 15.00 h of the following day.

Live pups were examined daily for clinical symptoms (including gross-morphological findings) during the clinical inspection of the dams. If pups showed particular findings these were documented with the dams concerned.

2. Body weight and food consumption

All parental animals were weighed on GD 0, 6, 14, 21, 28, 35, 42, 49, 56, 63 and 65 as well as on PND 0, 7, 14 and 21. The body weight change of the animals was calculated from these results.

Food consumption of parental animals was determined for the time periods GD 0-6, 6-14, 14-21, 21-28, 28-35, 35-42, 42-46, 46-49, 49-53, 53-56, 56-60, 60-63 and 63-65. No food consumption was determined during lactation.

Pup body weights were determined on the day after birth (PND 1) and on PND 7, 14, and 21.

3. Female reproduction and delivery data

For the females, the gestation index was calculated for F_1 litters according to the following equation:

$$\text{Female gestation index [\%]} = \frac{\text{Number of females with live pups on the day of birth}}{\text{Number of females pregnant*}} \times 100$$

* defined as number of females with implants *in utero*

The total number of pups delivered and the number of liveborn and stillborn pups were noted, and the live birth index was calculated for F_1 litters as follows:

$$\text{Live birth index [\%]} = \frac{\text{Number of liveborn pups at birth}}{\text{Total number of pups born}} \times 100$$

The implantations were counted and the post-implantation loss (in %) was calculated. To determine the number of implantation sites, the apparently non-pregnant uteri were stained for about 5 minutes in 10% ammonium sulfide solution according to the method of SALEWSKI.

$$\text{Post-implantation loss [\%]} = \frac{\text{Number of implantations – number of pups delivered}}{\text{Number of implantations}} \times 100$$

3. Litter data

All F_1 pups were examined as soon as possible on the day of birth to determine the total number of pups and the number of liveborn and stillborn members of each litter. Pups, which died before the first examination on the day of birth, were designated as stillborn pups.

The number of live pups/litter was calculated on the day after birth, and on lactation days 4, 7, 14, and 21. Furthermore, viability and lactation indices were calculated as follows:

$$\text{Viability index}\,[\%] = \frac{\text{number of live pups on day 4 after birth}}{\text{number of live pups on the day of birth}} \times 100$$

$$\text{Lactation index}\,[\%] = \frac{\text{number of live pups on day 21 after birth}}{\text{number of live pups on day 4 after birth}} \times 100$$

On the day of birth (PND 0) the sex of the pups was determined. The sex of the pups was finally confirmed at necropsy on PND 21.

$$\text{Sex ratio}\,[\%] = \frac{\text{number of live male or female pups on day 0/21}}{\text{number of live male and female pups on day 0/21}} \times 100$$

4. Hematology and clinical chemistry

On the day of sacrifice of the parental females (PND 28), blood was drawn in the morning from non-fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.

The following hematological and clinical chemistry parameters were determined for all surviving test group animals:

Hematology (PND 28 parental females):					
	Red blood cells		*White blood cells*		*Clotting potential*
✓	Erythrocyte count (RBC)	✓	White blood cell count (WBC)	✓	Platelet count (PLT)
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)		
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells (differential)		

Clinical chemistry (PND 28 parental females):					
	Electrolytes		*Metabolites and proteins*		*Enzymes:*
✓	Calcium	✓	Albumin	✓	Alanine aminotransferase (ALT)
✓	Phosphorus (inorganic)	✓	Bilirubin (total)	✓	Aspartate aminotransferase (AST)
		✓	Cholesterol	✓	Alkaline phosphatase (ALP)
		✓	Creatinine	✓	γ-glutamyl transferase (γ-GT)
		✓	Globulin (by calculation)		
		✓	Glucose		
		✓	Protein (total)		
		✓	Triglycerides		
		✓	Urea		

Hormones (PND 28 parental females):					
✓	11-desoxycorticosterone = 21-hydroxyprogesterone	✓	Corticosterone	✓	18-hydroxycorticosterone
✓	11-desoxycortisol	✓	Cortisol	✓	18-hydroxy-11-deoxycorticosterone
✓	Progesterone	✓	Estradiol	✓	Aldosterone
✓	Androstenedione	✓	Testosterone	✓	Dihydrotestosterone

5. Sacrifice and pathology

Parental animals

Four weeks after parturition (PND28) and one day after last treatment, blood was sampled in the morning from sows, which were subsequently killed by decapitation. The exsanguinated animals were necropsied and assessed by gross pathology, with special attention paid to the reproductive organs. Animals which died intercurrently or were sacrificed in a moribund state were necropsied as soon as possible after their death and assessed by gross pathology.

Organ weights were determined for adrenal glands, brain, kidneys, liver, ovaries, pituitary gland, spleen, thyroid (with parathyroid) glands and uterus.

Histopathological examinations were performed on reproductive organs from all control and high-dose females (cervix uteri, ovaries, oviducts, pituitary gland, uterus and vagina). Adrenal glands and gross lesions from all animals were histopathologically examined.

In addition two guinea pigs from the high-dose group that died or were sacrificed in a moribund state before scheduled sacrifice were histopathologically examined in the same way as the control / high-dose animals necroscopied on PND 28.

The scope of pathological examinations is summarized in the following table:

Pathology (PND 28 parental females): *The following organs were collected (column C), weighed (W) and examined histopathologically (H);* *✓: all groups, #: all animals of control and high dose groups*											
C	W	H		C	W	H		C	W	H	
✓	✓	✓	adrenals	✓	✓		liver	✓	✓		spleen
✓	✓		brain	✓	✓		kidneys	✓	✓		thyroid w. parathyroid gland
✓	✓	#	ovaries[$]	✓		#	oviducts	✓	✓	#	uterus (& cervix uteri)*
✓		#	vagina	✓	✓	#	pituitary		✓		body (anesthetized)
✓		✓	gross lesions								
[$] for animals that died intercurrently the ovaries were fixed in neutral buffered 4% formaldehyde. * The uteri of all cohabited female guinea pigs were examined for the presence and number of implantation sites											

The organs or tissues were fixed in neutral buffered 4% formaldehyde or in modified Davidson's solution. The hematoxylin-eosin (HE) stained slides were examined and assessed by light microscopy.

Pup necroscopy observations

On PND 21, pups were sacrificed under isoflurane anesthesia with CO_2. After sacrifice, all pups were examined externally, eviscerated and their organs were assessed macroscopically. All pups without any notable findings or abnormalities

were discarded after their macroscopic evaluation. Animals with notable findings or abnormalities were further evaluated on a case-by-case basis (e.g., histopathological evaluation or special staining), depending on the findings noted.

II. RESULTS AND DISCUSSION

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

One high-dose F_0 female (No. 150, 90 mg/kg bw/d) was found dead (GD 52) after showing piloerection and vaginal discharge, one high-dose female (No. 138) was sacrificed moribund (GD 71) after showing hypothermia, piloerection and lateral position.

One control animal (No. 12), one low-dose female (No. 221, 15 mg/kg bw/d) and one high-dose female (No. 243) were sacrificed prematurely (GD 46, 57, 27, respectively) because of abortion.

Table 2/55 Mortality

Parameter / Dose group	0	1	2	3
Epoxiconazole dose level (mg/kg bw/d)	0	15	50	90
Number of sows, mated	27	25	27	27
Number of sows, pregnant	27	25	27	27
… sacrificed after early delivery / abortion	1	1	0	1
… sacrificed moribund / found dead	0	0	0	2

2. Clinical signs of toxicity

Apart from the findings described in the mortality section no clinical findings were noted in any females of all dose groups during the gestation period or during lactation of F_1 litters.

C. PARENTAL BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption (see Table 2/56 and Figure 2/6)

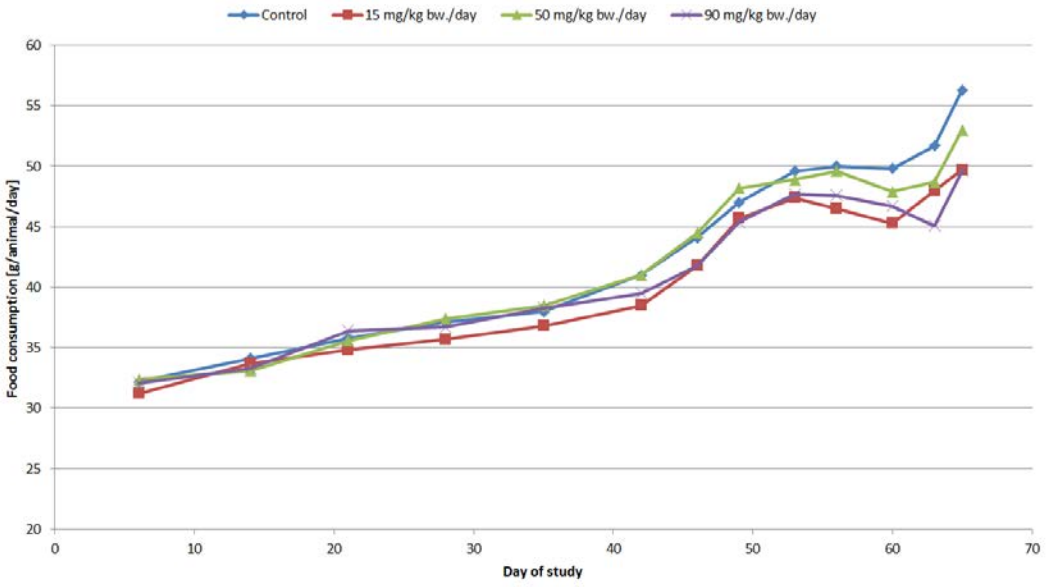
Food consumption of the high-dose F_0 females (90 mg/kg bw/d) was statistically significant below control (12 -13%) during last week of gestation. Food consumption of the low- and mid-dose F_0 females (15 and 50 mg/kg bw/d) was comparable to the control animals during premating, gestation and lactation. The statistically significantly decreased food consumption in the low-dose females during GD63 - 65 was considered not to be related to treatment.

Table 2/56 Mean food consumption

Parameter: mean food intake (g/animal)	Epoxiconazole (mg/kg bw/d)			
	0	15	50	90
GD 49 -53	49.6	47.4	48.9	47.7
Δ%		-4.4%	-1.4%	-3.8%
GD 53 - 56	50.0	46.5	49.6	47.6
Δ%		-7.0%	-0.8%	-4.8%
GD 56 - 60	49.8	45.3	47.9	46.7
Δ%		-9.0%	-3.8%	-6.2%
GD 60 - 63	51.7	48.0	48.7	**45.1****
Δ%		-7.2%	-5.8%	**-12.8%**
GD 63 - 65	56.3	**49.7***	53	**49.6***
Δ%		**-11.7%**	-5.9%	**-11.9%**

Statistical analysis: *p ≤ 0.05; **p ≤ 0.01 (Dunnett-test, 2-sided)

Figure 2/6 Food consumption



2. Body weight and body weight gain (see Figure 2/7)

Body weights of the high-dose F_0 females (90 mg/kg bw/d) were below control at the end of gestation (approx. 6% below control), although the difference was not statistically significant. Body weight gain of the high-dose females was statistically significantly decreased during gestation (approx. 15% below control). Body weights/ body weight gain of all low- and mid-dose females (15 and 50 mg/kg bw/d) were not influenced by the treatment during gestation. During lactation, body weights / body weight gain of sows were not influenced by the treatment.

Figure 2/7 Body weight development

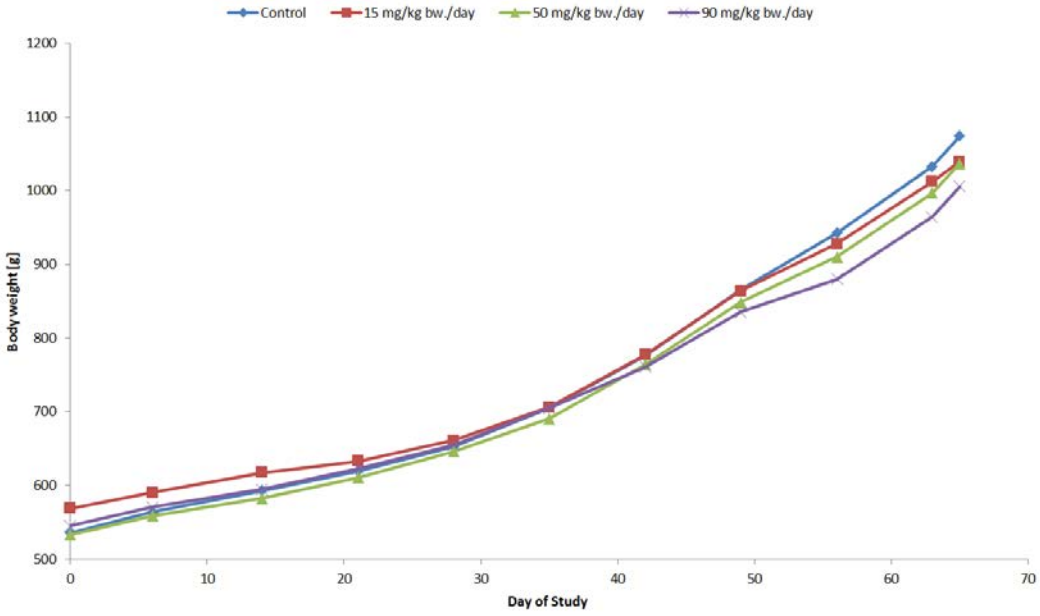


Table 2/57 Body weight change in F_0 females

Parameter: mean food intake (g/animal)	Epoxiconazole (mg/kg bw/d)			
	0	15	50	90
Gestation				
GD 0 - 6	27.9	21.6	26.3	24.8
Δ%		-22.6%	-5.7%	-11.1%
GD 6 - 65	509.0	452	478.1	**433.1***
Δ%		-11.2%	-6.1%	**-14.9%**
GD 0 - 65	538.1	474.1	504.5	**458.6***
Δ%		-11.9%	-6.2%	**-14.8%**
Lactation				
PND 0 - 7	8.9	11.1	-4.1	1.5
PND 7 - 14	-9.2	-8.2	-6.3	2.2
PND 14 - 21	9.2	4.0	11.5	5.4
PND 0 - 21	8.9	7.0	1.1	9.1

Statistical analysis: *p ≤ 0.05; **p ≤ 0.01 (Dunnett-test, 2-sided)

D. FEMALE REPRODUCTION AND DELIVERY DATA

All presumed pregnant guinea pigs which were placed into the study delivered pups or had implants in utero. The mean duration of gestation was equal in all test groups (69 days). All gestational parameters and indices were comparable between control and test substance treated groups.

Table 2/58 Reproduction and gestational parameters of female guinea pigs

Dose	[mg/kg bw/d]	0	15	50	90
Animals per dose		27	25	27	27
- pregnant	[n]	27	25	27	27
- early delivery / aborted	[n]	1	1	0	1
- killed moribund / found dead	[n]	0	0	0	2
- delivering	[n]	26	24	27	24
Females with liveborn		26	24	27	24
- **Gestation index**	[%]	100	100	100	100
- with stillborn pups	[n]	1	2	0	3
- with all stillborn	[n]	0	0	0	0
Duration of gestation	[days]	69 ± 1	69 ± 1	69 ± 1	69 ± 1
Implantation sites, total	[n]	89	85	85	72
- dto per dam	[n]	3.4	3.6	3.1	3.0
Post implantation loss	[n]	0.15	0.42	0.19	0.29
- dto per litter	[mean %]	4.17	9.58	5.86	6.94
Pups delivered	[n]	85	75	80	65
- per dam	[mean n]	3.3	3.1	3.0	2.7
- liveborn	[n]	84	73	80	62
- liveborn per dam	mean n]	3.2	3.0	3.0	2.6*
- **Live birth index**	[%]	99.2	97.9	100	96.4

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test 2-sided)

The number of delivered pups and the average litter size was comparable between control, mid and low-dose groups (0, 15 and 50 mg/kg bw/d). The number of liveborn pups and appeared to be lower in the high-dose group (90 mg/kg bw/d). This apparent decrease is attributed to 2 decedents and one abortion in this dose group, which are considered to be a result of maternal toxicity. This correlation to maternal toxicity is justifiable since neither the average number of implants nor post-implantation loss, gestation and live birth indices were significantly influenced by the high dose of the test material.

The rates of liveborn and stillborn F_1 pups were evenly distributed about the groups. The respective values reflect the normal range of biological variation inherent in the strain used in this study.

Thus, epoxiconazole did not adversely affect gestation and delivery of the F_0 generation parental females.

E. PUP DATA

1. Pup survival and sex ratio (see Table 2/59)

The test substance did not influence pre-weaning pup survival in any of the treated groups (15, 50 and 90 mg/kg bw/d). The sex ratio was also comparable between control and treatment groups.

Table 2/59 Pup survival and sex ratio

Dose		[mg/kg bw/d]	0	15	50	90
Animals per dose			27	25	27	27
Number of litters		[n]	26	24	27	24
- with liveborn pups		[n]	26	24	27	24
- with stillborn pups		[n]	1	2	0	3
- with all stillborn		[n]	0	0	0	0
Pups delivered		[n]	85	75	80	65
- liveborn		[n]	84	73	80	62
- stillborn		[n]	1	2	0	3
Pups alive Day 4			83	73	80	60
- Viability index		[%]	98.1	100	100	95.8
Pups alive Day 21		[n]	83	73	78	59
- Lactation index		[%]	100	100	98.5	99.2
Sex ratio	PND 0	[% live males]	37 / 83 = 45%	29 / 73 = 40%	40 / 80 = 50%	27 / 60 = 45%
	PND 21	[% live males]	37 / 83 = 45%	29 / 73 = 40%	40 / 78 = 51%	26 / 59 = 44%

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test 2-sided)

2. Pup clinical observations

There were no test substance-related clinical findings in pups of any dose group.

3. Pup body weight data (see Figure 2/8 and Figure 2/9)
No test compound-related influence on F1 pup body weights and pup body weight gain were noted in any of the dose groups (15, 50 and 90 mg/kg bw/d).

Figure 2/8 Body weight development of male pups

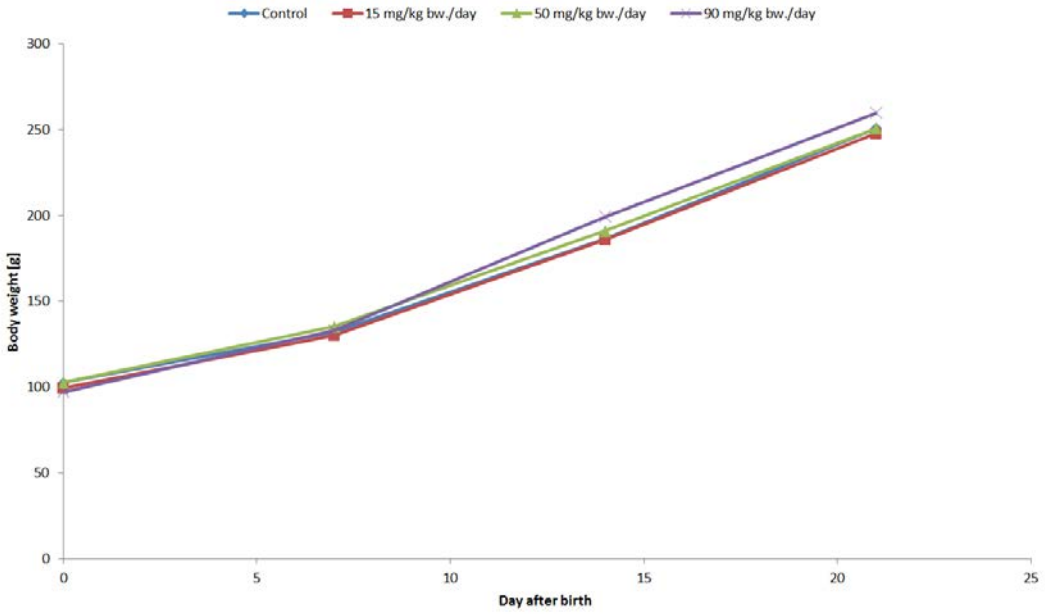
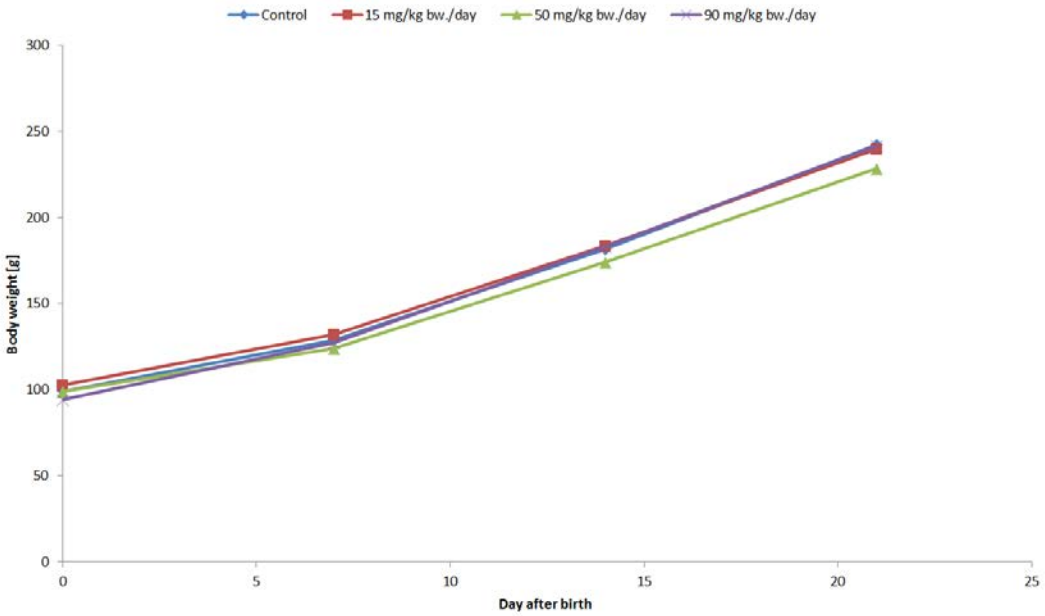


Figure 2/9 Body weight development of female pups



4. Pup necroscopy observations (see Table 2/60)

A few F_1 pups showed spontaneous findings at gross necropsy, such as partly cannibalized, post mortem autolysis, empty stomach, distended stomach, extended intestine and a blind uterine horn. These findings were not considered to be associated to the test substance.

Table 2/60 Pup necropsy observations

Dose [mg/kg bw/d]		0	15	50	90
Pups examined	[n]	37M + 48F	29M+46F	40M + 40F	30M + 35F
Nothing abnormal detected	[n]	37M + 47F	29M+46F	40M + 37F	26M + 35F
Partly cannibalized	[n]				1M
Post-mortem autolysis	[n]				2M
Stomach empty				2F	1M
Stomach + intestine extended		1F			
Blind uterine horn	[n]			1F	

* $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test 2-sided)

F. TERMINAL EXAMINATIONS OF PARENTAL F_0 FEMALES

1. Hematology (Table 2/39)

In all test groups (groups 1, 2 and 3, 15, 50 and 90 mg/kg bw/d) the mean corpuscular hemoglobin concentration (MCHC) was higher compared to controls. The measured red blood cell parameters (i.e., red blood cell (RBC) counts, hematocrit and hemoglobin values) were not altered. Therefore, the changes in MCHC (= hemoglobin / hematocrit) were regarded as incidental and not treatment-related.

Table 2/61 Hematology in F_0 females (selected parameters)

	Epoxiconazole dose (mg/kg bw/d)			
	0	15	50	90
N	26	24	27	24
Red blood cell count [tera/L]	4.99 ± 0.33	4.89 ± 0.39	4.78 ± 0.67	4.93 ± 0.32
Hemoglobin [mM]	7.7 ± 0.5	7.7 ± 0.5	7.7 ± 0.6	7.7 ± 0.4
Hematocrit [L/L]	0.394 ± 0.027	0.385 ± 0.034	0.382 ± 0.035	0.385± 0.024
Mean corpuscular Hb conc (MCHC) [mM]	19.68 ± 0.36	**20.18 ± 1.35***	**20.12 ± 0.43****	**20.09 ± 0.46****
MCV (fL)	78.9 ± 1.8	78.8 ± 2.3	78.7 ± 1.9	78.2 ± 2.4
MCH (fmol)	1.55 ± 0.04	1.59 ± 0.11	1.58 ± 0.04	1.57 ± 0.06
Reticulocytes [%]	1.2 ± 0.6	1.0 ± 0.5	1.0 ± 0.4	1.2 ± 0.8
Platelets [giga/L]	601 ± 112	547 ± 134	510 ± 185	559 ± 76
White blood cell count (WBC) [giga/L]	7.19 ± 1.18	6.81 ± 1.55	6.86 ± 1.85	6.84 ± 1.50
Abs. basophil count (BASOA) [giga/L]	0.08 ± 0.03	0.08 ± 0.03	0.09 ± 0.05	0.06 ± 0.03
Rel. basophil count (BASO) [%]	1.1 ± 0.4	1.1 ± 0.4	1.3 ± 0.7	0.9 ± 0.4
Abs. large unstained (LUCA) [giga/L]	0.22 ± 0.13	0.21 ± 0.12	0.23 ± 0.17	0.22 ± 0.12
Rel. large unstained (LUC) [%]	3.0 ± 1.6	3.1 ± 1.6	3.3 ± 2.3	3.2 ± 1.8

Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis + Wilcoxon test, 2-sided)

2. Clinical chemistry

No treatment-related changes of clinical chemistry parameters were measured. In females of test group 1 (15 mg/kg bw/d), cholesterol and triglyceride levels were higher and in dose group 3 (90 mg/kg bw/d) cholesterol values were lower compared to controls. All mentioned values were not altered dose-dependently and therefore the changes were regarded as incidental and not treatment-related.

Table 2/62 Clinical chemistry in F_0 females (selected parameters)

	Epoxiconazole dose (mg/kg bw/d)			
	0	15	50	90
N	26	24	27	24
Total bilirubin [µM]	0.68 ± 0.27	0.66 ± 0.31	0.60 ± 0.44	0.63 ± 0.56
Cholesterol [mM]	0.68 ± 0.32	**0.81 ± 0.28***	0.64 ± 0.30	**0.50 ± 0.23***
Triglycerides [mM]	0.45 ± 0.18	**0.59 ± 0.19****	0.51 ± 0.21	0.65 ± 0.36

Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$ (Kruskal-Wallis + Wilcoxon test, 2-sided)

4. Hormone changes [see Table 2/63]
In females of test group 3 (90 mg/kg bw/d) progesterone and 21-hydroxyprogesterone (i.e., 11-desoxycorticosterone) levels and in females of test group 2 (50 mg/kg bw/d) 21-hydroxyprogesterone levels were higher compared to controls. Androstenedione, testosterone, dihydrostestosterone as well as aldosterone values in nearly all samples were below the limit of quantification and therefore were not evaluated statistically.

Estradiol levels were not significantly affected by treatment.

Table 2/63 Hormones in F_0 females (PND28)

Dose group		0	1	2	3
Dose level (mg/kg bw/d)		0	15	50	90
Progesterone	[nM]	4.56 ± 5.15	6.65 ± 6.24	7.17 ± 6.16	**8.41 ± 5.39****
21-OH-progesterone	[nM]	0.67 ± 0.58	0.71 ± 0.41	**0.97 ± 0.62***	**1.23 ± 0.61*****
18-OH-corticosterone	[nM]	1.67 ± 0.94	1.35 ± 0.46	1.50 ± 0.75	1.29 ± 0.54
11-deoxycortisol	[nM]	1.63 ± 1.42	1.35 ± 1.58	1.25 ± 0.73	1.79 ± 1.68
Corticosterone	[nM]	8.54 ± 8.81	6.23 ± 8.71	6.06 ± 4.14	7.77 ± 5.62
Cortisol	[nM]	1533 ± 826	1382 ± 590	1325 ± 513	1366 ± 792
Estradiol	[pM]	1.13 ± 2.45	1.12 ± 2.71	0.88 ± 1.83	4.15 ± 15.85

* $p \leq 0.05$; ** $p \leq 0.01$; ***$p \leq 0.001$ (Kruskal-Wallis + Mann-Whitney-U-test; estradiol: … + Wilcoxon-test, 2-sided)

5. Gross necropsy observations
Yellow, white or red foci were observed in the liver of 1/25 animals in test group 1 (15 mg/kg bw/d), 6/27 animals in test group 2 (50 mg/kg bw/d) and 5/27 animals in test group 3 (90 mg/kg bw/d).

Few to single ovarian cysts ranging from 1 to 7 mm in diameter were more evident in the control group (6/27 animals) in comparison to test group 3 (90 mg/kg bw/d) in which cysts were macroscopically visualized in only 1 of 27 animals.

All other gross lesions observed in the test animals occurred singularly and were considered to be incidental and not related to treatment.

6. Organ weights [see Table 2/64**]**
When compared to the control group 0 (set to 100%), statistically significant decreases were found for **uterus** weights in group 1 (15 mg/kg bw/d, -16%* absolute wt) and in group 3 (90 mg/kg bw/d, -17%** absolute wt, -19%** relative wt). The absolute and relative weight decrease of the uterus showed no dose-response relationship. Furthermore, no clear histopathological correlate was found in the uterus of test group 3 (90 mg/kg bw/d) that could explain the weight decrease. Therefore, the uterus weight decrease was regarded as incidental.

The **adrenal weights** when related to body weight were statistically significantly increased in groups 2 and 3 (50 and 90 mg/kg bw/d), without showing a clear dose-dependent response. However, due to histopathological findings in the adrenals of these groups, the change was regarded as treatment related.

Although no microscopic examination was performed in the **livers**, the statistically significant relative weight increase in test group 3 (90 mg/kg bw/d) was regarded as treatment-related but not adverse and most probably responds to an adaptive effect.

All other weight parameters did not show relevant differences when compared with the control group.

Table 2/64 Organ weight in F0 females (PND 28)

Dose group		0	1	2	3
Dose level (mg/kg bw/d)		0	15	50	90
N		26	24	27	24
Terminal body wt	[g]	735.489	727.774 [99%]	715.367 [97%]	736.321 [100%]
Abs uterus wt	[g]	1.959	**1.651*** **[84%]**	1.727 [88%]	**1.617**** **[83%]**
Rel. uterus wt	[%]	2.827	2.894 [85%]	3.048** [90%]	**2.991**** **[81%]**
Abs. liver wt	[g]	22.973	22.414 [98%]	23.304 [101%]	25.157 [110%]
Rel. liver wt	[%]	3.120	3.109 [100%]	3.251 [104%]	**3.423**** **[110%]**
Abs. adrenal gland wt	[mg]	476.0	504.625 [106%]	520.0 [109%]	509.375 [107%]
Rel. adrenal gland wt	[%]	0.065	0.070 [107%]	**0.073**** **[113%]**	**0.070*** **[108%]**
Abs. ovary wt	[mg]	118.808	119.792 [101%]	116.37 [98%]	119.25 [100%]
Rel. ovary wt	[%]	0.016	0.017 [103%]	0.016 [101%]	0.016 [101%]

* $p < 0.05$; ** $p<0.01$ (Kruskal-Wallis H and Wilcoxon-test, 2-sided)

6. Histopathology [see Table 2/65]
Treatment-related findings were observed in the **adrenal glands**: Multifocal areas of cytoplasmic change were detected in the deep zona fasciculata increasing in incidence and severity from test group 2 (50 mg/kg bw/d) to test group 3 (90 mg/kg bw/d). In these areas the cells of the zona fasciculata lost their large vacuoles and displayed slight basophilic and foamy appearance. Other cells contained small, needle-shape cytoplasmic crystals, resembling cholesterol crystals. In addition, activated sinusoidal cells and minimal mononuclear infiltrating cells were also observed. Compared with the control group 0, the vacuolation of the zona fasciculata in test group 2 and 3 (50 and 90 mg/kg bw/d) was increased. The vacuolation in test group 1 (15 mg/kg bw/d) was comparable to the control group.

Table 2/65 Histopathological findings of the adrenal glands

Dose group	0	1	2	3
Dose level (mg/kg bw/d)	0	15	50	90
No. of animals examined [n]	27	25	27	27
Cytoplasmic change, (multi)focal			10	14
… grade 1			8	3
… grade 2			2	11
Vacuolation, cytoplasmic, diffuse	27	25	27	27
… grade 1	1	1	0	3
… grade 2	6	6	2	0
… grade 3	16	14	7	11
… grade 4	4	4	18	13

Only **livers** with macroscopic foci (1/25 in the low dose, 6/27 in the mid dose and 5/27 in the high dose) were examined by light microscopy. These findings correlated with acute focal necrosis (coagulation necrosis type accompanied by minimal or no inflammatory response and occasional, minimal dystrophic mineralization). Since no signs of hepatotoxicity were found in the clinical chemistry of these animals, the acute necrosis was attributed to the isoflurane anesthesia performed for the blood sampling before the animals were sacrificed for necropsy.

Ovarian cysts visualized macroscopically in controls animals correlated with cystic rete ovarii. This finding is reported in the literature to be commonly observed throughout the normal estrus cycle of the guinea pig. Unlike follicular or luteal cysts, rete ovarii cystic structures lack follicular or luteal cells and display single ciliated cells in their epithelium that ranges from columnar to flattened depending on the dilation grade. They do not secrete hormones but might be hormone-dependent.

One control animal (No. 12), one low-dose female (No. 221, 15 mg/kg bw/d) and one high-dose female (No. 243) were sacrificed prematurely because they aborted (GD 46, 57, 27, respectively). One high-dose F_0 female (No. 150, 90 mg/kg bw/d) died intercurrently (GD 52), most likely while it aborted as it became obvious by

the retention of a dead fetus in its vagina, another high-dose animal (No. 138) was sacrificed in a moribund state (GD 71) because it was unable to deliver. All of these animals showed similar alteration in the uterus, cervix and vagina. Namely, animal No. 150 showed necrosis, hemorrhage and severe inflammatory changes affecting mainly the uterus. Animal No. 138 showed similar but less severe changes predominantly in the cervix. In both animals, large congestive mesometrial vessels invaded by trophoblast were observed in the uterus and in the oviducts. All other animals with abortion (No. 12, 221 and 243) showed similar characteristics, the uterus being more severely affected (hemorrhage, necrosis and presence of trophoblast cells) than the vagina (edema, congestion or infiltrates). All other histopathological findings were either single observations, or were biologically equally distributed between control and treated animals. All of them were considered to be incidental and/or spontaneous in origin.

III. SUMMARY AND CONCLUSIONS

Epoxiconazole was administered to pregnant Dunkin-Hartley guinea pigs by stomach tube daily from implantation (GD 6) through weaning (PND 21) until one day before necropsy, at dosages of 0, 15, 50 and 90 mg/kg bw/d.

The only relevant **clinical observation** in the high-dose group was a higher number of abortions and abortion-related mortality in comparison to the control (3 cases vs. 1). In the pregnant high-dose (90 mg/kg bw/d) females, a dose-related intermittent reduction of food consumption as well as significant reductions of body weights/body weight gain were noted, predominantly towards the end of gestation. The abortions may be considered as a consequence of a latent maternal stress at the high dose level which is in line with the mild food consumption/body weight reductions and becomes morphologically evident with the vacuolation of the adrenal cortex (see pathology). However, in the prenatal developmental toxicity study with guinea pigs (see chapter 2.14; Schneider et al. 2011b; DocID 2011/1229838), also three abortions occurred among the 27 control group guinea pigs. Thus, these 3 abortions at the high dose level of this study may also possibly be caused by the general stress of daily gavage administration, and not be test substance related at all. More experience with gavage studies in guinea pigs is needed to make a sound decision.

Hematology and clinical chemistry investigations revealed no treatment-related effects in any of the test substance-treated groups, but hormone changes were noted at the mid- and high-dose level. In the high dose females (90 mg/kg bw/d) increased progesterone and 21-hydroxyprogesterone levels were measured, those of the latter hormone were still higher in female guinea pigs of the mid-dose group (50 mg/kg bw/d). The cause for these hormonal changes is not known. Taking into account the changes in the adrenals (see pathology below) an effect (direct or indirect) on steroid synthesis is possible. A variable onset of the sexual cycle after pregnancy and lactation in the females may also be an explanation for this observation on progesterone levels.

Pathological examinations revealed the **adrenal gland** as the target organ of epoxiconazole in guinea pigs. Mostly, the adrenal cortex was affected, showing a minimal to slight cytoplasmic change in multifocal areas of the deep *zona fasciculata* and an overall increased vacuolation of the whole *zona fasciculata* in the mid- (50 mg/kg bw/d) and high-dose group (90 mg/kg bw/d). The vacuolation affected both test groups equally; the cytoplasmic change was slightly more severe in the high-dose group (90 mg/kg bw/d). The cytoplasmic change was characterized by cells displaying a foamy, slight basophilic appearance or containing cholesterol crystals. Associated with this finding activated sinusoidal cells and minimal mononuclear infiltrates were observed. Altogether, these changes correlated with a significant increase of the relative weight in the adrenal glands in test groups 2 (50 mg/kg bw/d) and 3 (90 mg/kg bw/d) and might reflect either an alteration in the steroid hormone production of the adrenals or potential consequences of maternal stress at mid- and high-dose level. In the **liver**, a statistically significant relative weight increase was noted in test group 3 (90 mg/kg bw/d). A treatment-related adaptive effect appears to be the most likely explanation for this weight increase, but was not confirmed by microscopic examination of the affected organ.

There were no indications from clinical examinations as well as gross and histopathology, that the gavage administration of epoxiconazole adversely affected gestation, parturition or lactation of the parental females up to and including a dose of 90 mg/kg bw/day. Gestation, in particular gestation length, parturition, lactation and weaning as well as sexual organ weights and gross and histopathological findings of these organs were comparable between the guinea pigs of all test groups.

An apparent lower number of liveborn pups is attributed to two decedents and one abortion in this dose group, which are considered to be a result of maternal toxicity. This correlation to maternal toxicity is justifiable since neither the average number of implants nor post-implantation loss, gestation and live birth indices were significantly influenced by the high dose of the test material. Thus, this finding is not considered to be associated to the test substance.

All data recorded during gestation and lactation in terms of embryo-/fetal and pup development gave no indications for any developmental toxicity in the offspring up to a dose level of 90 mg/kg bw/day. Up to this dose level, the test substance did not adversely influence pup viability and pup body weights.

In conclusion, the mid and high dose of 50 and 90 mg/kg bw/d caused signs for an altered steroid hormone production of the adrenals, which was (at least) at the high-dose level related to stress and maternal toxicity in pregnant guinea pigs. Thus, the no observed adverse effect level (NOAEL) for maternal toxicity is 15 mg/kg body weight/day.

No effects were noted on gestation, parturition and up-bringing of offspring, at the tested dose levels up to 90 mg/kg bw/d.

Test substance-related, specific adverse effects on pre- and postnatal development of offspring were not observed in this study, at the tested dose levels up to 90 mg/kg bw/d.

STUDY RELEVANCE

The pre-postnatal reproduction toxicity study in guinea pigs is considered relevant for the overall relevance and reliability assessment of findings from reproduction toxicity and developmental toxicity studies performed with epoxiconazole in rats, especially in the context of reproduction and developmental toxicity classification of epoxiconazole for human health.

The current legal classification of epoxiconazole for reproduction toxicity and developmental toxicity is based on findings from epoxiconazole studies performed with rats. However, already in 1999, specialized experts from the SCP (Scientific Committee on Plants) considered in the case of the pyrimidine fungicide fenarimol, that the "… aromatase inhibition by fenarimol and its specific effects as seen in small rodents is not relevant to the human species". Further, based on guinea pig studies that had been conducted with fenarimol, the SCP concluded that "…the guinea pig model seemed to be the model of choice for defining the level of risk for the human for the effect of aromatase inhibition…".

The pre-postnatal developmental toxicity study with guinea pigs was performed to clarify whether rat effects - typically induced by azoles in rat reproduction toxicity studies and associated with aromatase-inhibition - are species-specific or also observable in a species recommended as animal model to assess the reproduction toxicity of aromatase inhibitors. Therefore, it was of special interest to look for effects on gestation length, the birth process (evidence of dystocia), on live litter size and on viability in this guinea pig study. In the rat two-generation study, these parameters were affected at a dose level of about 23 mg/kg bw/d [Hellwig 1992; see summary in RAC Background Document (2010)]. In the present investigation in guinea pigs, epoxiconazole was administered by oral gavage at dose levels of up to 90 mg/kg bw/d. There were no treatment-related effects on gestation length, parturition, post-implantation loss, pup survival or pup development. Thus, the present pre-postnatal reproduction study in guinea pigs with epoxiconazole provides relevant data which should be taken into account when assessing the relevance of certain, aromatase-associated findings obtained in rat reproduction toxicity studies with epoxiconazole.

In the light of the previous expert evaluation of the aromatase inhibitor fenarimol, it can be concluded that aromatase inhibition by epoxiconazole and its specific effects as seen in small rodents is likely to be not relevant to the human species.

For an overall relevance assessment of all new data, and for a weight-of-evidence evaluation of all epoxiconazole data relevant for developmental toxicity classification, please refer to Chapters 3 and 4.

2.1.6 Prenatal developmental toxicity study in rats III

STUDY REFERENCE

Report:	Schneider S., Strauss V., Fabian E., van Ravenzwaay B. 2010c BAS 480 F (Epoxiconazole) Prenatal Developmental Toxicity Study in Wistar Rats Oral Administration (Gavage) - Subcutaneous Co-administration of Estradiol cyclopentylpropionate BASF DocID 2011/1229839 Date of report: 15-Sep-2011
Guidelines:	There are no guidelines for this mechanistic study. Reference was made to: (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to (EC) No 1907/2006 of European Parliament and of Council on the REACH - Part B No. B.31 No. L 142; OECD 414; OPPTS 870.3700
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz)

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1a. Test Material	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.
1b. Test Material	Estradiol cyclopentylpropionate (ECP)
Description:	Solid, white with a yellow cast
Lot/Batch #:	049H1244
Purity:	99.8%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 04-Feb-2013.
2. Vehicle control:	1% aqueous Carboxymethylcellulose (1% CMC)

3. Test animals:

Species:	Rat
Strain:	Wistar [Crl:WI (Han)]
Sex:	Female
Age:	Sexually mature, virgin rats aged 10-12 weeks
Weight (pregnant rats GD 0):	220.5 ± 7.99 g (range 202.6 - 240.6 g)
Source:	Charles River Lab., Germany
Acclimatization period:	at least 5 days before mating
Diet:	Kliba maintenance diet for mouse/rats "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type M III Makrolon cages (Becker & Co, Castrop-Rauxel, Germany), floor area about 800 cm² with Lignocel fibre dustfree bedding (SSNIFF, Soest, Germany) and wooden gnawing blocks (Abedd Lab. and Vet. Service GmbH, Vienna) offered for enrichment
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work: 21-Sep-2010 to 11-Aug-2011 (in-life dates: 27-Sep-2010 (start treatment of 1st cohort, gestation day (GD) 6) to 15-Oct-2010 (Sacrifice of the 5th cohort, GD 20)

2. Animal assignment and treatment:

After an acclimatization period of at least 5 days, untreated animals were paired at the test facility with an untreated male animal from about 15:30 until 7:30 in the morning of the following day. When sperm were microscopically detected in the vaginal smear, the females were considered being impregnated and transferred into the study. This day was referred to as gestation day 0 (GD 0), the following day as GD 1. Epoxiconazole in 1% aqueous carboxymethylcellulose suspension (1% CMC) or the vehicle 1% CMC only was administered once daily to groups of 19-20 sperm-positive females animals by oral gavage from GD 6 to 15, at the dose level of 180 mg/kg bw/d. The volume administered each day was 10 mL/kg body

weight. The calculation of the administration volume was based on the most recent individual body weight. The estradiol cyclopentylpropionate (ECP) formulations were administered at dose levels of 0 (corn oil), 1 or 2 µg/animal by subcutaneous injection, once a day from GD 6 to 15 in parallel to the epoxiconazole treatment. The volume administered each day was 0.2 mL/animal. These ECP dose levels were selected based on results obtained in the initial epoxiconazole developmental toxicity study with ECP administration (see chapter 2.1.2, Schneider et al. 2010; DocID 2010/1062088). At terminal sacrifice on GD 20, seventeen to eighteen females per group had implantation sites.

Table 2/66 Test groups and doses

Test group	Dose epoxiconazole (mg/kg bw/d)	Concentration epoxiconazole (mg/100 mL)	Volume (mL/kg bw)	Dose ECP (µg/animal/d)	Volume (mL/animal)	No. of animals (mated)
0	0 (1% CMC)	0	10	0 (corn oil)	0.2	19
1	0 (1% CMC)	0	10	1	0.2	20
2	0 (1% CMC)	0	10	2	0.2	20
3	180	1800	10	0 (corn oil)	0.2	20
4	180	1800	10	1	0.2	20
5	180	1800	10	2	0.2	20

3. Test substance preparation and analysis:

Dose formulations of epoxiconazole in 1% CMC were prepared at the beginning of the administration period and thereafter at intervals of 7 days, which took into account the period of established stability. Application suspensions were prepared by weighing appropriate amounts of the test substance in calibrated beakers and suspending the test substance in 1% CMC using a high-speed homogenizer. A magnetic stirrer was used to keep the preparations homogeneous during treatment of the animals. The administered ECP dose formulations were prepared by dilution of an ECP / corn oil stock solution.

The analytical verification of test substance stability in the vehicle for at least 7 days at room temperature was carried out before the study was initiated, using a similar batch of the test substance.

Epoxiconazole concentration analyses were performed twice at the beginning and towards the end of the study. The homogeneity of the 180 mg/kg bw/d dose suspension was verified at the beginning of the study by taking 3 samples from the top, middle and bottom of the beaker while a magnetic stirrer was running. The results of these analyses are given in the table below.

Also ECP concentration control analyses were performed twice during the study period. Since ECP was completely miscible with corn oil, the solutions were considered to be homogenous without further analysis.

Relative standard deviations of maximum 0.7% indicated the homogenous distribution of epoxiconazole in the dosing suspensions. The actual nominal test-item concentrations were in the range of 100.4 to 104.5% of the target nominal concentrations and thus in the acceptable range.

Analysis of preparations for homogeneity and content of epoxiconazole					
Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]	% of nominal concentration	Mean ± SD
1% CMC	26.09.2010 [Homogeneity and concentration control analyses]	0	n.d.	---	---
		1.8	1.860	103.3	103.6 ± 0.7
			1.856	103.1	
			1.880	104.5	
	03.10.2010 [Concentration control analyses]	0	n.d.	---	
		1.8	1.807	100.4	

The mean values of estradiol cyclopentylpropionate in corn oil were found to be in the range of 86.3 – 107.5% of the nominal concentration.

Analysis of preparations for content of estradiol cyclopentylpropionate				
Vehicle	Date of sampling	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]	% of nominal concentration
Corn oil	24.09.2010	1	0.9535	95.4
		2	1.726	86.3
	11.10.2010	1	1.075	107.5
		2	1.920	96.0

4. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following tables:

Statistics for clinical and fetal examinations	
Parameter	**Statistical test**
Food consumption [a)], body weight, body weight change, corrected body weight gain (net maternal body weight change), carcass weight, weight of unopened uterus, number of corpora lutea, number of implantations, number of resorptions, number of live fetuses, proportions of pre-implantation loss, proportions of post-implantation loss, proportions of resorptions, proportion of live fetuses in each litter, litter mean fetal body weight, litter mean placental weight	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, females pregnant at terminal sacrifice, number of litters with fetal findings	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Proportions of fetuses with malformations, variations and/or unclassified observations in each litter	Pairwise comparison of the dose group with the control group using the WILCOXON-test (one-sided) for the hypothesis of equal proportions

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, treatment and post-treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

Statistics for pathology and clinical pathology	
Parameter	**Statistical test**
Hematology, clinical chemistry, hormone concentrations	Non-parametric one-way analysis using KRUSKAL-WALLIS test (two-sided). If the resulting p-value was equal or less than 0.05, a pair wise comparison of each dose group with the control group was performed using the WILCOXON test (two-sided) for the hypothesis of equal medians.

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily. If such signs occurred, the animals were examined several times daily (GD 0-20).

2. Body weight and food consumption

All animals were weighed on GD 0, 1, 3, 6, 8, 10, 13, 15, 17, 19 and 20. The body weight change of the animals was calculated from these results.

In addition, the corrected body weight gain was calculated after terminal sacrifice (terminal body weight on GD 20 minus weight of the unopened uterus minus body weight on GD 6).

Food consumption was determined on GD 1, 3, 6, 8, 10, 13, 15, 17, 19 and 20.

Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on GD 21 were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

3. Hematology and clinical chemistry

On the morning of GD 15, blood was drawn from non-fasted, isoflurane anesthetized animals from the retro-orbital plexus. The blood sampling procedure and the subsequent analysis of the blood and serum samples were carried out in a randomized sequence.

The following hematological and clinical chemistry parameters were determined for all animals:

Hematology:					
	Red blood cells		*White blood cells*		*Clotting Potential*
✓	Erythrocyte count (RBC)	✓	White blood cell count (WBC)	✓	Platelet count (PLT)
✓	Hemoglobin (HGB)	✓	Neutrophils (differential)		
✓	Hematocrit (HCT)	✓	Eosinophils (differential)		
✓	Mean corp. volume (MCV)	✓	Basophils (differential)		
✓	Mean corp. hemoglobin (MCH)	✓	Lymphocytes (differential)		
✓	Mean corp. Hb. conc. (MCHC)	✓	Monocytes (differential)		
✓	Reticulocytes	✓	Large unstained cells (differential)		
Hormones:					
✓	Progesterone (PROG)				
✓	Androstenedione (ANROS)				
✓	Estradiol (E2)				

4. Sacrifice

On GD 20, the dams were sacrificed after blood sampling in randomized order by cervical dislocation (after isoflurane anesthesia) and the fetuses were removed from the uterus. Dams were subsequently assessed by gross pathology. The uteri and the ovaries were removed and the following data were recorded:

- Weight of the unopened uterus
- Photograph of the unopened uterus with all contents in situ (raw data archive)
- Number of corpora lutea
- Number and distribution of implantation sites classified as
  - live fetuses or
  - dead implantations
    a. early resorptions (only decidual or placental tissues visible or positive staining according to SALEWSKI in uteri from apparently non-pregnant animals and the empty uterus horn in the case of single-horn pregnancy)
    b. late resorptions (embryonic or fetal tissue in addition to placental tissue visible
    c. dead fetuses (hypoxemic fetuses which did not breathe spontaneously after the uterus had been opened)

Based on the above the following parameters were calculated:

$$\text{Conception rate [\%]: } \frac{\text{Number of pregnant animals}}{\text{Number of fertilized animals}} \times 100$$

$$\text{Pre-implantation loss [\%]: } \frac{\text{Number of corpora lutea - number of implantations}}{\text{Number of corpora lutea}} \times 100$$

$$\text{Post-implantation loss [\%]: } \frac{\text{Number of implantations - number of live fetuses}}{\text{Number of implantations}} \times 100$$

5. Examination of fetuses

When the uterus was opened the viability of the fetuses and the condition of placentae, umbilical cords, fetal membranes, and fluids were carefully examined in situ. After dissection from the uterus each fetus was sexed and external tissues and all orifices were examined macroscopically. The fetuses were weighed. The sex was determined by observing the distance between the anus and the base of the genital tubercle. Thereafter, the fetuses were sacrificed by subcutaneous injection of pentobarbital (Narcoren®; dose: 0.1 mL per fetus). After these examinations, approximately one half of the fetuses per dam were eviscerated, skinned and placed in ethanol, the other half was placed in Harrison's fluid for fixation.

Soft tissue examination of the fetuses

The fetuses fixed in Harrison's fluid were examined for any visceral findings and subsequently discarded.

Skeletal examination of the fetuses

The skeletons of the fetuses fixed in ethanol were stained according to a modified method of Kimmel and Trammell. Thereafter, the skeletons of these fetuses were examined under a stereomicroscope. After this examination the stained fetal skeletons were retained individually.

Evaluation criteria for assessing the fetuses

Fetal morphology findings were described using the glossary of Wise et al. (1997) as far as possible. Classification of these findings was based on the terms and definitions proposed by Chahoud et al. (Chahoud et al. 1999; Solecki et al. 2001 and 2003):

Malformation	A permanent structural change that is likely to adversely affect the survival or health
Variation	A change that occurs also in fetuses of control animals and is unlikely to adversely affect the survival or health. This includes delays in growth or morphogenesis that has otherwise followed a normal pattern of development.

Moreover, the term **"unclassified observation"** was used for those fetal findings, which could not be classified as malformations or variations (e.g. focal liver necrosis in fetuses).

II. RESULTS AND DISCUSSION

Note: Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. Only pregnant dams with scheduled sacrifice on day 20 p.c. were taken for the calculation of mean gravid uterine weights, mean net maternal body weight change (corrected body weight gain) and summary of reproduction data.

For the above reasons the following females were excluded from the above-mentioned calculations:

Epoxiconazole dose	0 µg ECP/rat	1 µg ECP/rat	2 µg ECP/rat	Comments
0 mg/kg bw/d	017	029, 036, 040	042, 053, 056	Not pregnant
180 mg/kg bw/d	064, 080	086, 089	106, 114, 118	Not pregnant
			115	Sacrificed moribund

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

There were no test-substance-related mortalities in any of the groups. One female from test group 5 (No. 115, 180 mg/kg bw/d epoxiconazole plus 2 µg ECP) was killed moribund because of an accident during the subcutaneous administration procedure.

2. Clinical signs of toxicity

One animal of dose group 3 (No. 74, 180 mg/kg bw/d + 0 µg/animal/d ECP) showed vaginal hemorrhage on GD 20.

There were no clinical findings in all other females during the whole study period.

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption [see Table 2/67 and Figure 2/10]

The mean food consumption in dose group 3 (180 mg/kg bw/d + 0 µg/animal/d ECP), dose group 4 (180 mg/kg bw/d + 1 µg/animal/d ECP) and dose group 5 (180 mg/kg bw/d + 2 µg/animal/d ECP) was statistically significantly reduced during the entire treatment period and shortly thereafter, i.e. on GD 6 through 17. The decrease was biggest on GD 6 - 8 (32 - 37% below control).

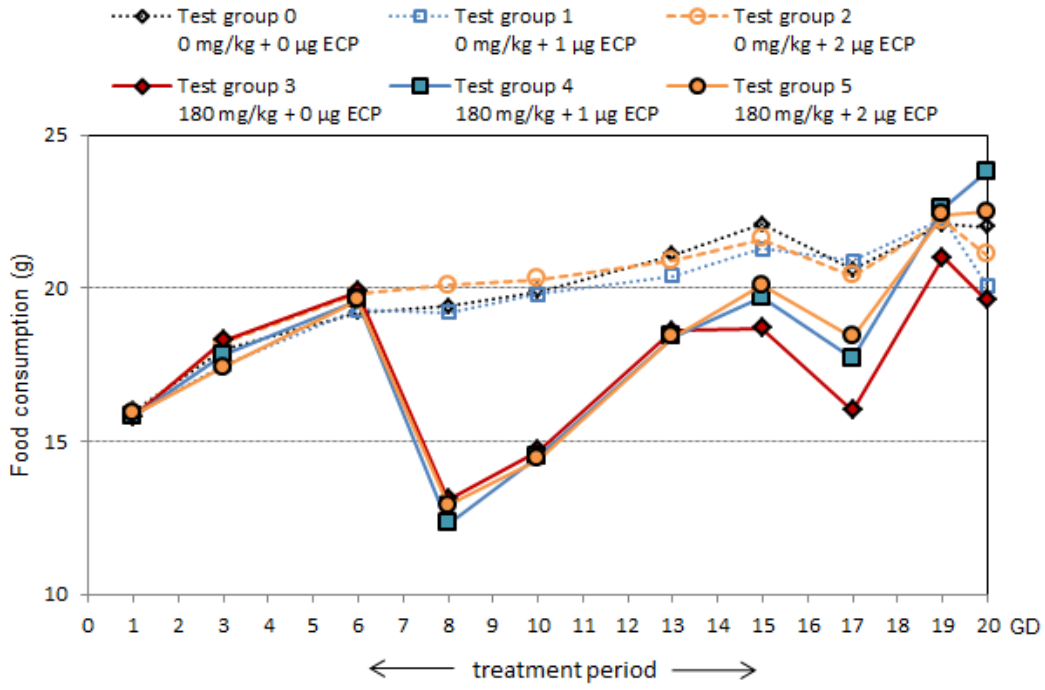
The food consumption in test groups 1 and 2 (0 mg/kg bw/d + 1 / 2 µg/animal/d ECP) was unaffected and did not show any statistically significant or biologically relevant differences in comparison to the controls.

Table 2/67 Food consumption

Parameter: mean food intake (g/animal)	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	0 µg ECP	1 µg ECP	2 µg ECP	0 µg ECP	1 µg ECP	2 µg ECP
GD 3 – 6	19.2	19.3	19.8	19.9	19.6	19.6
Δ%		0.5%	3.1%	3.6%	2.1%	2.1%
GD 6 – 8	19.4	19.2	20.1	**13.1****	**12.3****	**12.9****
Δ%		-1.0%	3.6%	**-32.5%**	**-36.6%**	**-33.5%**
GD 8 – 10	19.9	19.8	20.3	**14.7****	**14.5****	**14.4****
Δ%		-0.5%	2.0%	**-26.1%**	**-27.1%**	**-27.6%**
GD 10 – 13	21.1	20.4	20.9	**18.6****	**18.4****	**18.4****
Δ%		-3.3%	-0.9%	**-11.8%**	**-12.8%**	**-12.8%**
GD 13 – 15	22.1	21.3	21.6	**18.7****	**19.7****	**20.1***
Δ%		-3.6%	-2.3%	**-15.4%**	**-10.9%**	**-9.0%**
GD 15 – 17	20.6	20.9	20.4	**16.0****	**17.7****	**18.4***
Δ%		1.5%	-1.0%	**-22.3%**	**-14.1%**	**-10.7%**
GD 17 – 19	22.1	22.3	22.2	21.0	22.6	22.4
Δ%		0.9%	0.5%	-5.0%	2.3%	1.4%
GD 19 – 20	22.0	20.1	21.1	19.6	23.8	22.5
Δ%		-8.6%	-4.1%	-10.9%	8.2%	2.3%

* p < 0.05; ** p < 0.01 (Dunnett-Test, two-sided)

Figure 2/10 Food consumption



2. Body weight and body weight gain [see Table 2/68 and Figure 2/11]
The mean body weights in dose group 3 (180 mg/kg bw/d + 0 µg/animal/d ECP), dose group 4 (180 mg/kg bw/d + 1 µg/animal/d ECP) and dose group 5 (180 mg/kg bw/d + 2 µg/animal/d ECP) were statistically significantly reduced during the major part of the treatment period and shortly thereafter, i.e. GD 8 - 15 (groups 3 and 4) and GD 8 - 17 (dose group 5). In comparison to the control group, the average decrease was 5 -6%.

The mean body weight gain in dose group 3, 4 and 5 was statistically significantly reduced for the whole treatment period (GD 6 – 15, about 32 - 44% below the concurrent control value). The respective animals actually lost weight on GD 6 – 8. Body weight gain of dose group 3 was also reduced on GD 19 - 20 (about 54% below control) and for the period of GD 0 – 20 (about 20% below control).

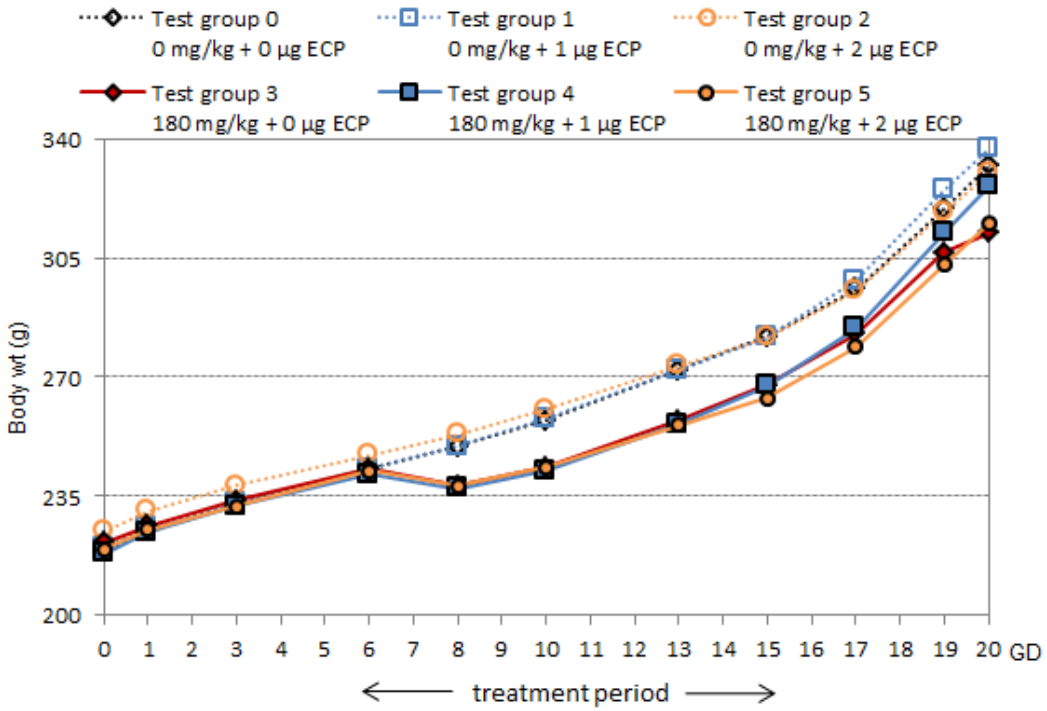
The body weight/body weight gain in test groups 1 and 2 (0 mg/kg bw/d + 1 / 2 µg/animal/d ECP) were unaffected and did not show any statistically significant or biologically relevant differences in comparison to the controls.

Table 2/68 Bodyweight change

Parameter: Body weight change (g/animal)	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	0 µg ECP	1 µg ECP	2 µg ECP	0 µg ECP	1 µg ECP	2 µg ECP
GD 3 – 6	10.3	9.8	9.0	9.1	9.4	10.3
Δ%		-4.9%	-13%	-12%	-8.7%	0.0%
GD 6 – 8	6.3	6.6	6.1	**-4.6****	**-4.0****	**-4.3****
Δ%		4.8%	-3.2%	**-173%**	**-163%**	**-168%**
GD 8 – 10	7.8	8.1	7.4	5.3	5.1	5.6
Δ%		3.8%	-5.1%	-32%	-35%	-28%
GD 10 – 13	14.6	14.4	12.9	13.7	13.7	12.5
Δ%		-1.4%	-12%	-6.2%	-6.2%	-14.4%
GD 13 – 15	9.8	9.5	8.2	10.4	11.3	7.9
Δ%		-3.1%	-16%	6.1%	15%	-19.4%
GD 15 – 17	14.0	16.9	13.7	14.9	16.8	15.2
Δ%		21%	-2.1%	6.4%	20.0%	8.6%
GD 17 – 19	23.8	26.4	22.9	24.0	28.1	24.1
Δ%		11%	-3.8%	0.8%	18%	1.3%
GD 19 – 20	12.5	12.1	11.6	**5.8***	13.7	12.0
Δ%		-3.2%	-7.2%	**-54%**	9.6%	-4.0%
GD 0 – 6	24.1	23.0	22.4	21.4	23.3	22.9
Δ%		-4.6%	-7.1%	-11%	-3.3%	-5.0%
GD 6 – 15	38.6	38.5	34.6	**24.8****	**26.1****	**21.7****
Δ%		-0.3%	-10.4%	**-36%**	**-32%**	**-44%**
GD 0 – 20	113.0	116.8	105.2	**90.9***	108.0	95.5
Δ%		3.4%	-6.9%	**-20%**	-4.4%	-15%

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

Figure 2/11 Body weight development



D. NECROPSY OBSERVATIONS

1. Corrected (net) body weight gain [Table 2/69]
The mean carcass weights in treatment groups administered epoxiconazole (dose group 3, 4 and 5) were statistically significantly reduced, in comparison to the control group, the average decrease was 4-7%. The net body weight change (GD 6 – 20) in these groups 5 was statistically significantly reduced (about 71%, 37% and 44% below the concurrent control value, respectively).

The mean carcass weights/corrected net body weight change in test groups administered 1 or 2 µg/animal/d estradiol cyclopentylpropionate without epoxiconazole (dose group 1 and 2) were unaffected and did not show any statistically significant or biologically relevant differences in comparison to the controls.

The weight of the gravid uterus was not significantly affected by treatment in any test group.

Table 2/69 Mean gravid uterus weights and net body weight change of pregnant rats administered epoxiconazole

Parameter: bw change (g/animal)	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	0 µg ECP	1 µg ECP	2 µg ECP	0 µg ECP	1 µg ECP	2 µg ECP
Gravid uterus (g)	62.4	67.9	56.2	61.9	68.1	58.1
Δ%		+9%	-10%	-1%	+9%	-7%
Carcass (g)	269.7	269.0	273.7	**250.5****	**258.0***	**256.9***
Δ%		0%	+1%	**-7%**	**-4%**	**-5%**
Net weight change from GD 6 (g)	26.5	26.0	26.6	**7.7****	**16.6****	**14.9****
Δ%		-2%	0%	**-71%**	**-37%**	**-44%**

* $p < 0.05$; ** $p < 0.01$ (Dunnett-test, 2-sided)

2. Hematology [Table 2/70]
In epoxiconazole (EXP) plus ECP co-treated rats (test group 4: 180 mg/kg bw/d EPX+1.0 µg/animal/d ECP; test group 5: 180 mg/kg bw/d EPX+2.0 µg/animal/d ECP) some calculated erythrocyte indices were altered (test group 4: decreased mean corpuscular volume (MCV) and mean corpuscular hemoglobin content (MCH); test group 5: decreased MCH and mean corpuscular hemoglobin concentration (MCHC)) without any change of the measured parameters (i.e., red blood cell counts, hematocrit and hemoglobin values). Therefore, these alterations of calculated parameters were regarded as biologically irrelevant.

Rats of test group 1 (1.0 µg/animal/d ECP) showed higher relative basophil counts compared to controls. Because this was the only changed parameter among differential blood cell counts, it was regarded as incidental rather than treatment-related.

In rats of test group 4 (180 mg/kg bw/d EPX+1.0 µg/animal/d ECP) relative reticulocyte counts were higher compared to controls without any deviation in the measured red blood cell parameters. Therefore, this alteration was regarded as maybe treatment-related but not adverse.

In rats of test group 2 (2.0 µg/animal/d ECP) relative reticulocyte counts as well as platelet counts were decreased. The mean platelet count in this group was within the historical control range of pregnant rats (platelet count: 802-1110 giga/L, and therefore was regarded as incidental and not treatment-related.

Table 2/70 Hematology in dams (GD 15)

Parameter	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
White blood cells [giga/L]	5.94	5.42	5.54	5.71	5.92	6.17
(Δ% ctrl)		-9%	-7%	-4%	0%	4%
Red blood cells [tera/L]	6.84	6.86	6.96	6.86	6.85	6.95
(Δ% ctrl)		0%	2%	0%	0%	2%
Hemoglobin [mmol/L]	7.6	7.5	7.9	7.5	7.4	7.5
(Δ% ctrl)		-1%	4%	-1%	-3%	-1%
Hematocrit [L/L]	0.360	0.356	0.369	0.356	0.353	0.360
(Δ% ctrl)		-1%	3%	-1%	-2%	0%
MCV [fL]	52.6	52.0	53.1	51.9	**51.6***	51.7
(Δ% ctrl)		-1%	1%	-1%	**-2%**	-2%
MCH [fM]	1.12	1.10	1.13	1.10	**1.08***	**1.08***
(Δ% ctrl)		-2%	1%	-2%	**-4%**	**-4%**
MCHC [mM]	21.20	21.13	21.38	21.12	21.02	**20.92***
(Δ% ctrl)		0%	1%	0%	-1%	**-1%**
Platelets [giga/L]	1109	1067	**1033**	979	1055	1148
(Δ% ctrl)		-4%	**-7%**	-12%	-5%	4%
Reticulocytes [%]	5.2	5.0	**4.7****	5.3	**6.0***	5.3
(Δ% ctrl)		-4%	**-10%**	2%	**15%**	2%
Rel. basophils [%]	0.0	**0.1***	0.1	0.0	0.1	0.0

* $p < 0.05$; ** $p < 0.01$ (Wilcoxon-Test, 2-sided)

3. Hormone changes [see Table 2/71]

In rats treated with epoxiconazole only (test group 3: (180 mg/kg bw/d + 0 µg/animal/d ECP) no estradiol was detected in rat sera on GD15. Co-treatment of EPX with ECP (test groups 4 and 5) increased the estradiol levels with increasing ECP dose, but estradiol concentrations were still substantially lower compared to the untreated control group.

In the epoxiconazole dose groups without or with ECP co-treatment (test group 3, 4 and 5) the progesterone concentrations where half of the control levels when analysed on GD15. High- dose treatment with ECP (test group 2: 2.0 µg/animal/d ECP) also led to lower progesterone levels.

Rats treated with EPX combined with a high dose ECP (test group 5) showed lower androstenedione concentrations compared to controls.

Table 2/71 Hormones in dams (GD 15)

Parameter	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Estradiol [pM]	20.13 ± 9.67	21.23 ± 13:92	17.78 ± 14:06	**0.00 ± 0:00****	**1.00 ± 4:24****	**2.43 ± 4.84****
(Δ% ctrl)		+5%	-12%	**-100%**	**-95%**	**-88%**
Progesterone [nM]	296.13 ± 87.70	301.35 ± 93.47	**249.88 ± 79.58***	**171.81 ± 54.29****	**143.09 ± 53.14****	**155.28 ± 75.40****
(Δ% ctrl)		+2%	**-16%**	**-42%**	**-52%**	**-48%**
Androstenedione [nM]	6.30 ± 1.41	6.18 ± 1.62	5.34 ± 1.67	7.85 ± 2.65	7.45 ± 2.15	**3.44 ± 1.91****
(Δ% ctrl)		-2%	-15%	+25%	+18%	**-45%**

* p < 0.05; ** p<0.01 (Wilcoxon-test, two-sided)

4. Gross necropsy observations

Findings related to embryo-fetal toxicity are summarized in section E.

The mean gravid uterus weights of all dams of all test groups were comparable to the control and not affected by treatment.

At necroscopy, no test substance-related findings were observed in dams of any test group. One animal of dose group 5 (180 mg/kg bw/d + 2 µg/animal/d ECP) had dilated renal pelves (No. 101). This observation was not considered to be associated to the test compound.

E. CESAREAN SECTION DATA [Table 2/72]

No significant differences between control and treated animals were observed for the conception rate, mean number of corpora lutea, implantation sites, preimplantation loss and the number of early resorptions.

The same is true for post-implantation loss, late resorptions and number of viable fetuses, with the exception of dose group 3 (180 mg/kg bw/d + 0 µg/animal/d ECP). In this epoxiconazole-only treated group a statistically significant increase of the post-implantation loss was observed (34.3% vs. 6.8% in control), which was mainly due to a higher number of late resorptions (31.2%). Consequently, the number of viable fetuses was also lower (-25%) in this test group.

A statistically significantly higher mean number of corpora lutea was noted for test group 1 (0 mg/kg bw/d + 1 µg/animal/d ECP). This is considered to be an incidental finding.

Table 2/72 Summary of reproduction toxicity data

Parameter	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Females mated	19	20	20	20	20	20
Dams pregnant	18	17	17	18	18	17
Female mortality	0	0	0	0	0	1
Dams with all resorptions	0	0	0	1	0	2
Dams with viable fetuses	18	17	17	17	18	14
Corpora lutea (mean)	13.6	15.4*	14.0	14.7	14.4	13.9
Implantation sites (mean)	12.7	14.1	12.5	13.0	13.8	12.1
Pre-implantation loss (mean %)	7.6	7.8	12.2	12.5	3.8	13.9
Post-implantation loss (mean %)	6.8	8.7	14.5	**34.3****	16.2	16.7
Resorptions, total (mean)	0.7	1.1	1.8	**4.3****	2.2	2.0
Resorptions, early (mean)	0.6	1.1	1.1	0.4	0.8	2.0
Resorptions, late (mean)	0.1	0.1	0.8	**3.8****	1.4	0.0
Dead fetuses	0	0	1	0	0	0
Live fetuses / litter (mean) [mean%]	12.0 [93.2%]	13.0 [91.3%]	10.6 [85.5%]	9.2 **[69.6%**]**	11.7 [83.8%]	11.6 [95.2%]
Sex ratio (% live males)	47.2	47.5	49.2	49.0	49.0	56.2
Fetal weight [g] (mean)	3.3	3.4	3.3	**3.0***	3.2	3.5
Placental wt [g] (mean) [Δ% ctrl]	0.47	0.47 [0%]	0.46 [-2%]	**0.87** [+85%]**	**0.75** [+60%]**	**0.61** [+30%]**

* $p < 0.05$; ** $p<0.01$ (Dunnett-test, two-sided)

The sex distribution of the fetuses in all treated groups was comparable to the control group.

The mean placental weights in dose group 3 (180 mg/kg bw/d + 0 µg/animal/d ECP), dose group 4 (180 mg/kg bw/d + 1 µg/animal/d ECP) and dose group 5 (180 mg/kg bw/d + 2 µg/animal/d ECP) were statistically significantly increased. The weight increase was biggest in epoxiconazole-only treated animals and became less distinct with increasing ECP dose. The mean placental weights were comparable to controls in groups 1 and 2 that received only ECP; differences observed in comparison to the control were neither statistically significant nor biologically relevant.

Mean fetal weights were statistically significantly reduced in test group 3 (180 mg/kg bw/d + 0 µg/animal/d ECP): about 9% below control). In all remaining test groups fetal weights were not influenced by epoxiconazole or by (co-) administered ECP. The minor differences between the groups reflect the usual fluctuation for this parameter.

F. EXTERNAL, VISCERAL AND SKELETAL EXAMINATION OF FETUSES

1. External examination

Fetal external malformations [Table 2/73]

External malformations were recorded for a significant number of fetuses and litters in test group 3 (180 mg/kg bw/d + 0 µg/animal/d ECP), test group 4 (180 mg/kg bw/d + 1 µg/animal/d ECP) and test group 5 (180 mg/kg bw/d + 2 µg/animal/d ECP). Apart from one gastroschisis in group 3, these numbers exclusively stand for cleft palates. One gastroschisis was also seen in test group 1 (0 mg/kg bw/d + 1 µg/animal/d ECP) and one cleft palate was found in test group 2 (0 mg/kg bw/d + 2 µg/animal/d ECP).

Table 2/73 Fetal external malformations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
	Fetuses (N)	216	221	182	157	210	162
	Litter (N)	18	17	17	17	18	14
Cleft palate	Fetal incidence	0 (0.0%)	0 (0.0%)	1 (0.5%)	30 (19%)	24 (11%)	23 (14%)
	Litter incidence	0 (0.0%)	0 (0.0%)	1 (5.9%)	**8 (47%)****	**10 (56%)****	**9 (64%)****
	Affected fetuses/litter	0.0	0.0	0.6	**17.6****	**10.6****	**14.4****
Gastroschisis	Fetal incidence	0 (0.0%)	1 (0.5%)	0 (0.0%)	1 (0.6%)	0 (0.0%)	0 (0.0%)
	Litter incidence	0 (0.0%)	1 (5.9%)	0 (0.0%)	1 (5.9%)	0 (0.0%)	0 (0.0%)
	Affected fetuses/litter	0.0	0.5	0.0	0.5	0.0	0.0
Total	Fetal incidence	0 (0.0%)	1 (0.5%)	1 (0.5%)	31 (20%)	24 (11%)	23 (14%)
	Litter incidence	0 (0.0%)	1 (5.9%)	1 (5.9%)	**8 (47%)****	**10 (56%)****	**9 (64%)****
	Affected fetuses/litter	0.0	0.5	0.6	**18.1****	**10.6****	**14.4****

* $p < 0.05$; ** $p<0.01$ (litter incidence: Fisher's Exact test, 1-sided; affected fetuses/litter, Wilcoxon-test, 1-sided)

Fetal external variations

One external variation (limb hyperextension) was observed in one fetus of dose group 5 (180 mg/kg bw/d + 2 µg/animal/d ECP). No relation to treatment is assumed for this single finding. For all remaining fetuses in all dose groups no external variations were observed.

Fetal external unclassified observations [Table 2/74]
Two unclassified external observations, blood coagulum around placenta and placentas fused, were recorded for single fetuses of test groups 2 - 5 and the control. A relation to dosing is not present if normal biological variation is taken into account. Therefore, a treatment-related effect is not assumed.

Table 2/74 Fetal external unclassified observations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Blood coagulum around placenta	Fetal incidence[1]	2 / 216	0 / 221	2 / 182	4 / 157	3 / 210	1 / 162
	Litter incidence[1]	1 / 18	0 / 17	1 / 17	3 / 17	2 / 18	1 / 14
Placenta fused	Fetal incidence[1]	0 / 216	0 / 221	0 / 182	0 / 157	1 / 210	0 / 162
	Litter incidence[1]	0 / 18	0 / 17	0 / 17	0 / 17	1 / 18	0 / 14
Total	Fetal incidence[1]	2 / 216	0 / 221	2 / 182	4 / 157	4 / 210	1 / 162
	Litter incidence[1]	1 / 18	0 / 17	1 / 17	3 / 17	3 / 18	1 / 14

[1] No. of fetuses (litters) with findings / no. fetuses (litters) examined

2. Soft tissue examination

Fetal soft tissue malformations
One fetus in dose group 2 (0 mg/kg bw/d + 2 µg/animal/d ECP) was found with "situs inversus". This finding was considered to be spontaneous in nature and without a relation to dosing.

Fetal soft tissue variations [Table 2/75]
Two soft tissue variations (dilated renal pelvis and dilated ureter) were observed in several fetuses of dose group 1, dose group 3 - 5 and control. This is a common finding in rat fetuses of that age and the incidences were neither related to epoxiconazole nor to ECP dose. Thus, no association to treatment is assumed.

Table 2/75 Fetal soft tissue variations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Dilated renal pelvis	Fetal incidence[1]	4 / 104	3 / 105	0 / 88	5 / 70	12 / 92	6 / 68
	Litter incidence[1]	3 / 18	3 / 17	0 / 17	3 / 17	7 / 18	4 / 14
Dilated ureter	Fetal incidence[1]	4 / 104	2 / 105	0 / 88	2 / 70	7 / 92	5 / 68
	Litter incidence[1]	3 / 18	2 / 17	0 / 17	2 / 17	5 / 18	3 / 14
Total	Fetal incidence[1]	4 / 104	3 / 105	0 / 88	5 / 70	12 / 92	6 / 68
	Litter incidence[1]	3 / 18	3 / 17	0 / 17	3 / 17	7 / 18	4 / 14
	Affected fetuses/litter	4.3	3.1	0.0	4.9	13.0	7.9

[1] No. of fetuses (litters) with findings / no. fetuses (litters) examined

Fetal soft tissue unclassified observations
No unclassified soft tissue observations were observed.

3. Skeletal examination

Fetal skeletal malformations [see Table 2/76 and Table 2/77]
Skeletal malformations were recorded for all fetuses in all dose groups and in the control. A variety of malformations (related to skull, sternum, vertebrae and limbs) were evenly distributed about groups 1 and 2 and the control, indicating no influence of ECP on incidence and pattern of skeletal malformations at the tested doses in general. Most malformations, however, were observed in the EPX (180 mg/kg bw/d) treated groups 3, 4 and 5, independent of the ECP co-treatment.

Table 2/76 Fetal skeletal malformations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Fetuses evaluated		112	116	94	87	118	94
Litters evaluated		18	17	17	17	18	14
Incidences							
Misshapen basisphenoid	Fetal	1	1		**12**	**31**	**23**
	Litter	1	1		**7***	**10****	**10****
Severely malformed skull bones	Fetal				**72**	**76**	**63**
	Litter				**16****	**15****	**14****
Small presphenoidal bone	Fetal	2			4	3	1
	Litter	1			3	1	1
Misshapen hyoid	Fetal					1	
	Litter					1	
Split basisphenoid	Fetal			1			
	Litter			1			
Severely malformed vertebral column	Fetal		1				
	Litter		1				
Misshapen thoracic vertebra[A]	Fetal					1	
	Litter					1	
Shortened scapula	Fetal		1				
	Litter		1				
Malpositioned and bipartite sternebra[A]	Fetal		2		2		
	Litter		2		2		
Cleft sternum	Fetal		1				
	Litter		1				
Absent tuberositas deltoidea	Fetal				**25**	**29**	**36**
	Litter				**9****	**11****	**12****
Small tuberositas deltoidea	Fetal				**18**	**29**	**15**
	Litter				**9****	**14****	**8****
Incomplete ossification of tuberositas deltoidea	Fetal			1	**5**	**21**	**8**
	Litter			1	**5***	**10****	**7****
Shortened humerus	Fetal		1				
	Litter		1				
Bent radius	Fetal		1				
	Litter		1				
Bent ulna	Fetal		1				
	Litter		1				
Bent femur	Fetal		1				
	Litter		1				

* $p < 0.05$; ** $p<0.01$ (litter incidence: Fisher's Exact test, 1-sided; affected fetuses/litter, Wilcoxon-test, 1-sided); [A] unchanged cartilage

Table 2/77 Total fetal skeletal malformations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Fetuses evaluate		112	116	94	87	118	94
Litters evaluated		18	17	17	17	18	14
Total	Fetal[#]	2 / 112	5 / 116	2 / 94	**84 / 87**	**113 / 118**	**89 / 94**
	Litter[#]	1 / 18	4 / 17	1 / 17	**17 / 17****	**18 / 18****	**14 / 14****
	Affected fetuses/litter	1.6	4.1	2.4	**95.6****	**95.7****	**94.6****

* $p < 0.05$; ** $p<0.01$ (litter incidence: Fisher's Exact test, 1-sided; affected fetuses/litter, Wilcoxon-test, 1-sided); [#] No. of fetuses (litters) with findings / no. fetuses (litters) examined; [A] unchanged cartilage

The most salient skeletal malformations in the latter test groups were severely malformed skull bones and tuberositas deltoidea abnormalities (absent, small, or incompletely ossified).

The malformations of skull bones were diverging in individual fetuses, but showed a common pattern usually comprising the following findings:

- absent or incompletely ossified palatine bones (resemble external cleft palate)
- small presphenoidal bone
- misshapen basisphenoid
- partly or completely absent alisphenoid bone and cartilage
- absent or incompletely ossified temporal bones
- incompletely ossified or (partly) absent temporal bone
- incompletely ossified horizontal bone
- completely or partly absent pterygoid bone
- partly absent maxilla
- incompletely ossified supraoccipital bone

Some of those findings are classified as variations when observed individually; however, assessed as the whole complex of skull abnormalities they constitute a malformation.

Fetal skeletal variations (Table 2/78 and Table 2/79)
In all groups including control skeletal variations with or without involvement of corresponding cartilaginous structures were noted. The majority of these skeletal variations appeared without a relation to treatment and did not reflect biologically relevant differences between the groups and/or can be found at a comparable frequency in the historical control data. The total incidence of skeletal variations is not different among the test and control groups.

Table 2/78 Total fetal skeletal variations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Total	Fetal incidence[1]	109 / 112	115 / 116	93 / 94	87 / 87	118 / 118	94 / 94
	Litter incidence[1]	18 / 18	17 / 17	17 / 17	17 / 17	18 / 18	14 / 14
	Affected fetuses/litter	97.6%	99.3%	99.0%	100.0%	100.0%	100.0%

[1] No. of fetuses (litters) with findings / no. fetuses (litters) examined

However, the incidence of a number of variations was increased in the epoxiconazole (180 mg/kg bw/d) treated groups 3, 4 and 5, independent of the ECP co-treatment. There were two groups of variations with a higher incidence in the epoxiconazole treated groups, one related to skull bones and one related to sternebra and ribs. All skeletal variations with statistically significant differences between the control and the treated groups and/or outside of the historical control range are compiled in Table 2/79.

Table 2/79 Occurrence of statistically significantly increased fetal skeletal variations (expressed as mean percentage of affected fetuses/litter)

Affected fetuses / litter (mean %)	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d			HCD Mean % (range)
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5	
	0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP	
Skull							
Incomplete ossification of palatal bone	0.8		2.9	3.4	**6.3***	**6.1***	0.4 (0.0 – 12.9)
Incomplete ossification of alisphenoid[#]				**3.2***	**12.2***	**14.5****	---
Incomplete ossification of temporal bone[#]				**6.6***	4.9	**5.6***	---
Stenebra / ribs							
Unossified sternebra, … unchanged cartilage	8.0	17.5	19.8	**48.7****	**47.6****	**18.8***	7.9 (0.8 – 39.0)
Misshapen sternebra, … unchanged cartilage	49.4	51.2	63.6	**71.2***	**76.7****	63.3	35.6 (7.7 – 65.3)
Bipartite ossification of sternebra, … unchanged cartilage	1.6		2.0	**8.1***	**9.8****	1.9	0.4 (0.0 – 4.6)
Supernumerary rib (14th), … cartilage present		2.7	1.2	**31.7****	**45.2****	**41.7****	6.4 (0.0 – 18.3)
Supernumerary rib (14th), … cartilage not present	26.8	43.1	31.0	**78.6****	**69.3****	**77.7****	48.7 (32.4 – 73.1)
Cervical rib, … cartilage present				0.8	**3.0***	**4.5***	0.3 (0.0 – 1.9)
Cervical rib, … cartilage not present	0.8	2.0	1.0	**45.5****	**50.8****	**42.9****	5.2 (0.0 – 14.7)

* $p < 0.05$; ** $p<0.01$ (affected fetuses/litter, Wilcoxon-test, 1-sided); HCD: historical control data;
[#] no historical control data available for this term at time of study report preparation

Fetal skeletal unclassified observations [Table 2/75]

Five isolated cartilage findings (bipartite processus xiphoideus, notched manubrium, branched rib cartilage, notched cartilage between basisphenoid and basisoccipital, and notched cartilage between alisphenoid and basisphenoid, without impact on the respective bone structures, which were designated as unclassified cartilage observations, were noted in all test groups. Distribution and pattern of these findings do not suggest a relationship to the treatment.

Table 2/80 Fetal total skeletal unclassified observations

		0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
		Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
		0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Fetuses evaluated		112	116	94	87	118	94
Litters evaluated		18	17	17	17	18	14
Incidences							
Total	Fetal	72 (64%)	72 (62%)	58 (62%)	46 (53%)	72 (61%)	67 (71%)
	Litter	16 (89%)	17 (100%)	17 (100%)	15 (88%)	18 (100%)	14 (100%)
	Affected fetuses/litter	60.4%	63.3%	68.3%	50.4%	61.3%	73.5%

* $p < 0.05$; ** $p<0.01$ (litter incidence, Fisher's Exact test, 1-sided)

The incidence of one cartilage finding - split cartilage between alisphenoid and basisphenoid - was increased in the epoxiconazole treated groups 3, 4 and 5, independent of the ECP co-treatment.

Table 2/81 Occurrence of statistically significant increases of fetal skeletal unclassified observations

Split cartilage between alisphenoid and basispenoid	0 mg epoxiconazole/kg bw/d			180 mg epoxiconazole/kg bw/d		
	Gr. 0	Gr. 1	Gr. 2	Gr. 3	Gr. 4	Gr. 5
	0 µg ECP	1.0 µg ECP	2.0 µg ECP	0 µg ECP	1.0 µg ECP	2.0 µg ECP
Fetal incidence [A]	0 / 112	0 / 116	0 / 94	7 / 87 (8%)	22 / 118 (19%)	8 / 94 (8.5%)
Litter incidence [A]	0 / 18	0 / 17	0 / 17	**6 / 17** (35%)**	**9 / 18** (50%)**	**5 / 14** (36%)**
Affected fetuses/litter	0%	0%	0%	**7.2%****	**20.8****	**8.1****

[A] No. of fetuses (litters) with findings / no. fetuses (litters) examined

* $p < 0.05$; ** $p<0.01$ (litter incidence: Fisher's Exact test, 1-sided; affected fetuses/litter: Wilcoxon-test, 1-sided)

SUMMARY OF ALL CLASSIFIED FETAL EXTERNAL, SOFT TISSUE AND SKELETAL OBSERVATIONS AND THEIR ASSESSMENT

Malformations

The isolated occurrence of one soft tissue malformation (situs inversus) in test group 2 (0 mg/kg bw/d + 2 µg/animal/d ECP) is considered to be an incidental finding. The most salient external and skeletal malformations were craniofacial abnormalities (cleft palate, malformed skull bones) and abnormalities of tuberositas deltoidea, which were present in the epoxiconazole (180 mg/kg bw/d) treated groups 3, 4 and 5, independent of the ECP co-treatment.

Other external and skeletal findings, such as gastroschisis, split basisphenoid, severely malformed vertebral column, misshapen thoracic vertebra, shortened scapula, shortened humerus, bent radius, bent ulna, bent femur, cleft sternum and malpositioned and bipartite sternebra occurred only in individual fetuses. The latter findings were considered to be of spontaneous origin because of their scattered occurrence in various test groups and the control, and/or their presence in the historical controls.

Table 2/82 Total fetal malformations (external, soft-tissue and skeletal)

Dose group		0	1	2	3	4	5
Epoxiconazole dose (mg/kg bw/d)		0	0	0	180	180	180
ECP (µg/d)		0	1	2	0	1	2
Litter	N	18	17	17	17	18	14
Fetuses	N	216	221	182	157	210	162
Fetal incidence	N	2	5	3	92	115	89
	(%)	(0.9)	(2.3)	(1.6)	(59)	(55)	(55)
Litter incidence	N	1	4	2	**17****	**18****	**14****
	(%)	(5.6)	(24)	(12)	**(100)**	**(100)**	**(100)**
Affected fetuses/litter	Mean%	0.9	2.2	1.5	**57.1****	**54.4****	**56.0****

** p<0.01 (litter incidence: Fisher's Exact test, 1-sided; affected fetuses/litter: Wilcoxon-test, 1-sided)

Variations

One **external variation** occurred in one fetus (limb hyperextension) in this study. No relation to treatment is assumed for this single finding.

Soft tissue variations, in the form of dilated renal pelvis/ureter occurred in all test groups (except group 2) including the controls without any relation to treatment.

Skeletal variations consisted primarily of transient delays/disturbances of ossification. The majority of the skeletal variations are equally distributed among the different test groups including controls, if normal biological variation is taken into account.

However, there were two groups of skeletal variations with a higher incidence in the epoxiconazole treated groups, one related to skull bones and one related to sternebra and ribs.

The ossification delays of skull bones are most likely no independent findings but correlate to the craniofacial abnormalities described in the malformations section.

Mild disturbances or slight retardations of the ossification process as described for the sternebra and ribs occur very frequently in GD 20 rat fetuses of this strain. They do not affect the morphology of the underlying structures as it becomes obvious by the unchanged underlying cartilages. They are often repaired during postnatal development. Taking all this into consideration, these findings were regarded to be of no toxicological relevance and are not classified as adverse events.

Table 2/83 Total fetal variations(external, soft-tissue and skeletal)

Dose group		0	1	2	3	4	5
Epoxiconazole dose (mg/kg bw/d)		0	0	0	180	180	180
Estradiol cyclopentylpropionate (µg/d)		0	1	2	0	1	2
Litter	N	18	17	17	17	18	14
Fetuses	N	216	221	182	157	210	162
Fetal incidence	N	113	118	93	92	130	100
	(%)	(52)	(53)	(51)	(59)	(62)	(62)
Litter incidence	N	18	17	17	17	18	14
	(%)	(100)	(100)	(100)	(100)	(100)	(100)
Affected fetuses/litter	Mean%	52.6	54.0	51.3	**58.1***	**61.7****	**62.3****

* p<0.05; ** p<0.01 (litter incidence: Fisher's Exact test, 1-sided; affected fetuses/litter: Wilcoxon-test, 1-sided)

Unclassified observations

Distribution and type of unclassified external and skeletal cartilage observations do generally not suggest a relation to treatment. The only exception is the increased incidence of split cartilage between alisphenoid and basisphenoid, which is, however, not an independent finding but most likely related to the craniofacial abnormalities described in the malformations section.

III. SUMMARY AND CONCLUSIONS

Epoxiconazole was administered (by oral gavage) at a standard dose of 180 mg/kg body weight/day to three groups each consisting of 20 mated female Wistar rats, on gestation day (GD) 6 through 15. Two of these 3 groups were co-administered either 1 or 2 µg/animal/day estradiol cyclopentylpropionate (ECP), which was injected subcutaneously. Three control groups, each consisting of 19-20 female rats, were dosed with the vehicle (1% carboxymethylcellulose suspension), two of which additionally received 1 or 2 µg/animal/day ECP by subcutaneous injection, from GD 6 through to GD 15.

Adverse clinical observations were neither recorded for the epoxiconazole-treated (180 mg/kg bw/d) groups 3, 4 and 5, nor for the groups administered ECP only

(groups 1 and 2, 1 and 2 µg/animal/d ECP). Epoxiconazole might have caused just one vaginal hemorrhage in the epoxiconazole-only treated group 3.

A significant epoxiconazole-related **clinical effect** in group 3, 4 and 5 dams was decreased food consumption (Table 2/67), which was reduced by an average of approximately 20% during the treatment period. Body weights/bodyweight gains (Table 2/68) were also decreased in all epoxiconazole treated groups. The effect was most distinct at the beginning of treatment (GD 6-8), where the animals actually lost weight. Additionally the corrected (net) body weight gain (Table 2/69) was markedly decreased in all epoxiconazole-treated groups. For all these parameters, the estradiol supplementation was not able to significantly attenuate the clinical effects caused by the high dose of 180 mg/kg bw/d epoxiconazole.

Regarding **clinical pathology** no hematological changes (Table 2/70) were observed in rats dosed from GD 6 until GD 15 with epoxiconazole alone (test group 3: 180 mg/kg bw/d epoxiconazole) or in combination with ECP (test group 4: 180 mg/kg bw/d epoxiconazole+1.0 µg/animal/d ECP; test group 5: 180 mg/kg bw/d epoxiconazole+2.0 µg/animal/d ECP). High dose administration of ECP (test group 2: 2.0 µg/animal/d ECP) led to a suppression of the erythroid cell development in bone marrow indicated by decreased relative reticulocyte counts, which is known to occur in rodents exposed to estradiol [e.g. Pololi-Anagnostou et al (1981].

Hormone measurements (Table 2/71) revealed that administered ECP doses of 1.0 and 2.0 µg/animal/d between GD 6 and GD 15 did not affect maternal serum estradiol concentrations (test groups 1 and 2) measured at GD 15. The ECP molecule does not cross react with the antibodies of the specific estradiol ELISA which was used in this study and was therefore not detectable in the serum samples. The cleavage of the ECP molecule to estradiol and a possible feedback triggered down-regulation of the endogenous estradiol synthesis in ECP treated rats seemed to result in similar serum estradiol concentrations like in control rats. In dams treated with epoxiconazole-only no serum estradiol levels were detected anymore proving the aromatase inhibiting capacity of epoxiconazole. In rats co-administered with epoxiconazole + ECP (test groups 4 and 5) increasing numbers of individuals with low levels of serum estradiol were identified dependent on the ECP dose. This is assessed to be related to ECP administration and its subsequent cleavage to estradiol.

High dose ECP treatment of dams between GD 6 and GD 15 (test group 2) led to slightly lower serum progesterone levels. Marked decreased progesterone levels were measured in rats dosed with epoxiconazole only (test group 3) with no additional effect of the co-treatment with ECP (test groups 4 and 5). Intraluteal estradiol levels sustain the progesterone production in corpora lutea of pregnant rats according to published literature [Gibori and Keyes, 1978].
Therefore, epoxiconazole as aromatase inhibitor might have caused decreased ovarian synthesis of progesterone via decreased intraluteal estradiol levels. The co-administered ECP dose may not have been sufficient to elevate the intraluteal estradiol levels substantially.

In rats of test group 5 (180 mg/kg bw/d EPX+2.0 µg/animal/d ECP) androstenedione levels were lower compared to controls but also compared to rats treated with 2.0 µg/animal/d ECP only (test group 2). Jackson et al [1989] described a direct inhibition of placental androgen production by estrogens.

No differences of toxicological relevance between the control and ECP co-exposed groups were determined for **reproductive parameters** such as conception rate, mean number of corpora lutea, mean number of implantations, pre- and post-implantation losses, live fetuses and fetal sex ratio.

In the epoxiconazole-only treated group 3 (180 mg/kg bw/d EPX + 0 µg/animal/d ECP) the mean post-implantation loss was significantly increased and the number of viable fetuses was lower, which was mainly the result of a higher number of late resorptions. Obviously, the dose of 180 mg/kg bw/d was high enough to cause a sufficiently profound placental malfunction, which lasted beyond the cessation of treatment on GD 15 and caused those fetal deaths. An interference of this high dose of EPX with normal placental function is also indicated by the distinctly increased placenta weights on GD 20 – five days after cessation of treatment. This effect, however, was attenuated by estradiol supplementation. Estradiol supplementation was shown to be effective in a previous study (see chapter 2.1.2; Schneider et al. 2010; 2011 = DocID 2010/1062088 and DocID 2011/1229836) and also prevented fetal deaths in the ECP co-treated groups in the present study.

Examination of the fetuses revealed fetal external and skeletal malformations in the epoxiconazole (180 mg/kg bw/d) treated groups 3, 4 and 5, independent of the ECP co-treatment. The most salient skeletal malformations in the epoxiconazole treated groups were severely malformed skull bones and abnormalities (absent, small, incompletely ossified) of *tuberositas deltoidea*. The malformations of skull bones were diverging in individual fetuses, but showed a common pattern of abnormalities affecting predominantly facial bones.

The findings in this study are in line with craniofacial abnormalities found after exposure to this this high dose of EPX (180 mg/kg bw/d) on GD 6 – 15 in previous rat studies. Estradiol supplementation neither influenced the pattern nor changed the incidence of the abnormalities in the skull and *tuberositas deltoidea*. However, even at a supplementation of 2 µg/animal/d ECP, the serum levels of this hormone were still 88% below control levels.

In conclusion, as known from previous studies, epoxiconazole adversely affected prenatal development of Wistar rats at 180 mg/kg bw/d, a dose which induced clear maternal toxicity in the form of reduced food consumption, body weight gain and estradiol depletion. Salient adverse developmental effects were fetal deaths as well as craniofacial and tuberositas deltoidea malformations. Supplementation of estradiol (1 or 2 µg/animal/d estradiol cyclopentylpropionate) resulted only in a very modest dose-related increase of serum estradiol levels, relative to the animals which received 180 mg/kg bw without supplementation. Nevertheless this modest increase of estradiol was sufficient to prevent fetal deaths and also reduced the extent of placental weight increase. However, under the study conditions the estradiol treatment regime chosen was not effective in

preventing craniofacial and tuberositas deltoidea abnormalities caused by high-dose epoxiconazole exposure.

STUDY RELEVANCE

This prenatal developmental toxicity study with pregnant rats was performed to clarify the mode of action for the induction of cleft palates, which had been observed in an early dose-range finding rat developmental toxicity study after excessive dosing of epoxiconazole (i.e. at a dose level of 180 mg/kg bw/d from GD 6-15 [Hellwig 1989; see summary in RAC Background Document (2010)].

New investigations in rats involving epoxiconazole / estradiol co-supplementation had indicated an association between aromatase inhibition and late fetal resorptions in rat fetuses via aromatase inhibition when epoxiconazole was administered to rats at dose levels of up to 50 mg/kg bw/d (a dose which was not sufficiently high to induce cleft palates). Therefore, to test the hypothesis of an association of aromatase inhibition and cleft palate formation, estradiol supplementation was combined with excessive epoxiconazole dosing in the present study. The study included also a detailed skeletal examination of the rat fetuses in order to better characterize the skeletal changes occurring at 180 mg/kg bw/d (in the range-finding study, only an external examination of the fetuses had been performed).

Relevance of the study for assessment of late fetal resorptions in rats

The increased late fetal resorptions induced by treatment of epoxiconazole at 180 mg/kg bw/d from GD 6-15 are related to a depletion of maternal estradiol, since incidences of late fetal resorptions are reduced to zero when epoxiconazole treated rats additionally receive estradiol supplementation during the same time period. Moreover, placental damage was indicated by a doubling of the placental weight increase, which could be partly reduced when estradiol is supplemented. Therefore, this study provides relevant mechanistic information for the induction of late fetal resorptions that should be taken into account in the overall assessment of all available data, especially in terms of judging on the evidence in support of a “secondary consequence of other effects” and also in terms of concluding on the relevance of the effect for humans.

Relevance of the study results for assessment of cleft palate formation in rats

Distinct maternal toxicity was observed at a dose level of 180 mg/kg bw/d (the only dose level tested in this study), and severe placental damage was indicated by a marked increase of placental weight. Cleft palates were increased in incidence confirming findings of the initial range-finding study [Hellwig 1989]. Although estradiol supplementation did not significantly reverse the marked reductions of measured estradiol plasma levels (sampling on GD15) and did not affect the incidence of the craniofacial malformations, it is highly likely that rat-specific, severe placental damage substantially contributed to the increased incidence of malformations by impairment of the placental barrier function. Moreover (and also independent of estradiol supplementation), again a substantial reduction of maternal progesterone levels was observed in this study (blood sampling on GD

15) confirming results of a previous epoxiconazole study [sampling on GD 20; Schneider 2002; see RAC Background Document (2010)].

This high-dose epoxiconazole study in rats therefore provides relevant mechanistic information for the induction of cleft palate that should be taken into account in the overall assessment of all available data, especially in terms of judging on the evidence in support of a "secondary consequence of other effects" and also in terms of concluding on the relevance of the effect for humans (see Chapters 3 and 4).

2.1.7 Prenatal developmental toxicity study with rat embryos

STUDY REFERENCE

Report:	Flick B., Schneider S., Menegola E., Mellert W. (2012a) BAS 480 F (Epoxiconazole) Morphological and immunohistochemical investigations in rat embryos gathered by C-section oral administration (gavage) BASF DocID 2012/1059618 Date of report: 29-Feb-2012
Guidelines:	No test guidelines are available for this mechanistic study.
GLP:	No

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material:	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.
2. Vehicle control:	1% aqueous carboxymethylcellulose (1% CMC)

3. Test animals:

Species:	Rat
Strain:	Wistar [Crl:WI (Han)]
Sex:	Female
Age:	Time-mated rats aged 10-12 weeks
Weight (pregnant rats GD 0):	170.8 ± 11.17 g (range 146.6 - 189.5 g)
Source:	Charles River Lab., Germany
Acclimatization period:	at least 6 days before treatment
Diet:	Kliba maintenance diet for mouse/rats "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type M III Makrolon cages (Becker & Co, Castrop-Rauxel, Germany and/or Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany), floor area about 800 cm² with dust free wooden bedding and wooden gnawing blocks (Abedd Lab. and Vet. Service GmbH, Vienna) offered for enrichment
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 08-Jul-2011 to 29-Feb-2012 [in-life dates: 08-Jul-2011 (arrival of animals), 14-Jul-2011 (start treatment on gestation day (GD) 6) to 19-Jul-2011 (last treatment and sacrifice, GD11)]

2. Animal assignment and treatment:

Female Wistar rats were paired by the breeder ("time-mated") and supplied on gestational day (GD) 0 (= detection of vaginal plug/sperm). The animals arrived on the same day (GD 0) at the experimental laboratory. The following day was designated as "GD 1". The animals were acclimatized to the laboratory conditions between start of the study (beginning of the experimental phase) and first administration (GD 6).

Epoxiconazole in 1% aqueous carboxymethylcellulose suspension (1% CMC) or the vehicle 1% CMC only was administered once daily to sperm-positive females animals by oral gavage from GD 6 to 11, at dose levels of 50, 100 or 180 mg/kg bw/d. The volume administered each day was 10 mL/kg body weight. The

calculation of the administration volume was based on the most recent individual body weight.

Table 2/84 Test groups and doses

Test group	Dose epoxiconazole (mg/kg bw/d)	Concentration epoxiconazole (mg/100 mL)	Volume (mL/kg bw)	No. of animals (mated)	No. of animals (pregnant)
0	0 (1% CMC)	0	10	6	5
1	50	500	10	3	2
2	100	1000	10	3	3
3	180	1800	10	6	6

3. Test substance stability in the vehicle:

The analytical verification of test substance stability in the vehicle for at least 7 days at room temperature was carried out before the study was initiated, using a similar batch of the test substance.

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt toxicity were performed at least once daily. If such signs occurred, the animals were examined several times daily (GD 0-11)

2. Body weight and food consumption

All animals were weighed on GD 0, 1, 3, 6, 8, and 11. The body weight change of the animals was calculated from these results.

Food consumption was determined for GD 0-1, 1-3, 3-6, 6-8, and 8-11.

3. Terminal examinations of the dams

On GD 11, dams were sacrificed by decapitation under isoflurane anesthesia. Dams were subsequently and assessed by gross pathology. The uteri were removed for preparation of the embryos.

4. Morphological and morphometric evaluation:

The uteri were opened and the embryos were separated from the decidua, yolk sac and amnion. Each embryo was evaluated for its growth and differentiation with the aid of a dissection stereomicroscope as described before (Klug et al., 1985 and Flick et al., 2009).

Evaluation of embryonic growth

The growth of the embryos was measured by determining the crown-rump length using an ocular micrometer.

Evaluation of embryonic differentiation

The differentiation of the embryos was assessed by counting the somites and evaluating the phenotype of the embryos based on a morphological score system (Klug et al., 1985). The development of the general embryonic shape, the differentiation of the neural tube, the head, eyes, ears, heart, fore and hind limbs, the caudal part of the trunk referred to as tail and the presence of blood in the embryo itself were assessed. Furthermore, the development of the yolk sac was estimated regarding its blood vessels system.

All embryonic findings were listed in tables according to the following definitions and evaluation criteria for assessing the embryos:

Anomaly / Dysmorphogenesis	***Variation***
A specific dysmorphogenesis of an embryo which is manifested in a disproportional development of single organ anlagen in comparison to the development of the whole embryo. It is a permanent structural change that would be likely to be detrimental to the normal structure, development, growth, and/or viability of an embryo.	*A structural change which is considered to have little or no detrimental effect on the embryo; may be transient and may occur relatively frequently in the control embryos. This includes delays in growth or differentiation that have otherwise followed a normal pattern of development.*

5. Immunohistochemical investigations of rat embryos:

The immunohistochemical investigation of the ex-vivo embryos was performed by Prof. Menegola at the University of Milan. For this purpose, embryos were harvested from pregnant rats on GD 11 at the BASF test facility, fixed and subsequently shipped to the University of Milan under conditions as described below.

Fixation of embryos

The uteri of three dams from each test and control group were transferred into phosphate buffered saline (PBS). As quickly as possible, the uteri were opened and the embryos separated from the decidua, yolk sac and amnion. All embryos of a dam were harvested, rinsed immediately in PBS (4°C), divided in groups of up to five embryos and fixed in Dent's fixative (DMSO: Methanol 1: 4) at -20°C overnight. After fixation the embryos were washed twice (30 min each) with cold (-20°C) methanol. After preparation the samples were stored in cold methanol (-20°C). Blinded samples were shipped to the University of Milan on dry ice for immunohistochemical investigations.

Immunostaining of embryos

Immunostaining of whole embryos was performed on samples provided by BASF. After washing in methanol, embryos were processed according to Menegola et al., 2003. Briefly, samples were incubated in hydrogen peroxide (5% in methanol), hydrated and incubated in balanced solutions containing fetal calf serum (Sigma). The selected primary antibody (anti-CRABP, ABR) was diluted 1:500. The

incubation time was 3 days at 4°C. The secondary antibody (anti-mouse IgG peroxidase, Boehringer) was diluted 1:40. The incubation was maintained overnight at 4°C. The colorimetric reaction was performed using 4-Cl-1-naphtol (Sigma) and 0.006% hydrogen peroxide as substrates. When stained tissues appeared dark brown the reaction was stopped with 30% ethanol. Stained samples were immediately photographed.

6. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
Food consumption[a)], body weight, body weight change	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality, frequency of dysmorphogenesis	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions
Crown-rump length, morphological score, number of somites	Simultaneous comparison of all test groups with the control group using analysis of variance and the Thamhane-2 post-hoc test (two-sided) for the hypothesis of equal means.
Yolk sac circulation	Pairwise comparison of each dose group with the control group using MANN-WHITNEY-U test for the hypothesis of equal medians

II. RESULTS

Note: Only pregnant dams were used for the calculations of mean maternal food consumption, body weight and body weight change. For the above reasons one control group female (No. 103) and one female of the low dose group (No. 107) were excluded from the above-mentioned calculations.

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

There were no test-substance-related mortalities in any of the groups.

2. Clinical signs of toxicity

There were no clinical findings in any of the test animals during the study period.

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption [see Table 2/85]
The mean food consumption of the dams in test groups 1 and 2 (50 and 100 mg/kg bw/d) was comparable to the control group. Decreased food consumption was observed between GD 6 and 11 (down to 85% of control) at 180 mg/kg bw/d.

Table 2/85 Food consumption

Parameter: mean food intake (g/animal)	Epoxiconazole (mg/kg bw/d)			
	0	50	100	180
Animals examined	5	2	3	6
GD 0 – 1	14.3	13.4	15.1	15.7
Δ%		-6%	6%	10%
GD 1 – 3	17.2	15.8	17.3	17.4
Δ%		-8%	1%	1%
GD 3 – 6	18.2	17.6	19.3	19.2
Δ%		-3%	6%	5%
GD 6 – 8	20.0	17.6	18.9	16.9*
Δ%		-12%	-6%	-16%
GD 8 – 11	19.5	17.5	18.8	16.8*
Δ%		-10%	-4%	-14%

* p < 0.05; ** p < 0.01 (Dunnett-Test, two-sided)

2. Body weight and body weight gain [Table 2/86]
The mean body weight of all test groups was comparable to the control group at all time points investigated. The mean body weight gain of dams from test group 2 (100 mg/kg bw/d) was significantly decreased between GD 6 and 8 (-96% of control), and in the top dose group (180 mg/kg bw/d, dams actually lost weight between GD 6 and 8 (-1.7 g vs. +7 g = -124%). The body weight gain between GD 8 and 11 was comparable to control in all test groups.

Table 2/86 Body weight change

Parameter: Body weight change (g/animal)	Epoxiconazole (mg/kg bw/d)			
	0	50	100	180
Animals examined	5	2	3	6
GD 3 – 6	10.5	10.3	10.9	9.8
Δ%		-2%	4%	-7%
GD 6 – 8	7.1	1.8	**0.3***	**-1.7****
Δ%		-75%	**-96%**	**-124%**
GD 8 – 11	16.3	16.3	16.1	18.2
Δ%		0%	-1%	12%

* p < 0.05; ** p < 0.01 (Dunnett-Test, two-sided)

D. NECROPSY

The gross pathological examinations did not reveal any abnormal findings in dams from treatment or control groups.

E. MORPHOLOGICAL INVESTIGATIONS IN RAT EMBRYOS [see Table 2/92]

Growth and differentiation parameters of 10 embryos from the control group and of 19 embryos from the 180 mg/kg bw/d dose group were examined. In addition, these embryos were examined for signs of dysmorphogenesis.

1. Evaluation of embryonic growth

The growth of the embryos was determined by measuring the crown-rump-length of the embryos. The crown-rump lengths of the embryos in the dose group 180 mg/kg bw/d and in control were comparable.

2. Evaluation of embryonic differentiation

The differentiation of the embryos was determined by evaluating the yolk sac, the number of somites, the total morphological score of organ anlagen and the incidence of dysmorphogeneses observable in rat embryos of GD 11. All determined mean values of these differentiation endpoints were comparable between high dose group (180 mg/kg bw/d) and control.

Table 2/87 Evaluation of growth and differentiation of GD 11 embryos including dysmorphogenesis

Test group	Dose [mg/kg bw/d	Number of embryos	Crown-rump length [mm]	Number of somites	Yolk sac	TMS	Incidence of dysmorpho–genesis
0	0	10	4.2 ± 0.3	32.3 ± 1.9	4.0 ± 0.0	37.2 ± 1.9	0%
3	180	19	4.1 ± 0.4	32.1 ± 1.7	3.8 ± 0.7	37.9 ± 1.6	5%

TMS = total morphological score of organ anlagen observable on GD 11

One of 19 embryos in the high dose group showed a neural tube defect and a hole in the yolk sac which were assessed as dysmorphogenesis. Because the neural tube defect of this embryo was positioned in the region of the back laying right below the hole of the yolk sac, it was most likely that the neural tube defect was caused by the open yolk sac. A hole in the yolk sac is most likely spontaneous in nature. Thereby, both anomalies observed in one embryo of the high dose group, leading to an incidence of dysmorphogenesis of 5% in this dose group, were considered not to be treatment related.

3. Immunohistochemical investigation in rat embryos

The fixed embryos were shipped to the laboratory of Prof. Menegola (University of Milan) and subsequently processed as described above.

During the staining process of the first 35 embryos from 8 dams it was discovered that the visualization of the neural crest cells (NCCs) was only possible in 17 embryos from four dams. In these embryos the immunostained masses at the level of the first and second branchial arches were well compacted and separated corresponding to the physiological situation. This subgroup of GD 11 embryos appeared to be at a relatively early stage of development. The other 18 embryos harvested on GD 11, which appeared to be at a relatively advanced stage of development, could not be immunostained successfully. The expected dark brown staining in the tissues did not emerge as expected. It was assumed by Menegola´s laboratory that these ex vivo embryos were developed too far and a compact distribution of the NCCs was not given anymore. A visualization of NCCs was not possible with the used protocol at the given developmental stage of these ex vivo rat embryos. Because only one half of the embryos could be evaluated, a bias of the assessment cannot be excluded. Therefore, the involved parties decided not to evaluate the remaining embryos in this study.

III. SUMMARY AND CONCLUSIONS

Epoxiconazole in 1% aqueous carboxymethylcellulose suspension (1% CMC) or the vehicle 1% CMC only was administered once daily to sperm-positive females animals by oral gavage from GD 6 to 11, at dose levels of 50, 100 or 180 mg/kg bw/d. On GD 11, rat embryos were explanted from the treated pregnant rats.

About 50% of the embryos of control and top dose group were subjected to morphological investigations. The remaining embryos were shipped to the University of Milano and subjected to immunohistochemical investigations by Prof. Menegola. These examinations were performed to clarify whether the craniofacial malformations in rat fetuses seen in previous studies after high-dose treatment with 180 mg/kg bw/d epoxiconazole are preceded by dysmorphogenesis of the branchial apparatus; such a pathogenic pathway has been hypothesized to be involved in the induction of craniofacial malformations by triazoles (Menegola et al., 2006).

Under the conditions of this study epoxiconazole caused adverse effects/findings in gestating Wistar rats at 180 mg/kg bw/d and to a minor degree at 100 mg/kg bw/d after oral administration from implantation to gestational day 11 (GD 6-11). Decreased food consumption, transient body weight loss or decreased body weight gain were indicative of marked maternal toxicity at 180 mg/kg bw/d. In the three dams treated with 100 mg/kg bw/d, marginal effects on body weight development were detectable on GD 6-8.

No test substance-related findings were observed in the morphology of the embryos at the epoxiconazole dose of 180 mg/kg bw/d. The development of the exposed embryos was comparable to control embryos with regard to all endpoints of growth (grown-rump-length) and differentiation (number of somites and total morphological score – summarizing the scored development of different organ anlagen). Furthermore, no significant increase of dysmorphogenesis was observed in comparison to control. In contrast to a corresponding in vitro study (Menegola E, 2012; DocID 2012/1058203; see chapter 2.3.3.1) investigating rat embryos of GD 11.5 after a 48-hour in vitro exposure to epoxiconazole, abnormalities and

delays in development of branchial arches, optic and optical vesicles, somites and the neural tube were not observed under in vivo conditions in this study.

For the immunohistochemical investigations with embryos from this study, Prof. Menegola applied the established protocol for immunohistochemical detection of neural crest cells using antibodies against cellular retinoic acid binding protein (anti-CRABP) (Menegola et. al. 2003). Under the study conditions, neural crest cell (NCCs) migration and distribution could only be visualized in 17 of the 37 investigated ex vivo GD 11 embryos. Therefore test substance-related effects could neither be assessed nor be excluded.

STUDY RELEVANCE

In this investigation, pregnant rats were exposed to epoxiconazole by daily oral gavage administration from GD 6-11. Embryos were dissected by Caesarean sectioning on GD11 and assessed for morphological changes and for changes in neural crest cell migration. Thus, the in-vivo study was designed to generally correspond to the embryonic exposure conditions as employed in Whole-Embryo-Culture investigations by Menegola et al. (see chapter 2.2.1.2, Menegola 2012, DocID 2012/1058203). The study was further complemented by an in-vitro investigation, which determined maternal plasma concentrations and also the embryonic tissue concentrations of epoxiconazole after treatment under identical conditions (see chapter 2.2.2.3, Flick et al 2012, DocID 2012/1058202).

180 mg/kg bw/d was selected as epoxiconazole dose level, which is known to reproducibly induce a high incidence of cleft palates in fetuses following oral administration to pregnant Wistar rats during GD 6-15 (see chapter 2.1.6, Schneider et al. 2010; DocID 2011/1229839; Hellwig 1989 - see RAC Background Report (2010)).

Results of this in-vivo investigation can be directly compared with in-vitro findings by Menegola, using the in-vivo plasmaconcentration data that allows to correlate in-vivo dose levels with epoxiconazole concentrations tested in Whole Embryo Culture (WEC). The measured maternal plasma and embryonic tissue concentrations from a parallel in-vivo study corresponded to WEC concentrations that caused clear effects under in-vitro conditions.

Based on in-vitro data by Menegola and the available kinetic data (which allowed to correlate in-vitro effect concentrations to in-vivo dose levels), it was very surprising that epoxiconazole treatment of pregnant rats from GD 6-11 did not induce any evidence of dysmorphogenesis in GD 11 old embryos.

The in-vivo study thus belongs to a suite of in-vitro and in-vivo mechanistic investigations that were initiated to elucidate the mechanism underlying the observed increased incidence of cleft palate after high-dose level treatment of rats with epoxiconazole. Thus, the study is relevant for choosing the appropriate classification of epoxiconazole for developmental toxicity as part of an overall weight-of-evidence assessment.

2.2 HUMAN DATA

No epidemiological data on epoxiconazole relevant for assessement of developmental toxicity is available.

2.3 OTHER RELEVANT INFORMATION

2.3.1 IN VITRO STUDIES

2.3.1.1 Whole-Embryo-Culture investigation

STUDY REFERENCE

Report:	Menegola E. (2012) BAS 480 F (Epoxiconazole) Morphological and immunohistochemical investigations in rat embryos cultured in vitro BASF DocID 2012/1058203 Date of report: 15-Feb-2012
Guidelines:	There are no guidelines for this mechanistic study.
GLP:	No

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material:	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.
2. Vehicles:	Ethanol, pure (Sigma) Dimethylsulfoxide = DMSO (Sigma)
3. Positive control:	Triadimefon (purity not indicated in the report)
4. Experimental animals	
Species:	Rat
Strain:	Wistar
Sex:	Male and Female
Source:	Charles River, Calco, Italy

Diet:	ad libitum (Italiana Mangimi, Settimo Milanese, Italy)
Water:	Tap water, ad libitum
Housing:	maintained under pathogen-free conditions
Environmental conditions:	
Temperature:	22 ± 2 °C
Humidity:	55 ± 5%
Air changes:	no information in the report
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 8-Jun-2011 to 29-Jul-2011

2. Test system and outline of experiments [Table 2/88]

Epoxiconazole was tested in Whole Embryo Culture (WEC) of approx. 9.5 day old Wistar rat embryos that were exposed to the test substance for 48 hours. Beside of morphological and morphometric examinations, immunostaining of cultured rat embryos with anti-CRABP was performed in order to visualize neural crest cells.

Three sets of experiments were performed (see Table 2/88):

In a **Preliminary Test (PT),** the sensitivity of the Wistar rat strain was investigated under WEC conditions using 250 µM triadimefon, a concentration which is known to produce branchial arch abnormalities in 100% of the exposed CD rat embryos [di Renzo et al, 2011]. Wistar rat embryos were evaluated for morphological effects and results were compared with historical control data of the test facility that had been obtained in WEC studies with CD rat embryos. Since it was not clear whether the solubility of epoxiconazole in the standard solvent ethanol (ca. 30 g/L or 91 µM) might limit the detection of dysmorphogenic effects, a higher concentration of 250 µM epoxiconazole was tested using DMSO as vehicle in this preliminary test.

Table 2/88 Experimental test groups

Preliminary Test			Concentration range finding test			Main Experiment		
Test material	Test conc. [µM]	No. embryos used	Test material	Test conc. [µM]	No. embryos used	Test material	Test conc. [µM]	No. embryos used
Ethanol	0	5	Ethanol	0	5	Ethanol	0	35
Triadimefon + Ethanol	250	5	Epoxiconazole + Ethanol	3	5	Epoxiconazole + Ethanol	3	30
Epoxiconazole + Ethanol	91	5	Epoxiconazole + Ethanol	30	5	Epoxiconazole + Ethanol	10	30
Epoxiconazole + DMSO	250	5	Epoxiconazole + Ethanol	60	5	Epoxiconazole + Ethanol	30	20
			Epoxiconazole + Ethanol	91	5	Epoxiconazole + Ethanol	60	20
						Epoxiconazole + Ethanol	91	20

The goal of the **Concentration Range Finding Test (CRFT)** was to find effective non-lethal concentrations in WEC. Final concentrations of 3, 30, 60 or 91 µM were tested. The rationale of the selected concentrations was to maintain the limit concentration level and to aim for test concentrations comparable to the maximum plasma levels reached under in-vivo exposure conditions following oral administration of epoxiconazole to pregnant Wistar rats at dose levels of 5, 50, 100 and 180 mg/kg bw/d (see chapter 2.3.2.1, Fabian and Landsiedel, 2011; DocID 2011/1112619 = Project No. 02B0277/03B001).

Epoxiconazole was finally tested at selected concentrations (**Main Experiment, ME**) in at least four independent WEC experiments. The overall analysis of data was designed in order to statistically describe the concentration-dependent effects on morphometrical and morphological data. The identification of neural crest cell distribution was performed in order to verify the hypothetical pathogenic pathway related to the dysmorphogenesis of the branchial apparatus (the embryonic apparatus involved in facial morphogenesis). Embryonic tissue concentrations were determined in order to be able to compare to embryonic tissue concentrations reached after in-vivo epoxiconazole exposure of pregnant rats.

2. Mating procedure

Sexually mature Wistar rats were mated overnight. In case of a sperm-positive vaginal smear obtained in the morning of the next day, this day was set at Day 0 gestation (GD 0).

3. Caesarean sectioning for retrieval of embryos

At GD 9.5 pregnant females were sacrificed by carbon dioxide, uteri removed and placed in sterile Tyrode's balanced salt solution (Sigma, Italy). All the following procedures were performed under a sterile cabin. After incision of the uterine wall, the extraction of the embryos from the decidual mass was performed under dissecting microscopes.

4. Selection of embryos for Whole Embryo Culture (WEC)

Embryos dissected on GD 9.5 as described were selected following the evaluation of the neurulation stage. The embryonic stage characterized by embryos showing raised cephalic neural fold was considered as optimal.

5. Set up of Whole Embryo Culture (WEC)

Embryos that fulfilled the selection criteria were pooled together and randomly allocated to different test groups. 5 embryos were transferred into each culture flask (volume flask 25 mL) containing 5 mL of culture medium and cultured according to New [1978].

The culture medium consisted of heat-inactivated Wistar rat serum pooled from several donor rats, aliquots stored at -20 °C. At the time of use, penicillin/streptomycin antibiotics solution (Sigma, Italy) was added to the rat serum to obtain a concentration of 100 I.U: penicillin/mL serum and 100 µg streptomycin/mL serum. The culture medium was pooled before the start of the whole experiment.

The flasks, equilibrated with sterile gas (O_2 5%, CO_2 5%, rest N_2), were inserted into a roller (30 rpm) incubator (37.8°C). Flasks were re-equilibrated with sterile gas every 12 hours with gas mixtures of O_2 10%, CO_2 5%, rest N_2. in the morning of GD10; in the evening of GD10 and in the morning of GD11, flasks were re-equilibrated with sterile gas composed of O_2 20%, CO_2 5%, rest N_2, according to the method described by Giavini et al [1992].

Stock dilutions of epoxiconazole in DMSO (81 mg/mL), of epoxiconazole in ethanol (30 mg/mL) or of triadimefon (positive control) in ethanol were prepared directly prior to use and subsequently diluted with DMSO or ethanol to obtain appropriate end concentrations when applying a volume of 1 µL per mL culture medium.

6. Morphological and morphometric evaluation:

After 48 hours of culture, whole embryos in their annexes were evaluated under a dissection microscope. Yolk sac diameter, yolk sac vascularization, amnios features, embryo curvature and embryo vitality were evaluated in the whole conceptus, while the embryonic organ development, crown-rump length and head length and the number of somites were evaluated after removing the extraembryonic annexes. The morphological score system proposed by Brown and Fabro [1981]) was applied to evaluate developmental delays. Each embryo was photographed for documentation.

After morphological and morphometric investigations, selected embryos and their yolk sacs from the CRFT and ME experiments were frozen in liquid nitrogen and sent to BASF for determination of embryonic tissue concentrations. 8-11 embryos from each of the epoxiconazole treatment groups and from the solvent control group were processed for immunohistochemical analysis. An overview of the number of samples evaluated for embryonic tissue concentration and for immunohistochemical analysis is given in Table 2/89.

Table 2/89 Distribution of cultured embryos for post-culture processing

Test group	Epoxiconazole concentration [µM]					
	0	3	10	30	60	91
No. embryos for immunostaining	11	10	11	9	9	8
No. embryos for concentration analysis	18	19	12	14	14	13
No. yolk sacs for concentration analysis	29	25	23	23	23	20

7. Immunohistochemical analysis

Immunostaining of whole embryos was performed on samples immediately fixed in Dent's cold fixative (DMSO:methanol 1:4) and maintained at -20°C overnight. After washing in methanol, embryos were processed according to Menegola et al. [2003]. Briefly, samples were incubated in hydrogen peroxide (5% in methanol), hydrated and incubated in balanced solutions containing fetal calf serum (Sigma). The selected primary antibody (anti-CRABP, ABR) was diluted 1:500. The incubation time was 3 days at 4°C. The secondary antibody (anti-mouse IgG peroxidase, Boehringer) was diluted 1:40. The incubation was maintained overnight at 4°C. The colorimetric reaction was performed by using as substrates 4-Cl-1-naphtol (Sigma) and 0.006% hydrogen peroxide. When stained tissues appeared dark brown the reaction was blocked with ethanol 30%. Stained samples were immediately photographed.

8. Determination of embryonic tissue concentrations

The analysis for the test compound in the embryonic tissues and culture medium was performed by BASF SE, APD/EC – Consumer Safety Residues, Limburgerhof.

The embryonic tissue (yolk sac and whole embryo) was pooled in groups of up to 5 embryos and transferred into Safe-Lock Tubes. The Safe-Lock Tubes with the embryonic tissue were stored on ice until homogenization using a glass/teflon tissue grinder. Subsequently, the samples were stored in a freezer (-20°C). Following extraction with acetonitrile/formic acid 1000/1 (v/v), the embryonic tissue concentrations were determined by UPLC analysis.

5. Statistics:

Morphometrical data were statistically analyzed by using ANOVA followed by Tukey's post hoc test. Frequencies of abnormalities were compared in different groups by using the chi-square test. The level of significance was set at $p<0.05$.

II. RESULTS

A. PRELIMINARY EXPERIMENT

Sensitivity of Wistar rat strain

5 solvent control embryos and 5 embryos exposed to 250 µM triadimefon (positive control, in ethanol) were assessed for dysmorphogenesis in order to ascertain the sensitivity of the Wistar strain. Based on the results obtained and comparison with data produced in previous investigations of the test facility in CD rat embryos, it was concluded that Wistar embryo rats are as sensitive as CD rat embryos for dysmorphogenetic effects of triadimefon.

Choice of solvent for subsequent WEC experiments with epoxiconazole

Morphological evaluation of 5 embryos indicated that 91 µM epoxiconazole (max. concentration dissolvable in ethanol) is active in inducing dysmorphogenesis at the level of the branchial apparatus and specific developmental delays at the level of the otic vesicle and of the posterior neuropore. No generalized effects on development were reported. The embryos exposed to 250 µM epoxiconazole (dissolved in DMSO) showed in addition to these effects a generalized and severe developmental delay, often associated with tissue edema and diffuse dysmorphogenesis.

On the basis of the results obtained, it was clear that ethanol could be used as standard solvent in subsequent WEC experiments with epoxiconazole and that 91 µM would produce morphological effects after 48-h in-vitro exposure of Wistar rat embryos.

B. CONCENTRATION RANGE FINDING TEST (CRFT)

Wistar rat embryos (1 flask with 5 embryos/group) were exposed to concentrations of 3, 30, 60 or 91 µM epoxiconazole; a solvent control group was exposed to ethanol only. Concentration-related dysmorphogenic effects at the level of the branchial apparatus were observed in embryos exposed to epoxiconazole concentrations of 30, 60 and 91 µM.

C. MAIN EXPERIMENT

On the basis of the results obtained in the CRFT, 3, 10, 30, 60 and 91 µM were chosen as the epoxiconazole concentrations for testing in the main experiment.

1. Morphological analysis (Table 2/90)

In the main experiment, concentration-related dysmorphogenic effects were reported at and above an epoxiconazole concentration of 10 µM, treatment-related effects mainly comprised embryos with fusion of branchial arches, abnormal branchial arch II, and embryos with delayed or severely delayed otic vesicle.

Table 2/90 Dysmorphogenetic effects

		Epoxiconazole							FON
	µM	0	3	10	30	60	91	250	250
Evaluated embryos	N	34	29	23	23	23	26	5	3
Total embryos with anomalies	N	1	0	**8****	**11****	**16****	**24****	**5****	**3****
	(%)	2.94	0.00	**34.78**	**47.83**	**69.57**	**92.31**	**100.0**	**100.0**
Plurimalformed						1	1	3	
I-II branchial arches fused				3	2	8	16	2	3
II branchial arch anomalous				4	6	5	4		
II-III branchial arches fused					2	2	1		
Fused somites						1	1		
Hook-shaped tail								1	
Edema								1	
Swollen rhomboencephalon		1		1	1	1			1
Visceral yolk sac hemostasis							2		
Total embryos with developmental delays	N	15	14	**19****	**17****	**18****	**24****	2	**3****
	(%)	44.12	48.28	**82.61**	**73.91**	**78.26**	**92.31**	40.0	**100.0**
Delayed posterior neuropore		10	9	11	6	10	14	1	1
Severely delayed posterior neuropore		1	4	4	1	7	8	1	2
Delayed otic vesicle		4	4	12	8	3	3		
Severely delayed otic vesicle		3	1	2	6	9	12	1	3
Otic placode			1	1	1	3	8	1	
Delayed optic vesicle		2	4			1	1		
Severely delayed optic vesicle		1			1	2			

(Statistical analysis: ** p≤0.01)

2. Morphometric evaluation (Table 2/91)

Embryos exposed to the top concentration of 91 µM epoxiconazole showed a statistically significant reduction in the total morphometric score when compared to respective value of the solvent control. However, there were no statistically significant differences observed for individual morphometric parameters in epoxiconazole exposed groups (with the exception of an apparent reduction of somites at 10 µM; due to the lack of a concentration-response relationship, this reduction is considered to be incidental. Overall, the authors concluded that no generalized effects on embryonic development were evident as a result of epoxiconazole exposure.

Table 2/91 Morphometric results

	Morphometric parameters evaluated [mean ± SD]				
Test group	VYS diameter [mm]	Crown-rump length [mm]	Head length [mm]	Somite number	Total score
Solvent control (ethanol)	3.45 ± 0.23 (33)[A]	3.53 ± 0.18 (32)	1.63 ± 0.15 (34)	23.71 ± 1.62 (34)	39.18 ± 1.09 (34)
Epoxiconazole					
3 µM	3.54 ± 0.17 (29)	3.50 ± 0.19 (27)	1.64 ± 0.14 (28)	23.03 ± 1.76 (29)	38.86 ± 1.38 (29)
10 µM	3.37 ± 0.18 (23)	3.41 ± 0.21 (22)	1.58± 0.12 (23)	**21.70 ± 1.58**** (23)	38.39 ± 1.47 (23)
30 µM	3.45 ± 0.19 (23)	3.55 ± 0.25 (23)	1.64 ± 0.14 (23)	23.00 ± 1.76 (23)	39.04 ± 1.33 (23)
60 µM	3.59 ± 0.26 (22)	3.46 ± 0.24 (21)	1.65 ± 0.17 (22)	22.41 ± 1.47 (22)	38.55 ± 1.37 (22)
91 µM	3.45 ± 0.27 (25)	3.32 ± 0.21 (24)	1.53 ± 0.12 (25)	22.52 ± 1.85 (25)	**38.00 ± 1.38** (25)**
Triadimefon					
250 µM	3.46 ± 0.18 (3)	3.32 ± 0.16 (3)	1.48 ± 0.23 (3)	21.33 ± 1.53 (3)	38.00 ± 1.73 (3)

Statistical analysis: ** p≤0.01
[A] number of embryos evaluated

3. Immunostaining

After immunostaining (performed in order to visualize neural crest cell (NCCs) migration and distribution at the level of the craniofacial elements), controls showed the typical NCC distribution, previously described in explanted CD rats or developed in vitro (Menegola et al., (2003)**Fehler! Textmarke nicht definiert.**): immunostained masses at the fronto-nasal level and at the level of the branchial apparatus and around the otic vesicle. The totality of embryos exposed to epoxiconazole at and above 30 µM showed abnormal neural crest cell distribution at the level of the branchial apparatus.

4. Determination of embryonic tissue concentrations

The embryonic tissue consisting of embryos and their yolk sacs harvested from WEC experiments (CRFT and ME) were frozen in liquid nitrogen after morphological evaluation.

This morphological evaluation was performed while the embryos were placed in Tyrode's salt solution. A leaking of the test substance from the embryonic tissue into the surrounding salt solution during morphological evaluation cannot be excluded.

The residues in these embryonic tissues showed a non-linear dose dependent increase from 0.24 mg/L at the lowest tested concentration level up to 8.0 mg/L at

the highest tested concentration level. However, the concentrations determined in the embryonic tissues had been lower than the concentration of the test substance adjusted in culture medium. The concentration ratio between embryonic tissue and the culture media varied from 0.17 to 0.36 over the concentration range investigated.

Table 2/92 Epoxiconazole concentrations in Whole Embryo Culture medium and in embryonic tissue

Epoxiconazole concentration in the culture medium		Epoxiconazole residue in embryonic tissue	Concentration ratio Embryonic tissue / culture medium
[µM]	[mg/L]	[mg/L]	
0	0	0.036	---
3	0.99	0.24	0.36
10	3.30	0.87	0.26
30	9.89	2.1	0.21
60	19.79	3.3	0.17
91	30.01	8.0	0.27

III. SUMMARY AND CONCLUSIONS

The rational for performing the present in vitro study was based on evidence that a number of azole derivatives (including triadimefon) are able to affect rat embryo development in vitro by altering the migration and distribution of cephalic neural crest cells into branchial arches (the embryonic precursors of facial elements). The present study was consequently designed to investigate possible adverse effects of epoxiconazole on in vitro growth and differentiation of Wistar rat embryos. Additionally, the distribution of neural crest cells was assessed by immunostaining.

The number of embryos with anomalies was significantly increased at and above 10 µM. The most common change was fusion of branchial arches I and II and an anomalous branchial arch II. Fusion of branchial arched II and III occurred at and above concentrations of 30 µM; single cases of embryos with fused somites or single plurimalformed embryos were found at and above 60 µM epoxiconazole. Increased number of embryos with delayed or severely delayed otic vesicles were noted at and above 10 µM. The morphometric evaluation did not indicate any generalized effects on embryonic development. Immunostaining for neural crest cell migration revealed abnormal neural crest cell migration at the level of the branchial apparatus in embryos exposed to 30 µM or higher concentrations.

The epoxiconazole concentration was determined in embryonic tissue, which showed a concentration-dependent increase as expected. The tissue concentrations were determined in embryos after their morphological and morphometric assessment; during this time the embryos were immersed in Tyrode’s salt solution. It is likely that a certain degree of epoxiconazole diffused from the embryonic tissue in the salt medium during the morphological/morphometric assessment, therefore the measured embryonic tissue concentrations are probably

underestimated. The concentration ratio to the culure medium varied from 0.17 to 0.36.

Overall the following No-Observed- or Lowest-Observed-Effect Concentrations were obtained under the in-vitro conditions employed:

NOEC (dysmorphogenesis): 3 µM
LOEC (dysmorphogenesis): 10 µM

NOEC (abnormal neural crest cell migration): 10 µM
LOEC (abnormal neural crest cell migration): 30 µM

STUDY RELEVANCE

This in-vitro study provides information on the effects of epoxiconazole when rat embryos are directly exposed to epoxiconazole under in-vitro Whole-Embryo-Culture conditions during a critical time window of craniofacial development.

The study belongs to a suite of in-vitro and in-vivo mechanistic investigations that were initiated to elucidate the mechanism underlying the observed increased incidence of cleft palate after high-dose level treatment of rats with epoxiconazole. Therefore, the study is relevant for choosing the appropriate classification of epoxiconazole for developmental toxicity as part of an overall weight-of-evidence assessment (see chapters 3 and 4).

2.3.1.2 HERG Assay

STUDY REFERENCE

Report:	Hebeisen S. 2011 BAS 480 F (Epoxiconazole techn): Effect on HERG Tail currents recorded from stably transfected HEK 293 cells BASF DocID 2010/1155854 Date of report: 14-Jan-2011
Guidelines:	ICH S7A Safety pharmacology study for human pharmaceuticals, CPMP/ICH/539/00, CPMP (2000); ICHS7B The non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals, CHMP/ICH/423/02, CPMP (2005)
GLP:	Yes (laboratory certified by Swiss Federal Office of Public Health, CH-3003 Bern, Switzerland)

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material:	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	L72-185
Purity:	99.6%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 01-Apr-2015.
2. Vehicles:	Dimethylsulfoxide = DMSO, ≥99.5% purity (Sigma)
3. Positive control:	
1st positive control:	Ketoconazole
Description:	Solid, white
Lot/Batch #:	121H0524 (supplied by Sigma; product no. K1003)
Purity:	>99%
Expiry data:	31-Aug-2013.
2nd positive control:	E-4031 (selective I_{Kr} blocker, reference substance)

Description:	Solid, white
Lot/Batch #:	086K46165 (supplied by Sigma)
Purity:	>98%
Expiry date:	06-April 2010

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 18-Feb-2010 to 27-Apr-2010

2. Test system and outline of experiments

Background information on the test system

The rapid delayed rectifier current (I_{Kr}) is important for cardiac action potential repolarization. Suppression of I_{Kr} function by adverse drug effects can induce *long-QT syndrome* carrying elevated risk of life-threatening arrhythmias. A large range of therapeutic agents with diverse chemical structures have been reported to induce long-QT syndrome. These include antihistamines (e.g. terfenadine), gastrointestinal prokinetic agents (e.g. cisapride), psychoactive substances (e.g. amitryptiline, chlorpromazine, haloperidol, thioridazine) and others. Besides the acquired long-QT syndrome also genetic defects are known to cause the disease. Today it is believed that I_{Kr} is mediated by the potassium channel HERG, which is expressed in the heart but also neural tissue. Although acquired long QT syndrome theoretically could also arise from block of any other voltage-gated potassium channel involved in ventricular repolarization, so far all known agents displaying this adverse effect preferentially block the HERG channel. The large volume of the inner vestibule, the features of HERG' s S6 sequence as well as the fast C-type inactivation process seem to contribute to the unique properties of this potassium channel.

Recently it was speculated that blockade of HERG channel might cause teratogenic effects via hypoxia and/or generation of reactive oxygen species in embryos (Danielsson B.R. et al. 2007; Nilsson M.F. et al. 2010). It was therefore of interest to investigate whether epoxiconazole has the potential to block the HERG channel, and if yes, at which concentration.

The test system was chosen to assess the potential of epoxiconazole for QT-prolongation. Interaction of epoxiconazole with the HERG channel is examined in HEK-293 cells stably expressing this potassium channel. The test system is considered suitable for this purpose according to above cited test guidelines.

Preparation of dose formulations

DMSO stock solutions of 80 mM epoxiconazole and of 10 mM ketoconazole were prepared in advance of the experiments and stored until use at -10 to -30 °C. The test solutions were diluted from the stock solutions using bath solution shortly prior to the electrophysiological experiments and kept at room temperature (19°C to 30°C) when in use. The vehicle concentration was adjusted in all dose formulations to achieve a final concentration of 0.3%.

Test item concentrations

Epoxiconazole was tested at concentrations of 1.0, 10, 30, 80 and 160 µM, ketoconazole at concentrations of 0.3, 1.0, 3.0, 10 and 30 µM. The epoxiconazole

concentrations were chosen on the basis of a non-GLP study which tested a large concentration range. For ketoconazole, the tested concentrations were selected on the basis of published literature.

Analysis of dose preparations
Representative samples of the dose formulations were collected at the end of experimentation and stored at -10 to -30 °C until shipment on dry ice to Harlan Laboratories. All samples were analysed by HLPC/UV.

Experimental procedure
HEK 293 cells stably expressing the HERG channel were seeded in 35mm culture dishes at a density allowing single cells to be recorded. The culture dishes were placed on the dish holder of the microscope and continuously perfused (at approximately 1 ml/min) with a perfusion medium. All solutions applied to cells including the pipette solution were maintained at room temperature (19-30°C). After formation of a Gigaohm seal between the patch electrodes and individual HERG stably transfected HEK 293 cells (pipette resistance range: 2.0MΩ-7.0MΩ; seal resistance range: >1GΩ) the cell membrane across the pipette tip was ruptured to assure electrical access to the cell interior (whole-cell patch-configuration). If the quality of the seal was poor, the process of seal formation was repeated with a different cell and a new pipette.

As soon as a stable seal was established HERG outward tail currents were measured upon depolarization of the cell membrane to +20mV for 2s (activation of channels) from a holding potential of -80mV and upon subsequent repolarization to -40mV for 3s. This voltage protocol was run at least 10 times at intervals of 10s. If current density was judged to be too low for measurement, another cell was recorded. Once control recordings had been accomplished, cells were continuously perfused with a bath solution containing the test item concentrations. During wash-in of the test item the voltage protocol indicated above was run continuously again at 10s intervals until the steady-state level of block was reached. Complete cumulative dose-response analysis was accomplished per cell. Only data from cells treated with the test item epoxiconazole, ketoconazole or E-4031 were documented.

IC_{50} determination
Concentration-response curves were determined and the IC_{50} values were calculated. Each concentration of the test item or Ketoconazole were analyzed in three separate experiments (n=3). The whole cell configuration was established. After measurement of the control period, concentrations of the test item were applied to the perfusion bath. During wash-in of the test item the voltage protocol outlined above was run until the steady-state level of channel inhibition was reached.

The inhibition curve was constructed with a sigmoidal two-parameter equation:

$$current_{peak,relative} = 100 \Big/ 1 + 10^{[(\log IC_{50} - X) - H]}$$

… where “X” is the drug concentration, “IC_{50}” is the concentration of the test item at half maximum inhibition and “H” is the Hill coefficient.

Data compilation and statistical analysis

Values (in pA/nA) of the peak amplitudes of outward tail currents were generated for each voltage step. The recorded current amplitudes at the steady state level of current inhibition were compared to those from control conditions measured in the pre-treatment phase of the same cell. The amount of current block was calculated as percentage of control. To determine whether any observed current inhibition is due to a test item interaction with the HERG channel or due to current rundown, these residual currents were compared to those measured in vehicle treated cells. Data from three individual cells were collected and the corresponding mean values and standard errors calculated.

Data were analysed with Analysis of Variance (ANOVA) applying a multi-sample comparison of the mean test concentrations of each test group against control values (Dunnett test, 1-sided) was conducted (statistically significant if $p < 0.05$).

II. RESULTS

Stability of test item

The test item stock solutions of 80 mM BAS 480 F epoxiconazole and 10 mM ketoconazole were stored at -10 to -30°C (freezer). The application of the test item was completed within one working day. During this period, there was no observation indicating any instability of the test preparation. For all concentrations of ketoconazole and 1.0, 10.0 and 30 µM epoxiconazole the test item effects of the dose formulations analyzed remained stable over several hours at room temperature. During this period, there were no observations indicating any instability of test item solved in bath solution. For the two highest tested concentrations of epoxiconazole (80 and 160 µM) precipitation was observed with time. Because of this effect that also caused a decreasing effect of the test item on the HERG channel- these test solutions were freshly prepared before each application. The concentration control analyses for epoxiconazole test preparations were found to be in the range of 85.1% to 110% of the nominal concentrations. The results obtained confirmed the correct preparation and storage of the application preparations during the conduct of the study.

Tail current data

A total of 9 experiments were used for compilation of data and analysis. A summary of the experimentally determined relative tail current percentage (steady-state current (pA) / control current (pA) with the means and standard error means (SEM) from 3 experiments are summarized in the table below.

Table 2/93 HERG assay: Mean values of relative tail current inhibition

Test item concentration	Concentration [µM]	Relative tail current (%)			Mean ± SEM
		Experiment 1	Experiment 2	Experiment 3	
Vehicle (0.3% DMSO)	0	98.10	97.35	97.66	97.70 ± 0.22%
Epoxiconazole	1	94.12	95.71	101.28	97.04 ± 2.17%
	10	79.68	83.45	85.67	82.93 ± 1.75%**
	30	61.67	64.17	67.43	64.42 ± 1.67%**
	80	28.89	38.21	38.34	35.15 ± 3.13%**
	160	15.04	12.56	17.27	14.96 ± 1.36%**
Ketoconazole	0.3	94.90	96.49	96.48	95.95 ± 0.53%
	1	71.04	69.22	72.14	70.80 ± 0.85%**
	3	45.00	37.63	44.00	42.21 ± 2.31%**
	10	14.16	13.79	17.13	15.03 ± 1.06%**
	30	0.81	1.82	0.90	1.18 ± 0.32%**
E-4031	0.1	6.37	1.94	6.61	4.97 ± 1.52%**

Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, 1-sided)

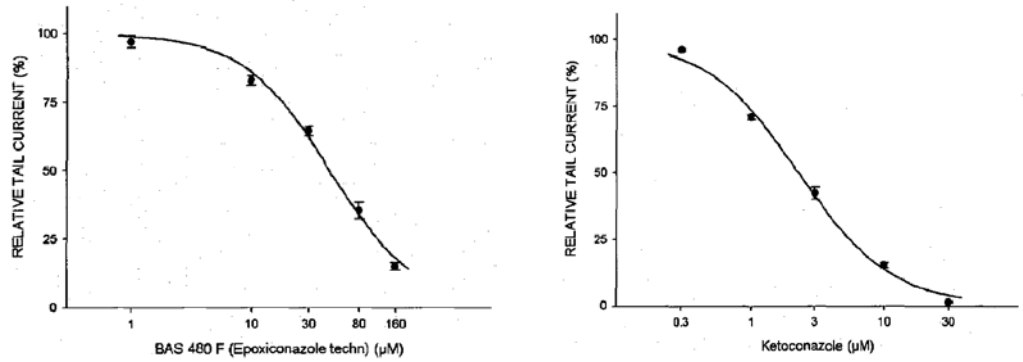
The IC_{50} determination using a best-fit two-parameter equation provided the following values:

Table 2/94 HERG assay: IC_{50} determination

Application	IC50 [µM]	Hill coefficient
Epoxiconazole	45.43	1.20
Ketoconazole	2.26	1.24

The obtained inhibition curves for epoxiconazole and ketoconazole are shown in Figure 2.1/12.

Figure 2.1/12 HERG assay: inhibition curves



III. SUMMARY AND CONCLUSIONS

The whole-cell patch-clamp technique was used to investigate the effect of epoxiconazole on HERG (human-ether-a-go-go related gene) potassium channels stably expressed in HEK 293 cells. Epoxiconazole was tested at concentrations of 1.0, 10, 30, 80 and 160 µM (n=3 cells) in order to determine compound effects on

HERG mediated current. For comparison the known HERG blocker ketoconazole was tested at concentrations of 0.3, 1.0, 3.0, 10 and 30 µM. Inhibition curves could be generated for both test compounds. They were best fit with an IC_{50} value of 45.43 µM (Hill coefficient: 1.20) for epoxiconazole and an IC_{50} of 2.26 µM (Hill coefficient: 1.24) for ketoconazole. Statistically significant reductions in the tail current were obtained at and above concentrations of 10 µM for epoxiconazole (about 83% remaining current) and 1 µM for ketoconazole (about 71% remaining current) No significant changes of the tail current were observed at an epoxiconazole concentration of 1 µM and at a ketoconazole concentration of 0.3 µM. The test system was validated using the reference item "E-4031" (selective I_{Kr} blocker), which effectively blocked the HERG tail current in this study at a concentration of 100 nM (about 5% remaining tail current).

STUDY RELEVANCE

The in-vitro study was performed to address the recent speculation that the blockade of the cardiac HERG potassium channel by triazoles might cause teratogenic effects via hypoxia and/or generation of reactive oxygen species in embryos (Danielsson B.R. et al. 2007; Nilsson M.F. et al. 2010). In the RAC 10 Meeting, Dr. Danielsson presented general but no epoxiconazole-specific data in support of the above outlined hypothesis. It was therefore of interest to investigate whether epoxiconazole has the potential to block the HERG channel, and if yes, at which concentration.

Overall, with an IC_{50} of 45.43 µM the in-vitro study results suggested a weak effect on the HERG channel, both in terms of direct comparison with compounds known to be potent HERG inhibitors and also by comparison with parallel tested ketoconazole (IC_{50} 2.26 µM).

However, it remains unclear whether the observed effect on the HERG channel seen in-vitro has any causal relationship to teratogenic effects observed in rats under in-vivo conditions.

In conclusion the results of the in-vitro study on inhibition of the HERG channel is considered relevant as supplemental information and should be considered as part of an overall weight-of-evidence assessment for selecting the appropriate classification of epoxiconazole for developmental toxicity (see chapters 3 and 4).

2.3.2 TOXICOKINETICS

2.3.2.1 Plasmakinetic and metabolism study in pregnant rats

STUDY REFERENCE

Report: Fabian E., Landsiedel R. (2011a)
^{14}C-BAS 480 F (Epoxiconazole) Kinetic study in pregnant Wistar rats
BASF DocID 2011/1112619
Date of report: 10-Jun-2011

Guidelines: Reference made to: OECD 414; OECD 417; OPPTS 870.7485; Commission Regulation (EC) No 440/2008; Japan/MAFF

GLP: Yes
(laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz

Report: Thiaener J., Kuhnke G., Glaessgen W.E. (2011)
Metabolism investigation of 14C-BAS 480 F (Epoxiconazole) in plasma of pregnant Wistar rats after oral administration
BASF DocID 2010/1031712
Date of report: 2-Aug-2011

GLP: Yes
(laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1a. Radiolabelled test material:	^{14}C-Epoxiconazole (BAS 480 F)
Lot/Batch #:	291-3101
Radiochemical purity:	99.7%
Specific activity:	6.3 MBq/mg
Label:	triazole-3(5)-C14

1b. Unlabelled test material:	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	8563

Purity:	97.0%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 30-Jun-2010.

2. Vehicle control: 1% aqueous Carboxymethylcellulose (1% CMC) or corn oil

3. Test animals:

Species:	Rat
Strain:	Wistar [Crl:WI (Han)(SPF)]
Sex:	Female
Age:	Young adult aged 10-12 weeks
Weight (pregnant rats GD 0):	about 190 to 229 g
Source:	Charles River Lab., Germany
Acclimatization period:	at least 4 days before mating
Diet:	Ground Kliba maintenance diet for mouse/rats "GLP", Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing in type M III Makrolon cages with dust-free wooden bedding (until GD 9); steel wire mesh cages (GD 9 until sacrifice), wooden gnawing blocks (Abedd Lab. and Vet. Service GmbH, Vienna) offered for enrichment
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	not mentioned in the report
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work: 02-Feb-2010 to 20-Apr-2010 (in-life dates: 02-Feb-2010 (arrival of animals), 01-Mar-2010)

2. Animal assignment and treatment:

After an acclimatization period of at least 4 days, untreated female rats were mated with an untreated male of the same breed (ratio female to male rats: 1 or 2 female rats with one male rat). The male rats were kept under the same conditions (air conditioning, feed, water) as the female rats of this study. Rats were paired from about 15:30 h of one day until about 7:30 h in the morning followed by preparation of vaginal smears. When sperm was detected in the vaginal smear, the

females were considered being impregnated and transferred into the study. This day was referred to as gestation day 0 (GD 0), the following day as GD 1.

The experimental design is outlined in Table 2/95. Each test group consisted of 4 pregnant rats. Dams from groups 4-7 received unlabeled epoxiconazole in 1% CMC suspension by daily oral gavage administration from GD 6 to 9, at dose levels of 5, 50, 100 and 180 mg/kg bw in a dose volume of 10 mL/kg bw. Rats from group 8 received 50 mg/kg bw/d unlabeled epoxiconazole in corn oil suspension from GD 6-9, the dose volume in this case was 2 mL/kg bw. Female rats from groups 0-3 remained untreated during GD 6-9.

On GD10, blood was sampled from rats of groups 4-7 and 8. Thereafter radiolabeled test substance preparations were administered to the pregnant rats from all dose groups 0-8. Blood samples (ca. 100-300 µL) were subsequently taken from the retroorbital sinus of each animal under isoflurane anesthesia at the following time points: 0.5, 1, 2, 4, 8, 24, 48, and 72 hours. A final blood sampling was performed at 96 hours upon exsanguination under isofluorane anesthesia, after which pups were removed from the uterus and killed by an overdose of Narcoren.

Table 2/95 Dose groups

Test group	Vehicle	Unlabeled epoxiconazole GD 6-9 (mg/kg bw/d)	Single radioactive epoxiconazole dose GD 10 (mg/kg bw/d)	MBq/animal	Ratio unlabeled / labeled	MBq/mg
Experiment 1						
0	1% CMC[#]	Untreated	5	10	pure radiolabel	6
1		Untreated	50	30	1.5 : 1	2.4
2		Untreated	100	30	4 : 1	1.2
3		Untreated	180	50	5 : 1	1
Experiment 2						
4	1% CMC[#]	5	5	10	pure radiolabel	6
5		50	50	30	1.5 : 1	2.4
6		100	100	30	4 : 1	1.2
7		180	180	50	5 : 1	1
Experiment 3						
8	Corn oil[$]	50	50	30	1.5 : 1	2.4

[#] dose volume 10 mL/kg bw; [$] dose volume 2 mL/kg bw

Rationale for dose selection:
Epoxiconazole dose levels were chosen to correspond to effect and no-effect levels that had been identified in previous prenatal developmental toxicity studies with Wistar rats. The highest dose level of 180 mg/kg bw/d was selected because cleft palates were induced at this dose level in previous investigations. The dose of 5 mg/kg bw/d represented a clear NOEL in developmental toxicity studies and 50 mg/kg bw/d was a NOAEL with regard to cleft palate formation and a LOAEL for the induction of late fetal resorptions in prenatal developmental toxicity rat studies. 100 mg/kg bw/d was selected as intermediate dose level.

3. Test substance preparation:

^{14}C-epoxiconazole and the non-radiolabeled test substance were prepared in aqueous carboxymethylcellulose. About 10 mL/kg body weight of a preparation were administered to rats by gavage. Specific activities of the test substance in the preparations of ^{14}C-epoxiconazole depended on the dose group. Details are given in the following sections.

Preparation of the unlabeled test substance

For the unlabeled test substance preparations in 1% CMC or corn oil, the specific amount of test substance was weighed, topped up with either 1% carboxymethylcellulose in highly deionized water or corn oil in a calibrated beaker and intensely mixed with a homogenizer. During administration, the preparations were kept homogeneous with a magnetic stirrer.

Preparation of the radiolabeled test substance

For the groups 0 and 4 (5 mg/kg bw epoxiconazole) pure radiolabeled epoxiconazole was prepared in 1% CMC. Therewith, the quantity of radioactivity per animal was about 10 MBq. For the higher dose levels of 50, 100 and 180 mg/kg bw/d, radiolabeled epoxiconazole was mixed with unlabeled epoxiconazole in order to achieve the intended specific activity of 2.4, 1.2 or 1 MBq/mg, respectively, resulting in administration of 30 MBq/rat for dose groups 1, 2, 5, 6, and 8, and 50 MBq/rat for the top dose groups 3 and 8.

For the preparation of ^{14}C-epoxiconazole in corn oil, a homogenous mixture was produced using magnetic stirrer, ultra-sonication and a homogenizer.

Homogeneity and concentration control analyses

Representative samples of the unlabeled test preparations were taken to determine the concentration of the test substance and the homogeneity. The stability of the test substance preparations were demonstrated before the start of the administration period. The measurements within the current study were performed by HPLC analyses.

The stability of the radioactive test substance in the vehicle was verified by analysis in the experiments. Radioactive test substance preparations were sampled before and at the end of the administration for verification of the concentrations, homogeneities and radiochemical purities by liquid scintillation counting and by HPLC analyses.

C. ANALYSES AND MEASUREMENTS

Whole blood samples were inverted several times to ensure homogeneity and then separated into plasma and blood cells by centrifugation.

1. Determination of total radioactivity

The total weight of the plasma sample was recorded, samples were mixed by shaking and aliquots were mixed with about 17 ml Hionic Fluorcocktail and analysed for total radioactivity by liquid scintillation counting.

2. Metabolic profiling

Pooled plasma samples were shipped to APD/EC Consumer Safety, BASF SE, Limburgerhof for metabolic profiling. Proteins were precipitated from the pooled plasma samples, and the protein precipitates from the time points of 2 h to 24 h were treated with Protease. The distribution of radioactive residues was determined, and supernatants after protein precipitation and solubilized radioactive residues after Protease treatment were analyzed by HPLC. Separation of peaks was performed via gradient elution on reversed-phase columns or on a HILIC column.

II. RESULTS

1. Homogeneity and concentration control analysis for the test substance preparations

The analytical investigations performed in the context of this study demonstrated the stability, homogeneity and correctness of the concentrations of unlabeled epoxiconazole administrations for dose groups 4-8 on GD 6-9 and radiolabeled ^{14}C-epoxiconazole) administrations for dose groups 0-8 on GD 10 in the vehicle for all performed experiments.

2. Plasma levels of ^{14}C-epoxiconazole

Plasma concentrations from pregnant rats that received a single oral dose with radiolabeled epoxiconazole on GD 10 are summarized in Table 2/96; plasma concentrations data from rats that were pre-exposed with 4 treatments of non-radiolabeled epoxiconazole during GD 6-9 followed by administration of ^{14}C-epoxiconazole on GD 10 are summarized in Table 2/97.

The total radioactive residues in pooled plasma samples of the dose groups with 5 mg/kg bw showed a continuous decrease with a second maximum at 8 h. After single dosing with 50 mg/kg bw, the highest levels were obtained at 0.5 h timepoint whereas the pre-dosed group of 50 mg/kg bw plasma levels peaked at 1 h and 8h. For the dose level of 100 mg/kg bw the plasma concentration of the single dose animals was at an approximately constant level up to 24 h (with slight relative maxima at 0.5 h and 24 h) and decreased afterwards, whereas the pre-dose group showed a continuous increase during the time period of 1 h to 8 h followed by continuous decrease. The high dose groups (single dose and pre-dose, 180 mg/kg bw) showed an increase up to 24 h and 8 h, respectively, with following decrease. For the two vehicles, 1% aqueous CMC and corn oil, at a dose level of 50 mg/kg bw, slightly higher maximum residue levels in plasma (after 4 h and 8 h) were observed when epoxiconazole was dosed in corn oil.

Table 2/96 Mean plasma concentration of radiolabel in pregnant rats after single oral administration of ^{14}C-epoxiconazole on GD10 (experiment 1)

Dose group	0	1	2	3
Dose (mg/kg bw/d)	5	50	100	180
Sampling time [h]	Plasmaconcentration [µg Eq/g plasma]			
0.5 h	1.26	9.52	11.70	6.96
1 h	0.99	7.97	11.63	7.94
2 h	0.77	6.74	10.47	8.34
4 h	0.70	6.64	10.69	9.68
8 h	0.74	6.47	11.21	11.14
24 h	0.37	4.21	11.40	17.63
48 h	0.18	1.61	3.91	10.85
72 h	0.11	0.95	1.71	3.75
96 h	0.07	0.55	1.01	1.76
120 h	0.04	0.36	0.64	0.91
144 h	0.03	0.18	0.39	0.67

Table 2/97 Mean plasma concentration of radiolabel in pregnant rats, single oral dose of ^{14}C-epoxiconazole on GD10 after 4 daily oral doses of unlabeled epoxiconazole from GD6-9

Dose group	4	5	8	6	7
Dose (mg/kg bw/d)	5	50	50	100	180
Vehicle	1% CMC	1% CMC	Corn oil	1% CMC	1% CMC
Sampling time [h]	Plasmaconcentration [µg Eq/g plasma]				
0.5 h	0.70	5.78	4.38	7.63	8.54
1 h	1.00	5.89	4.20	7.80	10.00
2 h	0.82	5.40	5.47	7.38	14.45
4 h	0.83	5.07	7.34	8.07	17.19
8 h	0.77	5.68	7.96	10.96	20.18
24 h	0.49	2.82	3.42	5.14	11.15
48 h	0.18	1.02	1.07	1.71	2.93
72 h	0.10	0.57	0.64	0.90	1.27
96 h	0.06	0.33	0.36	0.54	0.68
120 h	0.04	0.22	0.24	0.34	0.48
144 h	0.02	0.12	0.13	0.23	0.30

A comparison of the total radioactive residues in plasma of rats pre-dosed with unlabeled epoxiconazole with residue levels from rats that received a single dose of ^{14}C-epoxiconazole without pre-treatment showed no clear tendency: the time course of the residues in plasma of the low dose groups 0 and 4 (5 mg/kg bw) was comparable. Doses of 50 and 100 mg/kg bw resulted in slightly higher radioactive residue levels in plasma of the animals without pre-dosing and the high dose level

of 180 mg/kg bw led to somewhat higher maximum levels of the radioactive residues and a faster decrease in plasma of the pre-dosed animals.

The pharmacokinetic parameters derived from the plasma kinetics in pregnant Wistar rats after a single oral dose of ^{14}C-epoxiconazole on GD 10 are presented in the following table:

Table 2/98 Pharmacokinetic parameters, single oral dose of ^{14}C-epoxiconazole on GD10

Grp	Dose [mg/kg bw]	Vehicle	Tmax [hour]		Cmax [µg Eq/q]		Initial half life [hour]	Terminal half life [hour]	$AUC_{0\rightarrow\infty}$ [µg Eq x h / g]
0	5	1% CMC	0.5	8	1.26	0.74	2.18	30.11	30.35
1	50	1% CMC	0.5		9.52		3.13	26.91	284.11
2	100	1% CMC	0.5	24	11.70	11.40	6.60	25.50	596.32
3	180	1% CMC	24		17.63		---	23.72	957.20

The pharmacokinetic parameters derived from the plasma kinetics in pregnant Wistar rats after four daily oral doses of epoxiconazole on GD 6-9, followed by a single oral dose of ^{14}C-epoxiconazole on GD 10 are presented in the following table:

Table 2/99 Pharmacokinetic parameters, single oral dose of ^{14}C-epoxiconazole on GD10 after 4 daily oral doses of unlabeled epoxiconazole from GD6-9

Grp	Dose [mg/kg bw]	Vehicle	Tmax [hour]		Cmax [µg Eq/q]		Initial half life [hour]	Terminal half life [hour]	$AUC_{0\rightarrow\infty}$ [µg Eq x h / g]
4	5	1% CMC	1	4	1.00	0.83	---	31.78	31.86
5	50	1% CMC	1	8	5.89	5.86	(7.98)	31.79	202.17
6	100	1% CMC	1	8	7.80	10.96	(12.52)	27.87	351.34
7	180	1% CMC	8		20.18		---	30.08	653.72
8	50	Corn oil	0.5	8	4.38	7.96	---	24.14	244.18

AUC-values were derived which indicated an internal exposure correlated to the oral doses. Increasing the oral dose by a factor of 10 (from 5 to 50 mg/kg bw) resulted in an increase of the AUC values by factor of 9 and 6 (dose groups 0 versus 1 and 4 versus 5). Increasing the dose by a factor of 2 (from 50 to 100 mg/kg bw) resulted in an increase of the AUC values by a factor of about 2 (dose groups 1 versus 2 and 5 versus 6). Further increase of the dose by a factor of 1.8 (from 100 to 180 mg/kg bw) resulted in an increase of the AUC values by a factor of about 1.6 and 1.9 (dose groups 2 versus 3 and 6 versus 7). For the two vehicles 1% aqueous CMC (dose group 5) and corn oil (dose group 8), an about 1.2-fold higher AUC value was observed when epoxiconazole was dosed in oil.

3. Distribution of radioactive residues in plasma fractions

For the plasma samples taken shortly after dosing, the major part of the radioactive residues was found in the supernatants after protein precipitation. At later sampling occasions, increasing portions of the radioactive residues were precipitated from plasma with acidified acetonitrile. This increase was a little faster at lower dose levels. For further characterization of the residues associated with the protein fraction, selected protein precipitates (2 h to 24 h) were incubated with Protease which solubilized most of the radioactive residues. Treatment of two exemplary protein precipitates solely with buffer also released a considerable part of the radioactive residues. This indicated that residues had rather been associated with insoluble components than specifically bound to precipitated plasma proteins.

4. Plasma levels of unchanged epoxiconazole in different treatment groups

The total concentration of the parent compound epoxiconazole summarized from all plasma samples analyzed by HPLC for each individual time point (compiled in Table 2/100) showed a continuous decrease throughout the time course for the low dose groups (5 mg/kg bw) and the group having received a single dose of 50 mg/kg. In plasma of the pre-dosed animals at the dose level of 50 mg/kg bw, a maximum at 2 h with following decrease was observed.

For the 100 mg/kg bw dose level approx. constant concentrations of epoxiconazole were found up to 8 h followed by significant decrease.

In the case of the high dose groups (180 mg/kg bw) an increase of the parent concentration was detected within 8 h prior to decrease. The concentration of epoxiconazole revealed a delayed maximum with increasing dose level.

Pre-dosing of the animals for four days had no clear effect on the concentrations of parent epoxiconazole (compared to concentrations in rats administered only a single radiolabeled dose on GD10): the values for the parent compound were slightly higher in plasma of animals having received a single dose of 50 or 100 mg/kg bw compared to plasma of pre-dosed animals and lower at a single dose of 180 mg/kg bw compared to the respective pre-dosed group. The decrease of the concentration of epoxiconazole proceeds faster in plasma of the pre-dosed animals of the high dose groups. The concentration of epoxiconazole in plasma showed a slightly higher level after 4 h and 8 h when the active substance was dosed in corn oil at 50 mg/kg bw compared to using 1% CMC as vehicle.

Table 2/100 Plasma concentrations of epoxiconazole [µg/ml and % TRR]

Grp	Dose [mg/kg bw]	0.5 h	1 h	2 h*	4 h*	8 h*	24 h*	48 h	72 h	96 h
Epoxiconazole [µg/mL]										
0**	5	1.065	0.629	0.372	0.203	0.132	n.d.	n.a.	n.a.	n.a.
1	50	8.070	6.974	5.298	4.032	2.910	0.227	n.d.	n.a.	n.a.
2	100	7.900	7.609	7.074	8.061	6.393	3.094	n.d.	n.d.	n.a.
3	180	3.607	3.677	3.692	5.431	5.650	3.058	n.d.	n.d.	n.d.
4	5	0.947	0.508	0.312	0.196	0.106	0.006	n.a.	n.a.	n.a.
5	50	3.115	2.886	3.576	2.692	2.047	0.063	n.d.	n.a.	n.a.
6	100	5.356	5.354	5.680	5.255	5.903	0.107	n.d.	n.a.	n.a.
7	180	4.706	6.952	10.001	11.044	11.595	n.d.	n.a.	n.a.	n.a.
8	50 (corn oil)	3.338	2.676	3.417	4.115	3.311	0.083	n.d.	n.a.	n.a.
Epoxiconazole [%TRR]										
0**	5	76.9	58.3	40.2	25.8	15.4	n.d.	n.a.	n.a.	n.a.
1	50	67.6	65.2	56.6	57.0	39.1	4.9	n.d.	n.a.	n.a.
2	100	61.6	61.4	55.3	65.4	53.9	25.9	n.d.	n.d.	n.a.
3	180	44.9	45.4	42.3	53.2	47.1	20.4	n.d.	n.d.	n.d.
4	5	63.9	49.3	34.5	23.7	12.3	1.3	n.a.	n.a.	n.a.
5	50	62.2	55.5	65.0	54.9	30.4	2.0	n.d.	n.a.	n.a.
6	100	65.1	69.3	62.4	56.9	45.0	1.8	n.d.	n.a.	n.a.
7	180	51.1	56.7	60.5	54.8	47.1	n.d.	n.a.	n.a.	n.a.
8	50 (corn oil)	65.9	61.3	57.3	49.2	38.3	2.5	n.d.	n.a.	n.a.

n.a. = not analyzed by HPLC; n.d. = not detected

%TRR = radioactivity contained in an individual fraction compared to the Total Radioactive Residue in the particular sample / matrix worked up

* sum (residues in supernatants after protein precipitation plus residues solubilized by protease treatment of protein precipitates)

** additional extraction of residues from protein precipitates with water/acetonitrile mixtures (acidic and basic) at 4 and 8-hour timepoint (Dose group 0 only)

5. Biotransformation products and metabolic pathway [see Table 2/101 and Figure 2/13]

The identification of metabolites was based on co-chromatography experiments performed with selected plasma fractions and reference items. Some additional minor peaks in the supernatants after protein precipitation and a series of multiple polar components in the Protease solubilizates from the protein precipitates were characterized by their chromatographic properties. The unchanged parent epoxiconazole was the main component in the plasma samples from all dose groups at the beginning of the investigation period, and its concentration rapidly decreased. Latest 48 h after dosing no parent compound was detected any more. Metabolic transformation of epoxiconazole in the rat occurred mainly at two sites in the molecule: Hydroxylation of the chlorophenyl ring led to the formation of the metabolite 480M02 or / and its isomer 480M26. The oxirane ring was opened

hydrolytically to form the respective geminal diol, metabolite 480M05 or / and its isomers, followed by conjugation of the hydroxylated diol metabolite with glucuronic acid to form the metabolite 480M60 or with cysteine (or conjugation with glutathione and subsequent loss of glutamic acid and glycine) to form the metabolite 480M13 or / and isomers. The chlorophenyl ring was detached by cleavage of the diol 480M05 or the active substance at the C_2-bridge between the aromatic nuclei to form the metabolite 480M06 (detected only after protease treatment of protein precipitates from plasma samples). Free 1,2,4-triazole (metabolite 480M52) was mainly detected in the plasma samples obtained at the late sampling occasions and thus a final product of intense degradation of epoxiconazole. The proposed metabolic pathway of epoxiconazole in rats is shown in Figure 2/13.

Figure 2/13 Metabolic path of epoxiconazole in rats

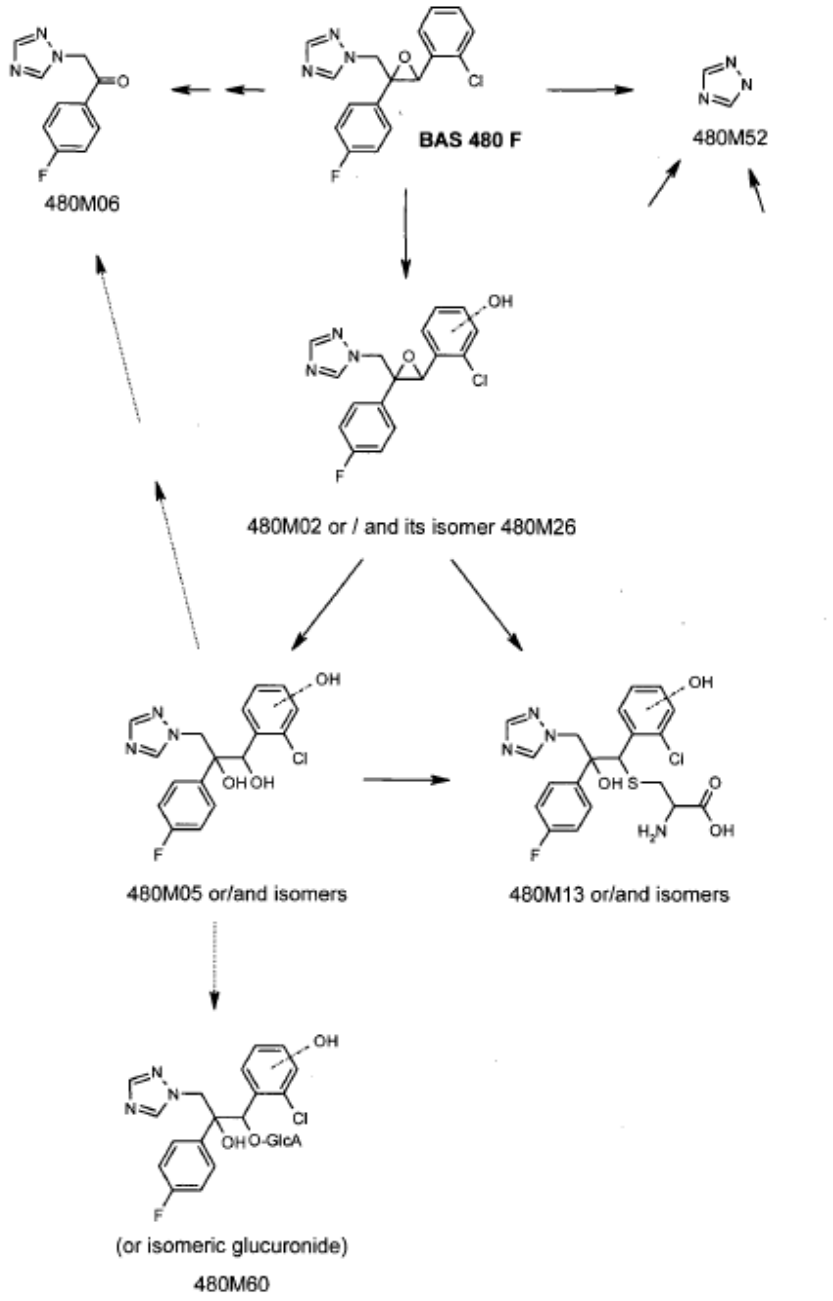


Table 2/101 Identification of radioactive residues in supernatants (after protein precipitation) from plasma samples

Vehicle: 1% CMC		Epoxiconazole		480M02 / 480M26	480M13 / 480M60	480M05	480M52	Total identified and/or characterised
mg/kg bw/d	time	µg/mL	%TRR	%TRR	%TRR	%TRR	%TRR	%TRR
Single dose administration of radiolabeled epoxiconazole on GD 10 - without pre-dosing from GD 6-9								
5	0.5 h	1.065	76.9	n.d.	3.1	11.5	6.3	97.8
	8 h	0.132	15.4	12.6	5.9	4.5	14.9	65.5
	24 h	n.d.	n.d.	11.5	6.0	n.d.	16.9	39.4
50	0.5 h	8.070	67.6	n.d.	2.6	5.6	3.5	82.0
	8 h	2.882	38.8	3.3	5.2	10.2	6.8	70.3
	24 h	0.227	4.9	11.7	5.3	1.5	16.4	49.3
	48 h	n.d.	n.d.	6.9	1.4	n.d.	18.0	29.8
100	0.5 h	7.900	61.6	n.d.	3.2	3.0	6.5	77.4
	8 h	6.188	52.2	n.d.	3.1	9.4	7.5	74.1
	24 h	3.094	25.9	11.3	6.4	5.2	15.6	64.4
	48 h	n.d.	n.d.	10.1	1.2	n.d.	29.8	41.8
	72 h	n.d.	n.d.	2.5	n.d.	n.d.	14.4	18.0
180	0.5 h	3.607	44.9	n.d.	10.6	7.4	14.3	83.2
	8 h	5.509	45.9	1.5	6.1	10.2	10.1	73.9
	24 h	3.058	20.4	12.4	8.9	8.0	12.3	64.1
	48 h	n.d.	n.d.	17.5	6.0	n.d.	32.3	55.7
	72 h	n.d.	n.d.	1.7	1.7	n.d.	29.1	32.4
	96 h	n.d.	n.d.	n.d.	n.d.	n.d.	11.9	11.9
Four day pre-dosing from GD 6-9 with administration of radiolabeled epoxiconazole on GD 10								
5	0.5 h	0.947	63.9	n.d.	n.d.	8.8	6.0	78.7
	8 h	0.106	12.3	10.8	4.4	3.6	9.4	47.9
	24 h	0.006	1.3	17.3	4.9	n.d.	16.1	41.3
50	0.5 h	3.115	62.2	n.d.	2.4	7.6	7.7	79.9
	8 h	1.990	29.5	4.4	2.9	6.1	9.7	58.0
	24 h	0.063	2.0	18.9	2.4	n.d.	27.1	50.4
	48 h	n.d.	n.d.	4.8	n.d.	n.d.	18.9	23.7
100	0.5 h	5.356	65.1	n.d.	3.4	6.2	7.5	82.1
	8 h	5.722	43.6	3.6	1.8	4.9	13.9	70.4
	24 h	0.107	1.8	13.0	n.d.	n.d.	41.0	56.8
	48 h	n.d.	n.d.	1.6	n.d.	n.d.	19.4	25.3
180	0.5 h	4.706	51.1	n.d.	4.4	3.0	12.9	76.4
	8 h	11.020	44.8	4.0	2.6	1.6	15.9	70.2
	24 h	n.d.	n.d.	8.7	1.6	n.d.	48.7	62.3
	48 h	n.d.	n.d.	n.d.	n.d.	n.d.	43.0	46.7

%TRR = radioactivity contained in an individual fraction compared to the Total Radioactive Residue in the particular sample / matrix worked up

III. SUMMARY AND CONCLUSIONS

Pregnant rats were administered a single oral dose of radiolabel led Epoxiconazole at dose levels of h 5, 50, 100 or 180 mg/kg bw on GD 10. AUC values of approx. 30, 284, 596 or 957 µg Eq x h/g were obtained, indicating dose proportionality (no evidence for impaired elimination). Cmax values occurred at later time points with increasing dose with evidence of enterohepatic circulation except for the high dose level. Slightly higher maximum plasma levels and AUC were obtained when corn oil was used as vehicle instead of 1% CMC (investigated at 50 mg/kg only). Changes in PK parameters of groups with predosing of unlabeled epoxiconazole from GD6-9 did not reveal any clear tendency for a change (AUC values of 32, 202, 351 and 654 µg Eq x h/g at the respective dose levels). Epoxiconazole was main component in plasma at early sampling time-points, followed by intense degradation (hydroxylation of chlorophenyl ring; hydrolytical opening of oxirane ring with subsequent glucuronic acid or glutathione conjugation reactions, cleavage from the chlorophenyl ring from parent or metabolized molecule; formation of 1,2,4-triazole. Epoxiconazole plasma levels seemed to decrease faster in predosed animals.

The study provided information on Tmax which were used to determine appropriate blood sampling time points for the prenatal developmental toxicity study for investigation of rat embryos (see Chapter 2.1.7; Flick et al. 2012; DocID 2012/1059618). Information on obtained Cmax values obtained for the cleft palate inducing dose of 180 mg/kg bw/d was considered in the selection of appropriate test concentrations for the Whole-Embryo-Culture experiments performed with epoxiconazole (see chapter 2.3.2.1, Menegola 2012; DocID 2012/1058203)

STUDY RELEVANCE

Studies on the biokinetics and metabolism of a test substance provide relevant information for the evaluation of test results from other toxicological studies and for the extrapolation of data from animals to man.

This toxicokinetic and metabolism study with epoxiconazole provides important supplemental information which can be used to interpret findings obtained in in-vitro investigations: the in-vivo study data allows an association between in-vivo external dose, in-vivo internal blood concentration and in-vitro test concentration. Thus, the data can be used to check the plausibility of obtained in-vitro results.

The study also provides the basis for a species comparison of the toxicokinetics and metabolic profile of epoxiconazole, together with the available parallel investigation in pregnant guinea pigs. By additional comparison of the observed toxicological profile of epoxiconazole in these two species, the toxicokinetic / metabolism data can inform whether the absence of late fetal resorptions or of cleft palate in guinea pig studies is explainable by differences in internal dose levels or by differences in metabolism.

In conclusion, the study belongs to a suite of in-vitro and in-vivo mechanistic investigations that were initiated to elucidate the mechanism underlying the

observed increased incidence of both cleft palate and late fetal resorptions after high-dose level treatment of rats with epoxiconazole. Thus, the study is relevant for choosing the appropriate classification of epoxiconazole for developmental toxicity as part of an overall weight-of-evidence assessment.

2.3.2.2 Plasmakinetic and metabolism studyin pregnant guinea pigs

STUDY REFERENCE

Report:	Fabian E., Landsiedel R. (2011b) Kinetic study of ^{14}C-BAS 480 F (Epoxiconazole) in pregnant guinea pigs oral administration (gavage) BASF DocID 2011/1112620 Date of report: 15-Jul-2011
Guidelines:	Reference is made to: OECD 414; OECD 417; OPPTS 870.7485; Commission Regulation (EC) No 440/2008; Japan/MAFF
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz

Report:	Thiaener J., Glaessgen W.E. (2011) Metabolism investigation of ^{14}C-BAS 480 F (Epoxiconazole) in plasma of pregnant guinea pigs after oral administration BASF DocID 2011/1108314 Date of report: 25-Jul-2011
Report:	Thiaener J. (2011) Report Amendment No. 1 to Final report: Metabolism investigation of ^{14}C-BAS 480 F (Epoxiconazole) in plasma of pregnant guinea pigs after oral administration BASF DocID 2011/1187839 Date of report: 02-Aug-2011
GLP:	Yes (laboratory certified by Landesamt fuer Umwelt, Wasserwirtschaft und Gewerbeaufsicht, 55116 Mainz

Note: The amendment to the report is a 3-page document (including cover page) it amends a sentence containing a data misinterpretations in three places of the original study report. Briefly, based on the observation of relatively high epoxiconazole concentrations in plasma sampled at early timepoints after dosing with at 90 mg/kg bw/d compared to levels obtained after dosing with 50 mg/kg bw/d, it was stated in the original report "…these data indicated a limited absorption or degradation of the parent compound BAS 480 F in the high dose group 4 during the first 8 h after application". In the amendment to the report, this sentence was corrected as follows: "… these data indicated a limited degradation of the parent compound…". The interpretation concerning the absorption of the parent compound was inserted into the report by mistake.

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1a. Radiolabelled test material:	^{14}C-Epoxiconazole (BAS 480 F)
Lot/Batch #:	291-3201
Radiochemical purity:	99.9%
Specific activity:	6.39 MBq/mg
Label:	triazole-3(5)-C14
1b. Unlabelled test material:	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.
2. Vehicle control:	1% aqueous Carboxymethylcellulose (1% CMC)
3. Test animals:	
Species:	Guinea pig
Strain:	Dunkin-Hartley, Crl:HA
Sex:	Female
Age:	Time-mated young adult, supplied Gestation Day (GD) 4
Weight at sampling (GD 10):	706 to 1072 g
Source:	Charles River Lab., Germany

Acclimatization period:	at least 2 days
Diet:	Kliba maintenance diet for rabbits & guinea pigs "GLP" meal, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum
Housing:	Individual housing, during acclimatization and during dosing of unlabeled test substance in Macrolon cages (20009; H Temp, PSU); during experiment with radiolabeled test substance in stainless steelwire mesh cages with grating
Enrichment:	PVC tunnel (during acclimatization and during administration of the unlabeled test substance
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	not mentioned in the report
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN

1. Dates of experimental work: 07-Apr-2011 to 09-May-2011 (in-life dates: 07-Apr-2011 (arrival of animals) to 08-May-2011, sacrifice of the animals)

2. Animal assignment and treatment:

Experimental design

Female Dunkin-Hartley guinea pigs were time-mated by the breeder and arrived at the test facility on Gestation Day (GD) 4. The experimental design is outlined in Table 2/102. Each test group consisted of 6 presumed pregnant guinea pigs. Females received unlabeled epoxiconazole in 1% CMC suspension by daily oral gavage administration from GD 6 to 9, at dose levels of 5, 15, 50 and 90 mg/kg bw in a dose volume of 10 mL/kg bw.

Rationale for dose selection:

Epoxiconazole dose levels were chosen to correspond to dose levels that had been investigated in previous prenatal developmental toxicity studies with guinea pigs. Dose levels of 5 and 50 mg/kg bw/d were also tested in a biokinetic / metabolism study in pregnant Wistar rats, the data can therefore be directly compared, and the top dose level of 90 mg/kg bw/d corresponds to half of the highest dose level (180 mg/kg bw/d) tested in aforementioned study in pregnant rats.

Blood sampling

On GD10, blood was sampled. Thereafter radiolabeled test substance preparations were administered to the females. Blood samples (ca. 100 µL) were subsequently

taken from the ear vein of each animal under isoflurane anesthesia at the following time points: 0.5, 1, 2, 4, 8, 24, 48, and 72 hours. A final blood sampling was performed at 96 hours upon exsanguination under isofluorane anesthesia.

Table 2/102 Dose groups

Test group	Vehicle	Unlabeled epoxiconazole GD 6-9	Single radioactive epoxiconazole dose GD 10			
		(mg/kg bw/d)	(mg/kg bw/d)	MBq/animal	Ratio unlabeled / labeled	MBq/mg
1	1% CMC[#]	5	5	5	1 : 1	1
2		15	15	15	1 : 1	1
3		50	50	25	2 : 1	0.5
4		90	90	45	2 : 1	0.5

[#] dose volume 10 mL/kg bw

3. Test substance preparation:

^{14}C-epoxiconazole and the non-radiolabeled test substance were prepared in 1% carboxymethylcellulose in highly deionized water (1% CMC). About 10 mL/kg body weight of a preparation were administered to guinea pigs by gavage. Specific activities of the test substance in the preparations of ^{14}C-epoxiconazole depended on the dose group. Details are given in the following sections.

Preparation of the unlabeled test substance

For the unlabeled test substance preparations in 1% CMC, the specific amount of test substance was weighed, topped up with either 1% CMC in a calibrated beaker and intensely mixed with a homogenizer. During administration, the preparations were kept homogeneous with a magnetic stirrer.

Preparation of the radiolabeled test substance preparations

For the groups 1 and 2 (5 and 15 mg/kg bw epoxiconazole) radiolabeled and unlabeled epoxiconazole was mixed and prepared in 1% CMC to achieve the intended specific activity of 1 MBq/mg. The final concentrations of epoxiconazole (sum of ^{14}C and ^{12}C-epoxiconazole) in the vehicle were 50 and 150 mg / 100 mL, respectively. The corresponding quantities of radioactivity administered to the animals was about 5 and 15 MBq, respectively.

For the groups 3 and 4 (50 and 90 mg/kg bw epoxiconazole) radiolabeled and unlabeled epoxiconazole was mixed and prepared in 1% CMC to achieve the intended specific activity of 0.5 MBq/mg. The final concentrations of epoxiconazole (sum of ^{14}C and ^{12}C-epoxiconazole) in the vehicle were 500 and 900 mg / 100 mL, respectively. The corresponding quantities of radioactivity administered to the animals was about 25 and 45 MBq, respectively.

The preparations were stirred and sonicated in order to produce homogeneous preparations.

Stability, homogeneity and concentration control analyses

The stability of the radioactive test substance in the vehicle was verified by analyses in the experiments.

Before start of and at the end of the administration, samples were taken to determine the amount of radioactivity in the preparation and to demonstrate the correct concentration of the test substance, the homogeneity of the test substance preparation as well as the radiochemical purity of the applied ^{14}C-epoxiconazole. These measurements were performed by LSC and HLPC analyses.

C. ANALYSES AND MEASUREMENTS

Whole blood samples were inverted several times to ensure homogeneity and then separated into plasma and blood cells by centrifugation.

1. Determination of total radioactivity

Plasma samples were obtained from non-coagulated blood by centrifugation. Aliquots of the plasma samples of each animal were used for the determination of the total radioactive residues in plasma. The total weight of the plasma sample was recorded, samples were mixed by shaking and aliquots were mixed with appropriate amounts of Hionic Fluor cocktail and analysed for total radioactivity by liquid scintillation counting.

Calculations are based on the specific activity of the radiolabeled test substance in the test substance preparation administered on GD 10 (radiolabeled dosing). The pre-treatment with unlabeled substance, leading in the organism to a potential change in specific activity is not considered within the current assessments.

2. Metabolic profiling

Pooled plasma samples were prepared by combining equal volumes from each animal of a certain dose group for the time periods of 0.5 h to 8 h and 24 h to 96 h, and the total radioactive residues in the pooled samples were measured within the present study. Proteins were precipitated from the pooled plasma samples with acidified acetonitrile, and the protein precipitates were incubated with Protease. The supernatants after protein precipitation and the solubilized residues after Protease treatment were analyzed by LSC and HPLC. Separation of peaks was performed via gradient elution on reversed-phase columns or on a HILIC column.

II. RESULTS

1. Homogeneity and concentration control analysis for the test substance preparations

The analytical investigations performed in the context of this study demonstrated the stability, homogeneity and correctness of the concentrations of unlabeled epoxiconazole administrations on GD 6-9 and radiolabeled ^{14}C-epoxiconazole) administrations on GD 10 in the vehicle for all dose groups.

2. Plasma levels of ^{14}C-epoxiconazole

Plasma concentrations from pregnant guinea pigs that were pre-exposed with 4 treatments of non-radiolabeled epoxiconazole during GD 6-9 followed by administration of ^{14}C-epoxiconazole on GD 10 are summarized in Table 2/103.

Table 2/103 Mean plasma concentration of radiolabel in pregnant guinea pigs, single oral dose of ^{14}C-epoxiconazole on GD10 after 4 daily oral doses of unlabeled epoxiconazole during GD6-9

Dose group	1	2	3	4
Dose (mg/kg bw/d)	5	15	50	90
No. of pregnant animals	6	3	5 [a]	5
Sampling time [h]	Mean plasmaconcentration [µg Eq/g plasma]			
0.5 h	0.52	1.16	2.48	3.01
1 h	0.59	1.69	4.11	4.87
2 h	0.52	1.52	4.61	6.12
4 h	0.51	1.48	4.60	8.40
8 h	0.45	1.01	5.04	8.54
24 h	0.36	0.78	3.78	6.06
48 h	0.19	0.43	3.05	3.40
72 h	0.16	0.29	1.69	2.58
96 h	0.12	0.20	1.19	1.47

[a] 1 guinea pig killed in moribund state 48 h after begin of blood sampling, data from this animal not considered

The total radioactive residues in pooled plasma samples of the dose groups with 5 and 15 mg/kg bw showed an increase up to 1 h after dosing (c_{max} values of 0.59 and 1.69 µg Eq/g, respectively) followed by a continuous decrease to values of 0.12 and 0.20 µg Eq/g at the last sampling time point of 96 h. The mean total radioactive residues in plasma of dose groups 3 and 4 (50 and 90 mg/kg bw) showed c_{max} values of 5.04 and 8.54 µg Eq/g at 8 h after dosing of the radiolabeled test substance with a continuous decrease thereafter to values of 1.19 and 1.47 µg Eq/g after 96 h.

The pharmacokinetic parameters derived from the plasma kinetics are presented in the following table:

Table 2/104 Pharmacokinetic parameters, single oral dose of ^{14}C-epoxiconazole on GD10 after 4 daily oral doses of unlabeled epoxiconazole from GD6-9

Grp	Dose [mg/kg bw]	Vehicle	Tmax [hour]	Cmax [µg Eq/q]	Initial half life [hour]	Terminal half life [hour]	$AUC_{0 \rightarrow \infty}$ [µg Eq x h / g]
1	5	1% CMC	1	0.59	---	45.16	31.91
2	15	1% CMC	1	1.69	---	36.75	64.47
3	50	1% CMC	8	5.04	---	41.97	352.63
4	90	1% CMC	8	8.54	---	35.60	484.45

AUC-values were derived which indicated an internal exposure correlated to the oral doses. Increasing the oral dose by a factor of 3 (from 5 to 15 mg/kg bw) resulted in an increase of the AUC values by factor of 2.0. Increasing the dose by a factor of 10 (from 5 to 50 mg/kg bw) resulted in an increase of the AUC values by a factor of 11.1. The increase of the dose by a factor of 18 (from 5 to 90 mg/kg bw) resulted in an increase of the AUC values by a factor of about 15.

3. Total Radioactive Residues (TRR)

The total radioactive residues in the pooled plasma samples were in accordance with the results calculated from the group mean values of the biokinetics study for the respective time periods. The residue levels in plasma showed a correlation to the applied oral doses. The residue values were determined at 0.502, 1.640, 4.305 and 7.651 µg/mL for the period of 0.5 h to 8 h after dosing and the dose groups of 5, 15, 50 and 90 mg/kg bw, respectively. The respective plasma levels for the time interval of 24 h to 96 h were 0.198, 0.534, 2.522 and 3.658 µg/mL.

4. Distribution of radioactive residues in plasma fractions

After workup of the pooled plasma samples from the early samplings (0.5 h to 8 h after dosing), the major part of the radioactive residues was found in the supernatants after protein precipitation (61.7% to 79.2% of the TRR). In the cases of the pooled samples of plasma collected 24 h to 96 h after dosing, higher portions of radioactive residues were precipitated from plasma with acidified acetonitrile, and the portions in the supernatants after protein precipitation were correspondingly lower (38.4% to 50.7% TRR). This altered distribution coincides with the disappearance of the parent compound epoxiconazole from both plasma fractions at later times (see below).

For further characterization of the residues associated with the protein fraction, the protein precipitates were incubated with Protease which solubilized most of the radioactive residues. No notable solid residues were found after Protease treatment and centrifugation. The portions of the unchanged parent compound detected by HPLC analysis of the residues solubilized by Protease treatment were very low. Precipitation of residues with acidified acetonitrile thus specifically concerned some biotransformation products of epoxiconazole (particularly metabolite 480M06).

5. Plasma levels of unchanged epoxiconazole and metabolites (Table 2/105)
The supernatants (after protein precipitation with acidified acetonitrile) from plasma sampled 0.5 h to 8 h after dosing showed metabolite patterns with two main components, the unchanged parent compound epoxiconazole and the metabolite 480M52 (1,2,4-triazole), accompanied by lower portions of the metabolite 480M13 or / and isomers or / and 480M60, the metabolite, the metabolite 480M05 or / and isomers or / and 480M54 and the metabolite 480M02 or / and its isomer 480M26. In the supernatants (after protein precipitation) from plasma sampled 24 h to 96 h after application, the parent compound was no longer present, and the portions of the metabolite 480M02 or / and its isomer 480M26 and particularly of the metabolite 480M52 had increased. The parent compound epoxiconazole was not detected in any fraction of the pooled samples of plasma collected 24 h to 96 h after dosing.

The metabolite 480M06 represented the main component in all Protease solubilizates from the protein precipitates, but was not detected in the supernatants (after protein precipitation). 480M06 residue levels increased with the dose level rising from 5 mg/kg bw to 50 mg/kg bw, but were relatively lower at a dose level of 90 mg/kg bw. The formation of the metabolite 480M06 thus seemed to be delayed in the case of the highest dose level. The parent compound epoxiconazole was only detected in two Protease solubilizates in low portions. The metabolite 480M52 (triazole) was found in both types of plasma fractions.

Table 2/105 Identification of radioactive residues from pooled plasma samples

		Epoxiconazole		480M52	480M13 / 480M60 [A]	480M05 / 480M54 [B]	480M02 / 480M26 [C]
mg/kg bw/d	**Pooled plasma time**	**µg/mL**	**%TRR**	**%TRR**	**%TRR**	**%TRR**	**%TRR**
… in supernatants after protein precipitation							
5	0.5 h to 8 h	0.077	15.4	14.1	4.2	8.2 + 7.5 = 15.7	4.6
15		0.184	11.2	15.7	4.7	8.1 + 10.0 = 18.1	3.3
50		0.501	11.6	13.6	7.0	(6.1 + 1.3) + 9.9 = 17.3	4.7
90		2.604	34.0	15.3	3.3	(5.1 + 1.0 + 0.6) + 7.3 = 14.0	4.1
5	24 h to 96 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
50		n.d.	n.d.	30.3	2.1	n.d.	9.3
90		n.d.	n.d.	34.6	n.d.	n.d.	16.1

		Epoxiconazole		480M52	480M06	Sum of HPLC peaks eluting	
						10-28 min	**31-57 min**
mg/kg bw/d	**Pooled plasma time**	**µg/mL**	**%TRR**	**%TRR**	**%TRR**	**%TRR**	**%TRR**
… in solubilizates after Protease treatment of protein precipitates							
5	0.5 h to 8 h	n.d.	n.d.	4.7	7.6	12.8	n.d.
15		0.015	0.9	2.4	5.9	7.8	3.4
50		n.d.	n.d.	n.d.	20.5	n.d.	7.8
90		0.130	1.7	2.1	3.8	9.6	0.6
5	24 h to 96 h	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15		n.d.	n.d.	10.5	15.5	22.0	n.d.
50		n.d.	n.d.	4.9	29.4	6.5	5.2
90		n.d.	n.d.	4.5	11.5	22.2	6.3

%TRR = radioactivity contained in an individual fraction compared to the Total Radioactive Residue in the particular sample / matrix worked up

[A] %TRR for 480M13 or/and isomers or/and 480M60

[B] %TRR for 480M05 / 480M54 represents a sum resulting from peaks that were identified in the report either as “480M05 or/and isomers” or “480M05 or/and isomers or/and 480M54”

[C] %TRR for 480M02 or/and its isomer 480M26

The total concentrations of the parent compound epoxiconazole, the metabolite 480M52 (triazole) and the sums of the conversion products of epoxiconazole calculated from the two analyzed fractions of all pooled plasma samples are compiled in Table 2/106.

Table 2/106 Summary of the total concentrations of radioactive residues, of unchanged parent compound, of metabolite 480M52 (1,2,4-triazole) and the sums of conversion products of ^{14}C-epoxiconazole detected in pooled plasma samples from pregnant guinea pigs treated with epoxiconazole

Vehicle: 1% CMC		^{14}C residues in plasma (TRR)		Epoxiconazole		480M52		Sum of conversion products of epoxiconazole	
mg/kg bw/d	**Pooled plasma time**	µg/mL	%TRR	µg/mL	%TRR	µg/mL	%TRR	µg/mL	%TRR
Four day pre-dosing from GD 6-9 with unlabeled epoxiconazole followed by administration of radiolabeled epoxiconazole on GD 10									
5	0.5 h to 8 h	0.502	100%	0.077	15.4%	0.094	18.8%	0.394	78.4%
15		1.640	100%	0.198	12.1%	0.296	18.1%	1.149	70.1%
50		4.305	100%	0.501	11.6%	0.587	13.6%	3.876	90.0%
90		7.651	100%	2.734	35.7%	1.333	17.4%	4.971	65.0%
5	24 h to 96 h	0.198	100%	0.000	0.0%	0.000	0.0%	0.176	89.1%
15		0.534	100%	0.000	0.0%	0.056	10.5%	0.462	86.5%
50		2.522	100%	0.000	0.0%	0.889	35.3%	2.441	96.8%
90		3.658	100%	0.000	0.0%	1.430	39.1%	3.547	97.0%

%TRR = radioactivity contained in an individual fraction compared to the Total Radioactive Residue in the particular sample / matrix worked up

The concentrations of epoxiconazole in the pooled samples of plasma collected 0.5 h to 8 h after application increased with the increasing doses applied, but the increase between dose group 3 and dose group 4 was markedly higher than the enhancement of the applied dose by a factor of 1.8. The relative portion of epoxiconazole was significantly higher in plasma of the high dose group 4. In plasma sampled 24 h to 96 h after application, no parent compound was detected any more, even at the highest dose level of 90 mg/kg bw. These data indicated a limited absorption or degradation of epoxiconazole in the high dose group 4 during the first 8 h after application. The concentrations of the metabolite 480M52 (triazole) and the sums of all conversion products of epoxiconazole showed an increase approximately corresponding to the rising dose levels.

6. Biotransformation products and metabolic pathway [see Figure 2/14]

The proposed metabolic pathway of epoxiconazole following administration by oral gavage to pregnant guinea pigs is shown in Figure 2/14.

In the guinea pig, metabolic transformation of epoxiconazole occurred mainly at two sites in the molecule: the chlorophenyl ring and the oxirane ring. Hydroxylation of the chlorophenyl ring led to the formation of the metabolite 480M02 (hydroxylated at the 5-position) or / and its isomer 480M26 (3-0H or 6-0H). According to the co-chromatography experiments, the main peak in the respective region of the chromatograms probably represented the metabolite

480M26. The oxirane ring was opened hydrolytically to form the respective geminal diol, metabolite 480M05 or / and its isomers. The hydroxylated diol metabolite 480M05 (or / and isomers) was conjugated with glucuronic acid to form the metabolite 480M60 or with cysteine to form the metabolite 480M13 or / and isomers (or conjugation with glutathione and subsequent loss of glutamic acid and glycine). Metabolite 480M54, an α-hydroxy methylthio compound, may be formed by degradation of the cysteine side chain. The (hydroxylated) chlorophenyl ring was detached by cleavage of the diol 480M05 or the active substance at the C_2-bridge between the aromatic nuclei to form the metabolite 480M06. Free 1,2,4-triazole (metabolite 480M52), which may have been cleft off at each step of the described pathway, was also detected in the plasma samples as one of the main components, particularly in pooled plasma sampled 24 h to 96 h after dosing. Some additional minor HPLC peaks were characterized on the basis of their chromatographic properties. No changes at the fluorinated aromatic ring of epoxiconazole were observed in the present study.

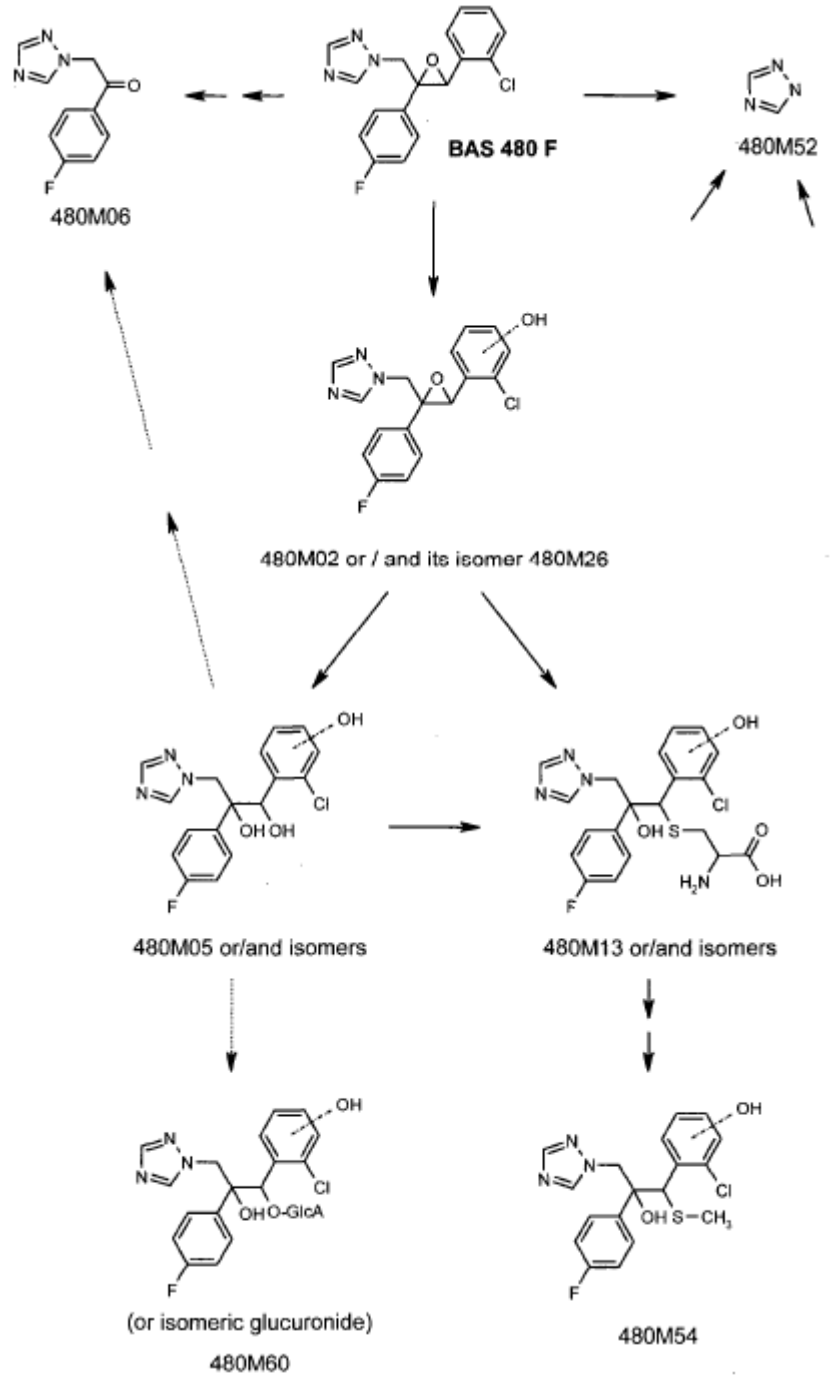
The metabolic pathway of epoxiconazole in pregnant guinea pigs was in good accordance with the pathway reported for pregnant Wistar rats with the only exception that a peak corresponding to metabolite 480M05 or / and isomers or / and 480M54 was additionally identified in plasma of pregnant guinea pigs.

III. SUMMARY AND CONCLUSIONS

The aim of the study was to generate information on the plasma kinetics and metabolism of radiolabeled ^{14}C-epoxiconazole in pregnant guinea pigs. It was of special interest to evaluate if the data on kinetics and metabolism of epoxiconazole in pregnant guinea pigs are comparable to data obtained in pregnant Wistar rats.

Guinea pigs were dosed with unlabeled epoxiconazole daily from GD6-9 (4 doses) and once with radiolabeled epoxiconazole on GD10 at dose levels of 5, 15, 50 and 90 mg/kg bw/d administered in 1% CMC . AUC values of approx. 32, 64, 352 or 484 µg Eq x h/g were obtained, indicating rough dose proportionality; Cmax and AUC values were very similar to rat data. The metabolic profile found in pregnant guinea pigs was comparable to rats.

Figure 2/14 Metabolic path of epoxiconazole in pregnant guinea pigs



STUDY RELEVANCE

Studies on the biokinetics and metabolism of a test substance provide relevant information for the evaluation of test results from other toxicological studies and for the extrapolation of data from animals to man.

The study provides the basis for a species comparison of the toxicokinetics and metabolic profile of epoxiconazole, together with the available parallel investigation in pregnant rats. By additional comparison of the observed toxicological profile of epoxiconazole in these two species, the toxicokinetic / metabolism data can inform whether the absence of late fetal resorptions or of cleft palate in guinea pig studies is explainable by differences in internal dose levels or by differences in metabolism.

In conclusion, the study belongs to a suite of in-vitro and in-vivo mechanistic investigations that were initiated to elucidate the mechanism underlying the observed increased incidence of both cleft palate and late fetal resorptions after high-dose level treatment of rats with epoxiconazole. Thus, the study is relevant for choosing the appropriate classification of epoxiconazole for developmental toxicity as part of an overall weight-of-evidence assessment.

2.3.2.3 Determination of epoxiconazole concentration in maternal plasma and embryonic tissue

STUDY REFERENCE

Report: Flick B., Schneider S., Richter M., Becker M., van Ravenzwaay B. (2012b)
BAS 480 F (Epoxiconazole) Analysis in plasma of pregnant Wistar rats and in tissue of GD 11 embryos oral administration (gavage)
BASF DocID 2012/1058202
Date of report: 29-Feb-2012

Guidelines: No test guidelines are available for this mechanistic study.

GLP: No

DETAILED STUDY SUMMARY AND RESULTS

I. MATERIALS AND METHODS
A. MATERIALS

1. Test Material:	Epoxiconazole (BAS 480 F)
Description:	Solid, white
Lot/Batch #:	COD-001118
Purity:	97.1%
Stability of test compound:	The test substance was stable over the study period under the storage conditions. The expiry date was 31-Dec-2011.
2. Vehicle control:	1% aqueous Carboxymethylcellulose (1% CMC)
3. Test animals:	
Species:	Rat
Strain:	Wistar [Crl:WI (Han)]
Sex:	Female
Age:	Time-mated rats aged 10-12 weeks
Weight (pregnant rats GD 0):	173.5 ± 9.14 g (range 157.3 - 189.4 g)
Source:	Charles River Lab., Germany
Acclimatization period:	at least 6 days before treatment
Diet:	Kliba maintenance diet for mouse/rats “GLP”, Provimi Kliba SA, Kaiseraugst, Switzerland, ad libitum
Water:	Tap water in bottles, ad libitum

Housing:	Individual housing in type M III Makrolon cages (Becker & Co, Castrop-Rauxel, Germany), floor area about 800 cm² with Lignocel fibre dustfree bedding (SSNIFF, Soest, Germany) and wooden gnawing blocks (Abedd Lab. and Vet. Service GmbH, Vienna) offered for enrichment
Environmental conditions:	
Temperature:	20 - 24 °C
Humidity:	30 - 70 %
Air changes:	15/hour
Photo period:	12 h light / 12 h dark (06:00 - 18:00 / 18:00 - 06:00)

B. STUDY DESIGN AND METHODS

1. Dates of experimental work: 19-May-2011 to 29-Feb-2012 (in-life dates: 19-May-2011 (arrival of animals), 25-May-2011 (start treatment on gestation day (GD) 6) to 30-May-2011 (last treatment and sacrifice, GD11))

2. Animal assignment and experimental procedure:

Female Wistar rats were paired by the breeder (“time-mated”) and supplied on gestational day (GD) 0 (= detection of vaginal plug/sperm). The animals arrived on the same day (GD 0) at the experimental laboratory. The following day was designated as “GD 1”. The animals were acclimatized to the laboratory conditions between start of the study (beginning of the experimental phase) and first administration (GD 6).

Epoxiconazole in 1% aqueous carboxymethylcellulose suspension (1% CMC) or the vehicle 1% CMC only was administered once daily to groups of 5 sperm-positive females animals by oral gavage from GD 6 to 11, at dose levels of 5, 50, 100 or 180 mg/kg bw/d. The volume administered each day was 10 mL/kg body weight. The calculation of the administration volume was based on the most recent individual body weight. At terminal sacrifice on GD 11, all of the sperm-positive dams under study were actually pregnant.

The time points for sampling maternal blood and embryonic tissue after dosing on GD 11 were selected based on the time (t_{max}) to reach peak plasma concentration of the test compound after oral administration (C_{max}), which were determined in a kinetic study of ^{14}C-radiolabelled epoxiconazole performed with pregnant Wistar rats (see chapter 2.3.2.2, Fabian and Landsiedel, 2011; BASF DocID 2011/1112619).

On the day of last treatment on GD 11, test group 4 was the group to be administered epoxiconazole first. Eight hours after administration, blood samples were obtained from these animals after decapitation under isoflurane anesthesia.

Group 0, 1, 2, and 3 were administered after group 4, in a time shifted manner. One hour after each administration, the dams were anesthetized with isoflurane and blood samples were obtained from the respective animals after decapitation. After sampling the blood, dams were necropsied. All embryos from each dam were removed from the uteri for analysis of epoxiconazole concentration.

Table 2/107 Test groups and doses

Test group	Dose epoxiconazole (mg/kg bw/d)	Concentration epoxiconazole (mg/100 mL)	Volume (mL/kg bw)	No. of animals (mated)	No. of animals (pregnant)	Timepoint of blood sampling after last treatment on GD 11
0	0 (1% CMC)	0	10	5	5	1 h
1	5	50	10	5	5	1 h
2	50	500	10	5	5	1 h
3	100	1000	10	5	5	1 h
4	180	1800	10	5	5	8 h

3. Test substance preparation and analysis:

The analytical verification of test substance stability in the vehicle for at least 7 days at room temperature was carried out before the study was initiated, using a similar batch of the test substance.

Epoxiconazole concentration control analyses were performed at the beginning of the study with samples of the epoxiconazole / 1% CMC preparations. The mean values of epoxiconazole in 1% Carboxymethylcellulose in highly deionized water were found to be in the range of 97.6 – 107.9% of the nominal concentration. These results demonstrated the correctness of the concentrations of epoxiconazole in 1% CMC.

Analysis of preparations for content of epoxiconazole							
Vehicle	Date of sampling	Sample No.	Nominal concentration [g/100 mL]	Analytical concentration [g/100 mL]			
				Sample I	Sample II	Mean	% of nominal concentration
1% CMC	24.05.2011 [concentration control analyses]	2	0	n.d.	n.d.	n.d.	---
		3	50	49.397	50.252	49.824	99.6
		4	500	497.393	491.912	494.652	98.9
		5	1000	983.607	968.231	975.919	97.6
		6	1800	2456.311	2460.724	2458.517	136.6
		2R*	0	n.d.	n.d.	n.d.	---
		6R*	1800	1942.479	1942.728	1942.603	107.9

The concentration of the top dose test preparation (group 4) and of the control group was re-analysed using retained samples (2R and 6R); the re-analysis results yielded values that corresponded to the expected nominal concentration, indicating that the first analysis of sample 6 was erroneous.

C. METHODS

1. Observations

The animals were examined for moribund condition or mortality twice daily on working days and once daily on weekends and public holidays. Cage side examinations for signs of morbidity, pertinent behavioral changes and overt

toxicity were performed at least once daily. If such signs occurred, the animals were examined several times daily (GD 0-11)

2. Body weight and food consumption

All animals were weighed on GD 0, 1, 3, 6, 8, and 11. The body weight change of the animals was calculated from these results.

Food consumption was determined for GD 0-1, 1-3, 3-6, 6-8, and 8-11.

3. Terminal examinations of the dams

On GD 11, the dams were sacrificed under isoflurane anesthesia by decapitation. Dams were subsequently assessed by gross pathology. The uteri were removed for the preparation of the embryonic tissues.

4. Analysis in maternal plasma and embryonic tissue

The analyses for determination of test compound concentration in maternal plasma and in the homogenized embryonic tissue samples were performed by BASF SE, APD/EC – Consumer Safety Residues, Limburgerhof.

Embryonic tissue

All embryos from each dam were harvested for analysis. The embryonic tissue (yolk sac, amnion and whole embryo) was pooled in groups of up to five embryos and transferred into Safe-Lock Tubes. The Safe-Lock Tubes with the embryonic tissue were stored on ice until homogenization using a glass/teflon tissue grinder. Subsequently, the samples were stored in a freezer (-20°C). Following extraction with acetonitrile/formic acid 1000/1 (v/v), the embryonic tissue concentrations were determined by UPLC analysis.

Maternal plasma

50 µL plasma samples from each dam were extracted with acetonitrile/formic acid 1000/1 (v/v) and analysed with UPLC.

Details of the UPLC analytical method are presented in the study report. The calculation of results was based on peak intensity measurements (peak area or peak height) using a calibration curve. The standard curve was obtained by plotting peak area or height versus the concentration of direct injection of epoxiconazole standards into UPLC-MS/MS respectively in the range of e.g. 0.050 ng/mL to 5.0 ng/mL. In a given injection run, the same volume was used for all samples and standards.

5. Statistics:

Where relevant, means and standard deviations of each test group were calculated. Statistical analyses were performed according to the following table:

Statistics for clinical examinations	
Parameter	**Statistical test**
Food consumption[a)], body weight, body weight change	Simultaneous comparison of all dose groups with the control group using the DUNNETT-test (two-sided) for the hypothesis of equal means
Female mortality	Pairwise comparison of each dose group with the control group using FISHER'S EXACT test (one-sided) for the hypothesis of equal proportions

a) For the parameter food consumption the "mean of means" was calculated and can be found in the relevant summary tables. The "mean of means" values allow a rough estimation of the total food consumption during different time intervals (pre-treatment, and treatment period); they are not exactly precise values, because the size of the intervals taken for calculation differs. For the "mean of means" values no statistical analysis was performed.

II. RESULTS

A. TEST SUBSTANCE ANALYSES

See Section B 3. above

B. OBSERVATIONS

1. Mortality

There were no test-substance-related mortalities in any of the groups.

2. Clinical signs of toxicity

There were no clinical findings in any of the test animals during the study period.

C. BODY WEIGHT AND FOOD CONSUMPTION

1. Food consumption [see Table 2/108]

The mean food consumption in dose group 4 (180 mg/kg bw/d) was statistically significantly reduced during the treatment period, i.e. on GD 6 through 11. The decrease was biggest on GD 6-8 (13.1 vs. 19.0 g = -31% below control). In group 3 (100 mg/kg bw/d) a similarly decreased mean food consumption value was recorded during GD6-8 (-27%), which however did not attain statistical significance.

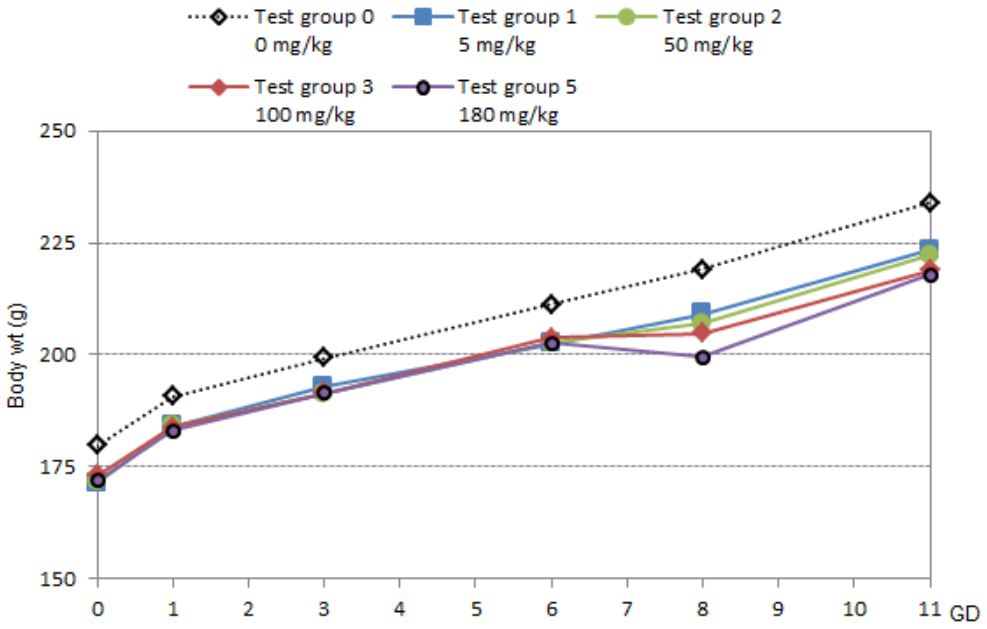
Table 2/108 Food consumption

Parameter: mean food intake (g/animal)	Epoxiconazole (mg/kg bw/d)				
	0	5	50	100	180
GD 3 – 6	18.7	17.1	17.9	18.2	18.5
Δ%		-9%	-4%	-3%	-1%
GD 6 – 8	19.0	17.9	16.6	13.9	13.1*
Δ%		-6%	-13%	-27%	-31%
GD 8 – 11	19.8	18.8	18.6	18.9	16.4**
Δ%		-5%	-6%	-5%	-17%

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

2. Body weight and body weight gain [see Figure 2/15 and Table 2/109]
There were no statistically significant differences in the mean body weight. At the top dose level, the mean body weight was reduced by 9% on GD8 and by 7% on GD11, which might be treatment related.

Figure 2/15 Body weight development



The mean body weight gain in dose group 3 and 4 was statistically significantly reduced at the beginning of the treatment period (GD 6–8, about 88 - 139% below the concurrent control value). At the top dose animals actually lost weight on GD 6 – 8. A non-significant decrease in weight gain was observed in group 2 at 50 mg/kg bw/d on GD6-8 (-44% below control). There were no statistically significant or biologically relevant differences regarding body weight change in Group 1 (5 mg/kg bw/d) when compared to the controls.

Table 2/109 Body weight change

Parameter: Body weight change (g/animal)	Epoxiconazole (mg/kg bw/d)				
	0	5	50	100	180
GD 3 – 6	12.0	9.6	11.5	12.5	11.1
Δ%		-20%	-4%	4%	-8%
GD 6 – 8	7.7	6.8	4.3	**0.9****	**-3.0****
Δ%		-12%	-44%	**-88%**	**-139%**
GD 8 – 11	15.0	14.2	15.3	14.1	18.1
Δ%		-5%	2%	-6%	21%
GD 0 – 11	54.3	52.0	50.7	45.8	45.9
Δ%		-4%	-7%	-16%	-15%

* $p < 0.05$; ** $p < 0.01$ (Dunnett-Test, two-sided)

D. NECROPSY

The gross pathological examinations did not reveal any abnormal findings in dams from treatment or control groups.

E. ANALYSES IN MATERNAL PLASMA AND EMBRYONIC TISSUE [see Table 2/92]

After 6 daily oral doses of epoxiconazole and sampling on GD11 at the presumed t_{max}, the mean residues in maternal plasma showed a non-linear dose dependent increase from 0.48 mg/L at the lowest tested dose level up to 9.2 mg/L at the highest tested dose level. The determined concentrations of the test substance in the corresponding embryonic tissues also showed a dose dependent increase from 0.23 mg/L up to 6.4 mg/L over the investigated dose range from 5 mg/kg bw/d to 180 mg/kg bw/d. The concentrations determined in the embryonic tissue were consistently lower than in the maternal serum. The concentration ratio between embryonic tissue and the maternal blood varied from 0.48 to 0.70 over the dose range investigated.

Table 2/110 Epoxiconazole concentrations in maternal plasma and embryonic tissue (embryo + amnion + yolk sac)

Test group	Epoxiconazole dose [mg/kg bw/d	Epoxiconazole concentration [mg/L]		Concentration ratio (Embryonic tissue / maternal plasma)
		Maternal plasma	Embryonic tissue	
1	5	0.48 ± 0.12	0.23 ± 0.08	0.48
2	50	4.5 ± 1.6	2.3 ± 0.7	0.51
3	100	6.6 ± 1.9	3.9 ± 1.1	0.59
4	180	9.2 ± 3.8	6.4 ± 3.8	0.70

III. SUMMARY AND CONCLUSIONS

Following the oral administration of 5, 50, 100 or 180 mg/kg bw/d. epoxiconazole to groups of pregnant Wistar rats from implantation to gestational day 11 (GD 6-11), the concentrations of epoxiconazole in maternal plasma sampled at approx. Tmax on GD 11 indicated an internal exposure that correlated with the oral dose levels. The concentrations of the test substance were also analysed in the embryonic tissue (representing yolk sac, amnion and the whole embryo). The following concentrations were determined in maternal plasma / embryonic tissue on GD 11 after repeated dosing: 5 mg/kg bw/d: 0.48 / 0.23 mg/L; 50 mg/kg bw/d: 4.5 / 2.3 mg/L; 100 mg/kg bw/d: 6.6 / 3.9 mg/L; 180 mg/kg bw/d: 9.2 / 6.4 mg/L. Thus under in-vivo conditions, a transplacental transfer of epoxiconazole was indicated; .the maternal plasma levels of epoxiconazole were about 1.5-2fold higher than the epoxiconazole content in embryonic tissue.

STUDY RELEVANCE

Together with the prenatal developmental toxicity investigation with rat embryos (see chapter 2.1.7, Flick et al. 2012; DocID 2012/1059618), this study provides important information for interpretation of the relevance of the Whole-Embryo-Culture study findings with epoxiconazole as reported by Menegola (see chapter 2.3.1.1; Menegola 2012; DocID 2012/1058203).

The study belongs to a suite of in-vitro and in-vivo mechanistic investigations that were initiated to elucidate the mechanism underlying the observed increased incidence of cleft palate after high-dose level treatment of rats with epoxiconazole. Thus, the study is relevant for choosing the appropriate classification of epoxiconazole for developmental toxicity as part of an overall weight-of-evidence assessment (see chapters 3 and 4).

2.3.3 ADDITIONAL RELEVANT INFORMATION

Literature review: Schneider S., Stinchcombe S., Hofmann T., 2012
Epoxiconazole (BAS 480 F): Relevance of guinea pigs as model for developmental and reproductive toxicity testing
BASF DocID 2011/1232616 (unpublished report)
Date of report: 28-February-2012

Report: Schneider S., Stinchcombe S., Rey Moreno M.C., Fegert I., Hofmann T., Strauss V. Groeters S., Fabian E., Richter M., van Ravenzwaay B. (2012):
"Species differences in developmental toxicity of epoxiconazole and relevance to humans"
(publication in preparation)

The guinea pig as model for azole reproductive and developmental toxicity testing

Guinea pigs were used as an alternative animal model for developmental and reproductive toxicity testing to examine the extent of species-specificity of epoxiconazole (BAS 480 F) such as effects on hormonal regulation of pregnancy, placentation, embryonic/fetal development and parturition, as they were identified in standard rat models.

The potential value of the guinea pig model for assessment of human reproduction toxicity hazards as an alternative to rodent models has long been recognized by the scientific and regulatory community. In the regulatory community this was specifically appreciated for azole compounds, as in 1999, the Scientific Committee on Plants (SCP) concluded in their interpretation of rat and guinea pig 2-generation reproduction toxicity studies conducted with the azole fenarimol that the aromatase inhibition by fenarimol and its specific effects as seen in small rodents is not relevant to the human species and that the guinea pig model seems to be the model of choice for defining the level of risk for the human for the effect of aromatase inhibition. (SCP/FENARI/005 - FINAL; Opinion adopted by the Scientific Committee on Plants on May 18, 1999).

The guinea pig is generally regarded as a most suitable model for elucidating the role of steroid hormones in pregnancy and parturition in humans (Sisk, 1976; cited in Batra et al., 1980). Also, in more recent publications the applicability of the guinea pig for regulatory reproductive toxicology testing is acknowledged. The authors conclude that the guinea pig can be used successfully for developmental and reproductive toxicology ("DART") studies in cases where traditional animal models are not relevant for human safety assessment (Rocca and Wehner 2009), or preferred the guinea pig as test species because of features "which are more similar to the human than is the case for other rodent species" (Hewitt et al., 2011).

Accordingly, there is a considerable number of developmental and reproductive toxicity studies with guinea pigs published in the literature, a non-exhaustive selection of published studies is given in the table below:

Table 2/111 Examples of published DART studies with guinea pigs

Chemical	Study type	Exposure and examination time points	Reference
Mifepristone	Male fertility study	Male exposure 4-wk premating until 3-5-day post mating with untreated females	Rocca and Wehner (2009)
	Embyro/fetal toxicity	Treatment from GD 4-30, exam GD 60	
	Female fertility study	Female exposure from GD30 of first pregnancy until GD30 of second pregnancy; exam on GD 30	
Phenylalanine	Embyrotoxicity	GD 1-17, examination of GD17 embryos	Kronick et al. (1987)
Ethanol	Pre/Postnatal toxicity	GD 43-62, exam of brain tissue weights from pups on PND10	Byrnes et al. (2001)
Ethanol	Embyro/fetal toxicity	GD2-68, 5 days/wk, 2 daily oral doses, exam on GD65	Hewitt et al. (2011)
Nonylphenol	Effects on the female reproduction tract	14-day s.c. treatment of adult non-pregnant female guinea pigs (intact or castrated)	Danzo et al. (2002)
p,p-'DDE			
Pentachlophenol			
Diethylstilbestrol			
Clophen A50 (PCB mixture)	Embyro/fetal toxicity	Dietary exposure from GD15-60	Brunström et al. (1982)
2,2',4,4',5,5'-hexachlorobiphenyl			
2,2',4,4',5,5'-hexachlorobiphenyl	Placental blood flow, embyro/fetal toxicity	Dietary exposure from GD45-63	Hedman et al. (1985)
Natalizumab	Embyro/fetal toxicity	a) Treatment from GD 4-30, every other day b) Treatment 4-wk premating through to GD 30, dosing on alternate days; necroscopy GD 59-62	Wehner et al. (2009)
Hyperthermia	Embryofetal toxicity	Hyperthermia induced on day between GD 11-14, two 2-h heating periods/day; sacrifice on GD 23-24	Cawdell-Smith et al. (1992)

A number of publications from the last 30 years reviewed the characteristics of guinea pig reproductive biology in comparison to a variety of species including humans. There is general consensus that the guinea pig is a promising alternative to murid rodents for reproductive and developmental toxicity testing, because the guinea pig displays several key physiological characteristics of gestation that more closely resemble human pregnancy than do currently established standard animal models. These physiological characteristics comprise hormonal regulation, placentation, trimester-equivalent gestation, extensive prenatal organ system development (including the brain growth spurt) and parturition. They are discussed and appropriate literature citations are given in the following paragraphs.

1) Overview

The anatomy and physiology of gestation as well as the birth process are very similar in rats and rabbits but very much different in humans. Recent publications have reinforced earlier observations that the guinea pig is a more appropriate model for many aspects of human pregnancy and parturition (Mitchell and Taggart, 2009; Hewitt et al., 2011) than murid rodents. Main species differences are summarized in the following table:

Table 2/112 Species-specific parameters relevant for parturition and pregnancy

	Rabbit	**Rat**	**Guinea pig**	**Human**
Gestation, days	32 ± 3	22 ± 1	67 ± 3	266 ± 14
Placenta	Hemodichorial, labyrinthine	Hemotrichorial, labyrinthine	Hemomonochorial, labyrinthine, discoid	Hemomonochorial, villous, discoid
Main source of estrogen	Ovaries	Ovaries	Non-pregnant: ovaries Pregnant: Placenta/fetus	Non-pregnant: ovaries Pregnant: Placenta/fetus
Source of progesterone	Corpus luteum	Corpus luteum	Corpus luteum, then placenta	Corpus luteum, then placenta
Progesterone withdrawal at parturition	Yes	Yes	No	No

Liggins and Thorburn (1994); Mitchell and Taggart (2009)

2) Placentation

Carter (2007) reviewed the strengths and weaknesses of animal models of human placentation and found that the guinea pig is a good alternative rodent model and among the few species known to develop pregnancy toxemia. The guinea pig is a well-established model for the study of placental transfer (Jansson and Persson, 1990) and fetal growth restriction (Carter, 1993). As in humans the placenta is hemomonochorial (Enders, 1965). Guinea pigs have a subplacenta, a structure that has no functional equivalent in the human placenta. However, it serves as a source of trophoblast invasion into the endometrium (decidua) and its arterial vessels (endo- and perivascular invasion) extending also to the myometrial and mesometrial spiral arteries, features which are similarly present in humans. It is reported that pregnancy toxemia occurs in guinea pigs and can be experimentally induced (Seidl et al., 1979; Golden et al., 1980). On the other hand, like murid rodents, guinea pigs retain a yolk sac placenta until term.

3) Hormonal regulation of pregnancy and parturition

In rats and mice, corpora lutea are the source of progesterone and are active throughout gestation. Moreover, the presence of corpora lutea is absolutely necessary for maintenance of gestation. Mating induces a prolactin surge in the pituitary, which is necessary for sustained corpora lutea activity. Subsequently, luteotrophic lactogens synthetized in the trophoblast bind to prolactin receptors of corpora lutea and maintain progesterone secretion throughout gestation (Malassiné

et al. 2003). In contrast, maintenance of gestation in guinea pigs is dependent on the activity of corpora lutea only during the first 4 weeks. Thereafter, progesterone is synthesized by the placenta (Csapo et al., 1981). This is analogous to humans, in which the corpus luteum is active only during the first trimester. Thereafter pregnancy is maintained by placental progesterone (P4) production, even after ovarectomy. Thus, endocrine mechanisms maintaining pregnancy are quite similar in humans and guinea pigs, but different to murid rodents.

Ovaries are the source of estrogen in non-pregnant and pregnant rats. In contrast, pregnant guinea pigs and pregnant humans produce estrogens mainly via the placenta (Batra et al., 1980; Hobkirk and Glasier, 1993). Estrogen synthesis in human placenta is dependent on synthesis of androgen precursors (Dehydro–epiandrostenedione) in maternal and fetal adrenals (feto-placental unit). Complete synthesis of estrogen in human placenta is not possible due to absence of 17α-hydroxylase (Fuchs and Fields, 1998; Strauss et al. 1996). In contrast, the rat placenta possesses 17α-hydroxylase, but lack aromatase. Thus, while the rat placenta is capable of producing androstenedione in the second half of pregnancy, it is not capable of producing estradiol (Matt and MacDonald, 1984; Sybulski, 1969; Townsend and Ryan 1970); placental androstenedione appears to serve as important precursor source for estradiol synthesis by the maternal ovaries (Jackson and Albrecht, 1986).

In humans and guinea pigs newborns have much more advanced stage of maturity as compared to rodents. There is a common occurrence of preterm birth, i.e. parturition in humans and guinea pigs is not as precisely regulated as in rodents but rather thought to be a multifactorial process (Mitchell and Taggart, 2009).

Prolonged gestation, delayed onset of parturition and dystocia are common findings in rat reproduction studies with azole fungicides. It is likely that the increased duration of pregnancy and birth difficulties observed in the 2-generation reproduction toxicity study in rats (BASF) and in the rat pre-/postnatal developmental toxicity study (Taxvig et al., 2007) result from interaction of epoxiconazole with the hormonal regulation of parturition. In pregnant untreated rats, progesterone levels drop sharply within a very narrow time window, which is one early key event for the onset of parturition.

Other than in rats, in pregnant women parturition occurs in the face of extremely high and unchanging or increasing levels of maternal plasma progesterone concentrations (Boroditsky, 1978; Tulchinsky, 1972). Several concepts exist that try to describe a “functional progesterone withdrawal” and the one for which most experimental evidence exists is the “paracrine hypothesis”. Accordingly, progesterone is synthesized within a local intrauterine network, including the fetal membranes (amnion and chorion) and maternal decidua, which controls progesterone concentrations in the underlying uterine muscle (myometrium). Significant changes in progesterone concentration may occur in this network without changes in the maternal progesterone blood level. Several investigators have shown that both estrogen and progesterone are produced by human fetal membranes and decidua. Prior to labor progesterone and estrone are produced, but following labor onset, the predominant products are inactive progesterone metabolites and the biologically active estradiol (Mitchell and Taggart, 2009). In

the normal nonpregnant female, estrogens are secreted in significant quantities only by the ovaries (although minute amounts are also secreted by the adrenal cortices). During early pregnancy, estrogen is at first produced in the corpus luteum but later in pregnancy the placenta will become the main source of estrogen. Placental estrogen formation is dependent upon 17α-hydroxylase activity of the fetus to provide its androgen precursors (Strauss, 1996).

During human pregnancy, tremendous quantities of estrogen are produced by placental aromatase and secreted; towards the end of pregnancy, the daily production of placental estrogens increases to about 30 times the mother's normal level of production (Guyton & Hall: Textbook of Medical Physiology; Liggins and Thorburn, 1994, Strauss, 1996). The fetus itself has an important role regarding hormonal activity. A significant steroid production and metabolism occurs in the fetal membranes (sulfohydrolase activity for estrone sulphate, production of increasing amounts of estradiol at the time of parturition) and 17ß-hydroxysteroid dehydrogenase (interconversion of estrone and estradiol), reduction of progesterone to 5α-, 3α-, and 3ß-reduced metabolites (Strauss, 1996). In humans estrogen is rather thought to be permissive than obligatory for the timing of parturition, stimulatory effects on the release of prostaglandins being the most prominent factor related to ripening of the cervix in preparation for birth (Liggins and Thorburn, 1994; Strauss, 1996).

Guinea pigs give birth in the presence of high circulating progesterone levels and without a requirement for prepartum luteolysis, like higher primates and humans and unlike rodents and rabbits (Challis et al., 1975; Thorburn and Challis, 1979). Moreover, circulating sex steroid hormone levels in guinea pigs and women follow analogous patterns during pregnancy, and responses to progesterone antagonist treatments are similar (Challis et al. 1971; Tulchinsky et al., 1972; Elger et al., 1986). As in humans, estrogen is produced within the pregnant uterus and the placenta (Batra et al., 1980; Hobkirk et al., 1993). The similarities extend to the role of prostaglandins. Prostaglandin administration induces labor and delivery in guinea pigs, like in women and other mammals (Elger and Hasan, 1985). Similar to humans, the guinea pig gestational tissues (amnion, visceral yolk sac, placenta and myo-endometrium) produce labor-promoting prostaglandins (PGE2 and PGF2α), and the prostaglandin output of the placenta and the amnion increases with advancing pregnancy (Moussard et al., 1986; Schellenberg and Kirkby, 1997). Intrauterine prostaglandins promote parturition by a mechanism that does not involve luteolysis and systemic progesterone withdrawal, which are essential actions of labor-promoting prostaglandins in rodents. The similarities in prostaglandin control of human and guinea pig gestational length, together with the similarities of progesterone action, indicate that guinea pigs can serve as a relevant non-primate model for studies where conclusions and inferences are extrapolated to the physiology and pathophysiology of human birth (Welsh et al., 2005).

4) Embryofetal development

Guinea pigs deliver precocial young after a long gestation. Thus many events that occur during human fetal development also occur during fetal life in guinea pigs. This is in contrast to rodents that have a short gestation and altricial young, where

many of these processes occur during postnatal development. In a recently published developmental toxicity study which examined the consequences of chronic maternal ethanol exposure on fetal development, the guinea pig was preferred as test species, "due to its trimester-equivalent gestation, extensive prenatal organ-system development including the brain growth spurt, and hemomonochorial placenta, which are more similar to the human than is the case for other rodent species." The authors considered that the guinea pig is a well established model for the study of ethanol teratogenesis (Hewitt et al., 2011). In an overview provided by Schardein et al. (1985) on species sensitivities and prediction of teratogenic potential the guinea pig was identified as being predictable for teratogenic effects of, among other compounds, various steroid hormones and Vitamin A analogues. Thus, presuming the published assumed cross species mechanism of action (Menegola et al., 2006), a potential susceptibility for potential teratogenic effects of azoles can be hypothesised for guinea pigs as well.

Why is the guinea pig not used as standard reproductive toxicity model for human risk assessment?

Experience with guinea pig reproductive toxicity studies is very limited. Historical control group data is scarce due to the limited number of studies conducted so far. Due to species-inherent properties (such as longer estrous cycles and less active sexual behavior of guinea pigs compared to rats, longer gestation periods), the duration of a 2-generation reproduction toxicity study in guinea pigs would take about 2-3 years (compared to about an in-life period of approx. 40 wk in a standard rat 2-generation reproductive toxicity study). Due to the longer pregnancy period of guinea pigs compared to rats, the maturity of born guinea pig pups is much more developed than in rats - this might limit the use of guinea pigs as model to study pup postnatal development in some cases. However, for those developmental stages that occur prenatally in humans and postnatally in rats, the guinea pig might be a more appropriate animal model for human hazard and risk assessment.

The actual lactation period is much shorter in guinea pigs than in rats, making the guinea pig a less sensitive predictor for toxicity resulting from lactational exposure. Due to physiological differences compared to rats, guinea pigs due not display developmental markers to easily determine the status of sexual maturity. The extent of manipulation of guinea pigs that would be required to obtain daily vaginal smears for the determination of menarchy onset would likely causes immense stress to the animals resulting in altered (not-substance related) adrenocortical hormone levels.

SUMMARY AND CONCLUSIONS

The guinea pig shares much more similarities with humans regarding pregnancy, gestational synthesis of estrogen and progesterone, intrauterine fetal development, and parturition when compared to rats. The species differences between murid rodents on the one hand, and guinea pigs and humans on the other, are most evident during the second half of gestation and initiation of parturition. This matches the time period in which adverse effects, namely late resorptions, prolonged gestation length, and dystocia occurred in rats but not in guinea pigs, although epoxiconazole was administered at maternally toxic dose levels to both species. In the light of the results obtained in recent studies with epoxiconazole and considering the remarkable species differences it is therefore very unlikely that the results in rats have predictive value for humans. Furthermore, the results of the new rat and guinea pig studies with epoxiconazole strengthen the evidence that the guinea pig is a model of first choice regarding effects on steroid hormone regulation during pregnancy with regard to humans.

With the guinea pig reproductive toxicity model it is possible to evaluate specific toxicological endpoints for which the rat is known not to be the appropriate/relevant model for extrapolation of the results to humans. Therefore, the guinea pig model is considered to be a very valuable and relevant complementary reproductive toxicity study model for special chemical classes (i.e. azoles) that are known to potentially influence steroid hormones levels involved in the regulation of pregnancy and parturition.

STUDY RELEVANCE

The literature review constitutes relevant supplemental information to justify the use of the guinea pig as superior animal model (compared to the rat) for human hazard and risk assessment of the reproduction toxicity of epoxiconazole. Therefore the review should be taken into consideration as part of an overall weight-of-evidence assessment for choosing the appropriate classification of epoxiconazole for developmental toxicity (see chapters 3 and 4).

3 OVERALL RELEVANCE OF THE PROVIDED INFORMATION

The RAC had justified their previous opinion from March 2010 on the harmonised classification of epoxiconazole on the basis of two main adverse effects in rat studies that were considered as critical for re-classification of epoxiconazole for developmental toxicity:

1) published rat data from 2007/2008 showing **post-implantation loss and resorptions** in the reported absence of maternal toxicity
2) increased incidence of **malformations as cleft palate in rats** based on study data already evaluated by ECB in 1997 / 2002-2003 / 2007-2008 and that formed the basis for the current harmonized classification for developmental toxicity as published in the CLP Regulation 1272/2008 (Repr. Cat. 3, R63 – DSD; Repr. 2, H361d).

During the RAC discussions, BASF had pointed out that studies with epoxiconazole are ongoing and planned to further investigate the endocrine disrupting potential (in compliance with the legal requirement to conduct such investigations as published in Commission Directive 2008/107/EC), and that results would be expected to be also of high relevance for the classification and labeling discussions. However, these data were not taken into account for the current RAC opinion for procedural reasons.

The new study data provides useful and relevant information for the choice of the appropriate reproduction toxicity classification of epoxiconazole. Robust study summaries of these investigations are included in this Additional Report. A conclusion on the relevance of the new information for developmental toxicity classification of epoxiconazole is provided below, including a clarification of the added value of the new information in comparison to already considered information in the RAC Opinion from March 2010.

Relevance of new information for developmental toxicity classification of epoxiconazole concerning the rat finding "post-implantation loss and resorptions"

The new data demonstrate that post implantation loss / late fetal resorptions resulting from treatment with 50 mg/kg bw/d epoxiconazole **does occur in the presence of distinct maternal toxicity** (clinical signs, statistically significant reduction in feed consumption during late gestation, statistically significant reduction of corrected body weight gain, clear evidence of anemia, changes of clinical chemistry parameters) [see Schneider et al. 2010a, 2010b]. In the studies published by Taxvig et al. (2007, 2008), "adjusted body weight" was reported only in the 2007 paper (without giving any details on how the data was adjusted); clinical signs and feed consumption data were not reported, and hematological or clinical-chemistry examinations were apparently not performed.

The new data shows that post-implantation loss / late fetal resorptions observed at 50 mg/kg bw/d occur in the presence of a marked decrease of the estradiol concentration in maternal plasma. Additional histopathological examinations revealed that epoxiconazole doses of 23 and 50 mg/kg bw/d administered from GD 7-18 and GD 7-21 caused a time- and dose-dependent degeneration of the placenta. Importantly, the severity of the placental damage is correlated with the occurrence of late fetal resorptions. Post-implantation loss / late fetal resorptions induced by 50 mg/kg bw/d epoxiconazole treatment from GD 7-21 could be prevented by co-treatment of pregnant rats with estradiol cyclopentylpropionate [Schneider et al. 2010a, 2010b, Schneider and Rey Moreno 2011, Schneider et al 2011b.]. Overall these new data provide clear evidence that marked depletion of maternal estradiol levels resulting from epoxiconazole-mediated aromatase inhibition is causally related to placental damage and to late fetal death in rats. In view of the known differences in the hormonal regulation of pregnancy between rats and humans, the **demonstrated mechanism of action for induction of late fetal resorptions in rats is considered to be of no or very limited relevance to humans.**

Further developmental toxicity data were generated using guinea pigs as appropriate animal model. As far as the hormonal regulation of pregnancy and parturition is concerned, guinea pigs and humans share much more similarities than rats and humans. Therefore, guinea pigs are a recommended animal model for investigating aspects of human pregnancy and parturition [see chapter 2.3.3 for an overview on species similarities and differences; Schneider et al. 2012a]. In prenatal developmental toxicity studies, pregnant guinea pigs received epoxiconazole daily by oral gavage administration during GD 6-63 at dose levels of up to 90 mg/kg bw/d; Caesarean sectioning was performed on GD 63. Under these study conditions, there was no evidence for any treatment-related increase of post-implantation loss / late resorptions, despite the fact that epoxiconazole was administered for a much longer time period at half the lethal dose and at almost twice the dose level that caused post-implantation loss in rat developmental toxicity studies. There was also no indication of any treatment-related placental damage in guinea pig studies [Schneider et al. 2011a, 2011b].

Supplemental toxicokinetic and metabolism investigations were performed with pregnant rats and pregnant guinea pigs, which included dose levels that caused developmental toxicity in rat studies [Fabian and Landsiedel 2011a, 2011b; Thiaener et al. 2011; Thiaener and Glaessgen 2011]. No significant species differences were found in these studies. Therefore, the absence of effects on the placenta or on post-implantation loss in prenatal developmental toxicity studies in guinea pigs is unlikely to have resulted from species differences in toxicokinetics or metabolism.

In a pre-postnatal developmental toxicity study, female guinea pigs were treated with up to 90 mg/kg bw/d epoxiconazole from GD 6 until the end of lactation (i.e., for a total treatment period of ca. 84 days) [see Schneider et al 2011c]. None of the azole-typical effects that had been observed in the rat two-generation study with 23 mg/kg bw/d epoxiconazole such as increased pregnancy duration, parturition difficulties, reduction of live litter size or reduced pup survival were seen in guinea pigs.

Overall, these findings with epoxiconazole are confirmatory scientific evidence in support of the SCP position [Scientific Committee on Plants 1999] that adverse reproduction toxicity effects in rats resulting from aromatase inhibition are species-specific and therefore unlikely to be relevant for humans.

Relevance of new information for developmental toxicity classification of epoxiconazole concerning the rat finding "malformations as cleft palate"

INVESTIGATIONS OF HYPOTHESES OF THE MODE-OF-ACTION FOR EPOXICONAZOLE-MEDIATED INDUCTION OF CLEFT PALATE

In the RAC Opinion from March 2010, several hypotheses for the mode of action of cleft palate formation by azoles were mentioned, specifically a) inhibition of embryonic CYP26 resulting in reduced degradation of endogenic retinoic acid which then causes dysmorphogenesis [Menegola et al. 2006] or b) blockade of the IKr potassium (HERG) channel, resulting in embryonic arrhythmia and hypoxia. However, RAC could not evaluate the relevance of these hypotheses because studies with epoxiconazole had been lacking.

Meanwhile, in-vitro studies with epoxiconazole are available that investigated these mechanisms [see Menegola 2012, Hebeisen 2011]. In order to put the in-vitro Whole-Embryo Culture investigations in perspective, the same endpoints were investigated under corresponding in-vivo conditions [see Flick et al. 2012a]. Finally, epoxiconazole concentrations in maternal plasma and in embryonic tissue were determined following in-vivo exposure of pregnant rats, so that administered dose levels could be directly related to internal epoxiconazole concentrations [see Flick et al. 2012b]. With these data it was possible to assess the extent of placental transfer of epoxiconazole during the early phase of organogenesis. Moreover, the toxicokinetic data also allowed relating epoxiconazole effect concentrations identified in in-vitro studies to results from corresponding in-vivo studies. The administered dose levels of 50 and 180 mg/kg bw/d corresponded to maternal plasma concentrations of ca. 4.5 and 9.2 mg/L or about 14 and 28 µM, respectively, when blood sampling was performed at around Tmax after repeated dosing.

The effect of epoxiconazole and of ketoconazole on HERG tail currents recorded from stably transfected HEK 293 cells (HERG-Assay) were investigated [Hebeisen 2011]. An IC_{50} value of 45.43 µM (Hill coefficient: 1.20) was obtained for epoxiconazole and an IC_{50} value of 2.26 µM (Hill coefficient: 1.24) was obtained for ketoconazole. Thus, in this investigation epoxiconazole displayed a 20fold lower potency compared to ketoconazole. According to published literature, there are no published reports of ketoconazole causing torsade de pointes (TdP) in humans when used alone. Also when comparing the IC_{50} value obtained for epoxiconazole with published data from pharmaceuticals, epoxiconazole would be considered as a weak inhibitor of HERG tail currents (Redfern et al. 2003). The relevance of this in-vitro finding with epoxiconazole remains unclear; it is difficult to follow the hypothesis that an unspecific effect such as hypoxia (speculated to be caused by repeated episodes of embryo-cardiac arrhythmia via HERG channel blockade) should elicit an increased incidence of one specific external malformation. In any case, the obtained The IC_{50} value ca. 45 µM is in support of a high-dose threshold effect (water solubility of epoxiconazole is 7 mg/L = 21 µM) that is probably of low practical relevance for humans.

Dysmorphogenesis of the branchial apparatus of embryos is hypothesized to be a pathogenic pathway that is involved in the induction of craniofacial malformations by triazoles (Menegola et al. 2006). To test this hypothesis, in-vitro (i.e., whole embryo culture) experiments with epoxiconazole were performed by Prof. Menegola [Menegola 2012]. Under these in-vitro study conditions, epoxiconazole caused dysmorphogenesis of the cultured embryos at and above cell culture concentrations of 10 µM. The No-Observed-Adverse-Effect-Concentration (NOAEC) for dysmorphogenesis was 3 µM. Abnormal neural crest cell distribution was noted at and above cell culture concentrations of 30 µM.

However, the in-vitro findings by Menegola could not be reproduced in rat embryos exposed to epoxiconazole under relevant in-vivo conditions: Female pregnant Wistar rats were exposed by repeated oral administration (gavage) from GD 6-11 to epoxiconazole at dose levels of 50, 100 and 180 mg/kg bw/d [Flick et al 2012a]. No substance-related adverse findings were observed in GD11 embryos at the dose level of 180 mg/kg bw/d when examined for signs of dysmorphogenesis. Furthermore, (normal) neural crest cell (NCCs) migration and distribution was visualized in 17 of the 37 investigated ex vivo GD 11 embryos, while immunostaining was unsuccessful for the remaining 20 fetuses. Therefore test substance related effects on NCC migration could not be assessed.

The reason for the discrepancy between in-vitro and in-vivo results is currently unclear. However, evidence obtained in a recently published in-vivo investigation by Mineshima et al [2012] is in line with the in-vivo findings of the epoxiconazole embryo study showing the absence of teratogenicity when exposure occurs between GD 6-11. Briefly, the time-dependent teratogenicity patterns of ketoconazole and Vitamin A palmitate were compared, including the occurrence of cleft palates. Pregnant rats were exposed to single doses of either vitamin A palmitate or ketoconazole on specific gestational days between GD 8 and 15, followed by Caesarean sectioning on GD 20 and examination of rat fetuses. Ketoconazole induced cleft palates only when treatment occurred between GD 12-14, while the most sensitive time window for Vitamin A was between GD 8-10; a second window with considerably lower sensitivity for cleft palate formation by Vitamin A palmitate was between GD 12-14. Thus, the available evidence indicates that the mechanism of cleft palate formation by ketoconazole is different from that by vitamin A.

New BASF rat developmental toxicity study data confirms that cleft palates induced at 180 mg/kg bw/d epoxiconazole occur in the presence of distinct maternal toxicity. The cleft palates were not prevented by estradiol co-treatment.

Groups of pregnant Wistar rats were administered epoxiconazole by daily oral gavage at dose levels of 180 mg/kg bw/d, from GD 6-15, in the vehicle 1% CMC. Additional treatment and vehicle control groups received daily subcutaneous injection of 1 or 2 µg/rat/day estradiol cyclopentylpropionate. Investigations required by OECD Test Guideline 414 were performed [Schneider et al 2010c]. Distinct maternal toxicity was observed in all epoxiconazole treatment groups (reduced feed consumption, body weight loss, drastically reduced corrected body weight gain between 37-71% of the control value, marked reductions in estradiol and progesterone). Post-implantation loss (late resorptions) was increased and fetal weights were decreased only in the epoxiconazole group without estradiol supplementation. Placental weights were markedly increased by epoxiconazole treatment, which indicated that severe placental damage had occurred. The placental weight increase was dose-dependently attenuated by estradiol co-administration, which corresponds to the observation that estradiol supplementation significantly decreased the extent of placental damage caused by 50 mg/kg bw/d epoxiconazole [Schneider et al. 2011]. The external malformations were significantly increased in all epoxiconazole groups (50-60% of the litters; highest values obtained in groups additionally receiving high estradiol level). Most of the malformations observed were craniofacial malformations (mainly cleft palate) and abnormal tuberositas deltoidea. The supplementation with estradiol did not have an effect on the incidence of malformations; measured maternal plasma estradiol and progesterone levels remained low.

New rat study data provides substantial evidence for a high-dose threshold for cleft palate formation, which is associated with marked maternal toxicity

Since cleft palate is a rare malformation in rats, it is difficult to assess the dose-response relationship and to reliably define a threshold dose for the occurrence of cleft palate on a study-by-study basis. However, the new BASF data [Schneider et al. 2010a, 2010b, 2010c] adds additional 1958 fetuses to the previous data base and therefore offers the opportunity to perform a more reliable analysis of the dose-response relationship for the occurrence of cleft palate, taking into account the complete toxicological data base of rat oral prenatal epoxiconazole studies. Together with the fetuses examined for external malformations from previously evaluated BASF studies, a total of 3971 rat fetuses from dams orally treated with epoxiconazole dose levels ranging from 5 to 180 mg/kg bw/d are now available for assessment (not including 280 rat fetuses that were examined in oral gavage developmental toxicity studies by Taxvig et al [2007, 2008]), but for which an examination for fetal malformations was not explicitly mentioned in the publications). A synopsis of oral prenatal developmental toxicity data for epoxiconazole that is available for analysis for the occurrence of cleft palate is presented in Table 3/1 below:

Table 3/1 Synopsis of data on cleft palate occurrence in oral prenatal developmental toxicity studies with epoxiconazole

Study ref.	Hellwig 1989	Hellwig 1990b	Schneider 2002	Taxvig 2007	Taxvig 2008	Schneider 2010a	Schneider 2010b	Schneider 2010c
Route	gavage	gavage	gavage	gavage	gavage	gavage	gavage	gavage
Treatment (GD)	6-15	6-15	6-19	7-21	7-21	7-18 + 7-21	7-21	6-15
DOSE [mg/kg bw/d]	Fetuses examined / fetuses with cleft palate							
5		370/0						
15		332/**1**		(81/0)				
20	300/**1**							
23						543/0		
45		297/0						
50				(128/0)	(72/0)	444/0	78/0	
50 + 0.5 ECP							131/0	
50 + 1.0 ECP							191/0	
60	241/0							
180	271/**136**		202/**3**					157/**30**
180 + 1 ECP								210/**24**
180 + 2 ECP								162/**23**

ECP = µg/animal estradiol cyclopentylpropionate

At dose levels below 180 mg/kg bw, a subset of 2969 fetuses were examined for external malformations in gavage developmental toxicity studies with epoxiconazole by BASF. Two fetuses with cleft palates were reported to have occurred in this subset. In both cases, these fetuses had multiple external malformations; therefore, there is an increased likelihood for a spontaneous occurrence rather than a specific effect. At the dose level of 180 mg/kg bw/d, a total of 1002 fetuses were assessed for external malformations, and 216 of them were found with cleft palate.

Taken together, the weight-of-evidence points to a clear high-dose threshold for the induction of cleft palate at 180 mg/kg bw/d and to a clear NOAEL of 45-60 mg/kg bw/d (based on the absence of cleft palates in 1343 fetuses examined for this dose range).

In the case of studies that involved treatment with epoxiconazole beyond GD 15 at dose levels of 50 mg/kg bw/d or higher dose levels, there is a certain likelihood that a higher incidence of cleft palates was “masked” (i.e. was not observable) due to the significant increases in late fetal resorptions induced by continued treatment during late pregnancy. This dilemma is not specific to epoxiconazole. Since the current OECD test guideline 414 (2001) requires treatment until shortly before scheduled birth, the incidence of substance-related malformations could theoretically be underestimated in the case of any chemical that significantly increases the post-implantation loss. However, in the case of the epoxiconazole data package, the new studies included epoxiconazole dose groups that were co-treated with estradiol cyclopentylpropionate, thereby reducing the occurrence of late resorptions to background levels (see Figure 3/1), and thereby “demasking” any potentially hidden fetal incidences of cleft palate..

Figure 3/1 Synopsis of post-implantation loss data on cleft palate occurrence in oral prenatal developmental toxicity studies with epoxiconazole

Dose levels (mg/kg bw/day)	**Early** resorptions	**Late** resorptions	**Treatment (GD)**
0	5.5	2.5	7 – 21
23	3.9	5.2	7 – 21
50	6.2	35.8**	7 – 21
50	21.5	30.1**	7 – 21
50 + 0.5*	6.3	6.5	7 – 21
50 + 1*	6.8	2.6	7 – 21
0	5.9	0.9	6 – 15
0 + 1*	8.3	0.5	6 – 15
0 + 2*	8.1	5.9	6 – 15
180	3.1	31.2**	6 – 15
180 + 1*	5.6	10.6	6 – 15
180 + 2*	16.7	0.0	6 – 15

* µg ECP/rat (estradiol cyclopentylpropionate)

50 mg/kg bw/d represents the LOAEL for the occurrence of late fetal resorptions in oral prenatal developmental toxicity tests with epoxiconazole treatment from GD 7-21 (NOAEL is 23 mg/kg bw/d, see Schneider et al. 2010a). No cleft palates were found in rats treated with epoxiconazole at 50 mg/kg bw and supplemented with estradiol (ECP), providing clear evidence that cleft palates were not induced at this dose level and did not remain undetected in Schneider at al. 2010a as a result of “masking” by post-implantation loss.

Cleft palates were not induced in guinea pig developmental toxicity studies when tested at up to 90 mg/kg bw/d (half the lethal dose in guinea pigs).

Since aromatase inhibitors are known to interfere with rat-specific hormonal regulation of pregnancy and parturition, the results obtained with epoxiconazole in rat prenatal and rat reproduction toxicity studies are considered to be of limited relevance for human risk assessment. Especially rat-specific massive placental damage in combination with marked reductions of progesterone and estradiol occurring during a sensitive time window of organogenesis may significantly contribute to the formation of craniofacial malformations. Such effects are not likely to occur in humans. Prenatal developmental toxicity studies with epoxiconazole were therefore performed with guinea pigs which have been considered to be the animal model of choice for human risk assessment of aromatase inhibitors [SCP 1999]. A total of 427 guinea pig fetuses were examined for external malformations in guinea pig prenatal developmental toxicity studies that involved epoxiconazole treatment from GD 6-63 at dose levels of up to 90 mg/kg bw/d. No cleft palates were found [Schneider et al. 2011a, 2011b]. There

was no evidence of relevant differences in toxicokinetics or metabolism of epoxiconazole that could explain the species-difference in the occurrence of cleft palates [Fabian and Landsiedel 2011a, 2011b, Thiaener et al 2011, Thiaener and Glaessgen 2011]. Thus the available new evidence is in line either with a rat-specific occurrence of cleft palate or with the existence of a species-independent high threshold dose for the occurrence of cleft palate, which is markedly toxic in rats and would be lethal in guinea pigs.

Overall, results obtained in mechanistic BASF studies so far are encouraging since they indicate a mode-of-action for the occurrence of developmental toxicity via rat-specific disruption of the hormonal regulation during pregnancy, which supports the general conclusion of limited human relevance for all results obtained in rat developmental toxicity studies. At this time, results from epoxiconazole and from relevant published investigations with similar substances point to a multi-factorial (and hence possibly substance-specific) process for the induction of craniofacial malformations in rats. In the case of epoxiconazole, the rat-specific massive placental damage occurring during a critical period of organogenesis is a likely key event involved in the mechanism of action for epoxiconazole-mediated cleft palate formation.

BASF plans to continue studying the endocrine disrupting potential of epoxiconazole (in compliance to requirements of Regulation 2008/107/EC) and try to further elucidate the mechanism underlying cleft palate formation in rats, taking into account possible alternative modes-of-action (e.g. Amaral et al. 2009, Mineshima et al. 2012).

4 COMPARISON WITH THE CLP AND DSD CRITERIA

According to the "Opinion of the Committee for Risk Assessment on a dossier proposing harmonized Classification and Labeling at Community Level" on the substance epoxiconazole; EC number: 406-850-2; CAS-No. 133855-98-8 (ECHA, 17 March 2010, CLH-O-0000000630-85-05/F), and based on consideration of "all the available data", two main adverse effects of epoxiconazole were identified and considered as critical for the classification decision:

1) **Post-implantation loss and resorptions**
2) **Malformations as cleft palate**

4.1 COMPARISON WITH THE CLP CRITERIA

1) Post-implantation loss and resorptions

The new data provides mechanistic information for the induction of post-implantation loss and late fetal resorptions in rat developmental toxicity studies with epoxiconazole. With the new epoxiconazole data **a specific maternally-mediated mechanism was demonstrated** (depletion of maternal estradiol via aromatase inhibition resulting in dose-dependent placental damage and late fetal resorptions; estradiol co-treatment prevented late fetal resorptions and significant reduced placental damage). According to the CLP classification criteria ***"…Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1"***.

Further developmental toxicity studies were performed with epoxiconazole in guinea pigs, a species which hormonal regulation of pregnancy is very similar to that of humans. Supplemental studies with pregnant animals demonstrated comparable toxicokinetics and metabolic profiles of epoxiconazole in rats and guinea pigs. However, in the guinea pig developmental toxicity studies, no placental damage and no post-implantation loss were induced despite administration of higher epoxiconazole dose levels over a considerably longer treatment duration that compared to the rat studies with epoxiconazole. Therefore, and **in view of the long-known fundamental differences between humans and murid rodents in the hormonal regulation of pregnancy**, and in combination with the provided clear experimental evidence based on epoxiconazole studies, **the perturbation of the rat hormonal regulation via aromatase inhibition by epoxiconazole that led to developmental toxicity is considered to be a rat-specific effect that is not predictive for developmental toxicity of epoxiconazole in humans.** According to the CLP criteria for Category 1B *("Presumed human reproductive toxicant"), "…the classification of a substance in this Category 1B is largely based on animal data. Such data shall provide clear evidence of an adverse effect [...] on development in the absence of other toxic effects, or if occurring with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However, **when there is mechanistic information that raises doubt about***

the relevance of the effect for humans, classification in Category 2 may be more appropriate".

A substance in Category 2 would be considered a "*suspected human reproductive toxicant*". Criteria for a classification in Category 2 as given in the CLP Regulation are: "*Substances are classified in Category 2 for reproductive toxicity when there is some evidence […] of an adverse effect on […] development, and where the evidence is not sufficiently convincing to place the substance in Category 1. […] Such effects shall have been observed in the absence of other effects, or if occurring together with other toxic effects the toxic effect is considered not to be a secondary consequence of the other toxic effects*".
However, the overall data available for epoxiconazole leaves little doubt that the observed post-implantation loss and late fetal resorptions in rat studies are not relevant for humans. Therefore, based on this effect a classification of epoxiconazole as "Suspected human reproductive toxicant" is considered inappropriate.

Guidance to decide on "no classification" according to the CLP Regulation (1.1.1.5) is as follows: *"For the purpose of classification for health hazards (Part 3) route of exposure, mechanistic information and metabolism studies are pertinent to determining the relevance of an effect in humans. […]* ***When there is scientific evidence that the mechanism or mode of action is not relevant for humans, the substance or mixture should not be classified.****"*
Slightly more detailed guidance is given in chapter 3.7.3.2: "*Toxicokinetic studies in animals and humans, site of action and mechanism or mode of action study results may provide relevant information which reduces or increases concerns about the hazard to human health. If it is conclusively demonstrated that the clearly identified mechanism or mode of action has no relevance for humans or when the toxicokinetic differences are so marked that it is certain that the hazardous property will not be expressed in humans then a substance which produces an adverse effect on reproduction in experimental animals should not be classified."*

Hence, following the decision logic for reproductive toxicity classification according to the criteria of the CLP Regulation, epoxiconazole does not require classification as reproductive toxicant on the basis of rodent-specific induction of post-implantation loss / resorptions, i.e. via a mechanism that is not relevant for humans.

2) Malformations as cleft palate

Based on the evaluated epoxiconazole data, the RAC concluded in the RAC Opinion issued in March 2010, comparing finding with CLP classification criteria:

"… the induction of a high incidence of cleft palates in the presence of maternal toxicity (Hellwig 1989) and the repeated observation of isolated cleft palates in rats at doses without maternal toxicity enable a ***clear identification of cleft palate as a developmental effect*** *of epoxiconazole. It is considered that induction of cleft palates cannot be attributed to maternal toxicity such as decreased food consumption or reduced body weight and* ***it cannot be considered secondary to other maternal toxic effects.**** *"RAC […] considers that the level of evidence for induction of cleft palates is in agreement with the criteria for CLP classification criteria Rep.Cat. 1B that "available data provide* ***clear*** *evidence of an adverse effect […] on development in the absence of other toxic effects or if occurring together with other toxic effects the adverse effect on reproduction* ***is considered not to be a secondary non-specific consequence of other toxic effects****". Besides, in the absence of relevant mechanistic information* ***it cannot be concluded "that there is a doubt about the relevance of the effect for humans implying that classification in category 2 may be more appropriate".***

* Remark from the data submitter: It is agreed that reductions in feed intake or of body weight (as was observed with epoxiconazole) do not offer a mechanistic explanation for the occurrence of cleft palate. Such changes can only be considered as more or less **clear indicators of maternal toxicity**. For most developmental effects, the underlying mechanism is unknown. In addition, in early-phase studies, it is usually difficult to predict before the start of the study which kind of developmental toxicity will occur and therefore, it is not practical to consider all kinds of imaginable maternal toxicity endpoints that could be relevant to explain the mode-of-action for a speculated developmental effect. In the case of epoxiconazole, the critical effect of cleft palate was observed in an early dose-range finding study with rats from 1989 and occurred at a toxic dose level, which was in excess of the maximum tolerated dose. In this study, the investigation of maternal toxicity did not go beyond the minimal data requirements of the OECD 414 requirements that were valid at the time. In this study, epoxiconazole caused clinical signs, marked changes in body weight gain and also reductions in food consumption. More elaborate investigations of the maternal toxicity induced by epoxiconazole were performed in later studies which showed that epoxiconazole additionally caused anemia and marked changes in hormone levels at the cleft-palate inducing dose of 180 mg/kg bw/d.

Based on the new level of information from all available studies with epoxiconazole, it can be concluded that **the increased incidence of cleft palate in rats represents clear evidence of an adverse effect on development which occur in the presence of other toxic effects.** As discussed in detail in Chapter 3, with the new data from rat developmental toxicity studies, a reliable dose-response analysis and estimation of a threshold effect dose level is possible, even for a rare malformation such as cleft palate, on the basis of data from almost 4000 fetuses examined for external malformations. With the new data, the overall weight-of-evidence is in support of the previous evaluation by the TC C&L Experts of the ECB that treatment related increases of the cleft palate incidence in

rat fetuses by epoxiconazole were induced only at the high dose level of 180 mg/kg bw/d and in the presence of marked maternal toxicity. Thus, the weight-of-evidence clearly points to a high-dose threshold effect.

Moreover, the available new mechanistic data provides evidence in support of a rat-specific effect. Hence, doubt can be raised for the human relevance of the finding of cleft palates in rats based on the following available evidence:

- Evidence for a rat-specific perturbed hormonal regulation of pregnancy during a critical time window of organogenesis (marked reductions of estradiol and of progesterone measured on GD 15)**;**
- the observation that the postulated mode-of-action for azole-mediated induction of craniofacial malformations via CYP26 inhibition could not be demonstrated for epoxiconazole under relevant in-vivo conditions in rats at dose levels known to induce cleft palates, reducing concerns for a species-independent mechanism as postulated for azoles by Menegola et al. (2006); indirect evidence that cleft palate induction in rats must be caused during a late stage of organogenesis (no evidence of embryo effects upon exposure from GD 6-11);
- a high likelihood that (rat-specific) fulminant placental damage at 180 mg/kg bw/d contributed to the induction of cleft palates during the critical time window in rats;
- the absence of cleft palates and of placental damage in guinea pig studies despite high-dose treatment.

In this regard, the following considerations for selecting the appropriate developmental toxicity classification of epoxiconazole in the CLP Regulation 1272/2008 are considered relevant for epoxiconazole:

According to the CLP classification criteria ***"…Classification shall not automatically be discounted for substances that produce developmental toxicity only in association with maternal toxicity, even if a specific maternally-mediated mechanism has been demonstrated. In such a case, classification in Category 2 may be considered more appropriate than Category 1".***

According to the CLP criteria for Category 1B *("Presumed human reproductive toxicant"), "…the classification of a substance in this Category 1B is largely based on animal data. Such data shall provide* ***clear*** *evidence of an adverse effect [...] on development in the absence of other toxic effects, or if occurring with other toxic effects the adverse effect on reproduction is considered not to be a secondary non-specific consequence of other toxic effects. However,* ***when there is mechanistic information that raises doubt about the relevance of the effect for humans, classification in Category 2 may be more appropriate***".

Since aromatase inhibitors are known to interfere with rat-specific hormonal regulation of pregnancy and parturition, the results obtained with epoxiconazole in rat prenatal and rat reproduction toxicity studies are considered to be of limited relevance for human risk assessment. Especially rat-specific massive placental damage in combination with marked reductions of progesterone and estradiol

occurring during a sensitive time window of organogenesis is highly likely to have significantly contributed to the formation of craniofacial malformations. Such effects are not likely to occur in humans. Therefore, the results obtained in guinea pig developmental toxicity studies with epoxiconazole are considered to be more reliable for human hazard assessment of the developmental toxicity. In guinea pig studies, there was no evidence of cleft palate formation up to the dose level of 90 mg/kg bw/d. In view of the still remaining uncertainties regarding the mode-of-action for cleft palate induction in rats, it is strongly recommended to retain the current legal classification of epoxiconazole for developmental toxicity in Category 2 (CLP) for the time being.

Hence, epoxiconazole classification in Category 2 can be justified against the CLP classification criteria, based on reasonable doubt about the relevance of the observed rat finding cleft palates for humans.

4.2 COMPARISON WITH THE DSD CRITERIA

1) Post-implantation loss and resorptions

Criteria of the DSD (Commission Directive 2001/59/EC =: 28th ATP to Council Directive 67/548/EEC)

Three categories are defined for substances that are toxic to reproduction. The placing of a compound in category 1 "Substances known to cause developmental toxicity in humans" is done on the basis of epidemiological data. Placing in category 2 "*Substances which should be regarded as if they cause developmental toxicity to humans*" or category 3 "*Substances which cause concern for humans owing to possible developmental toxic effects"* is done primarily on the basis of animal data.

Based on the DSD criteria epoxiconazole does not require a reproductive toxicity classification in category 1 since there is no evidence of corresponding adverse effects in humans from epidemiological data.

A weight-of-evidence evaluation is needed whether epoxiconazole requires a reproductive toxicity classification in category 2 or category 3 or whether epoxiconazole should not be classified for developmental toxicity on the basis of following considerations given in the DSD:

- "***Chemicals should not be classified as toxic to reproduction where such effects are solely produced as a non-specific secondary consequence of other toxic effects."***
- A classification in Category 3 would be warranted generally on the basis of "*results in appropriate animal studies which provide sufficient evidence to cause a strong suspicion of developmental toxicity in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects* ***but which are not a secondary non-specific consequence of the other toxic effects****, but where the evidence is insufficient to place the substance in category 2"*

- *"In common with most other types of toxic effect, substances demonstrating reproductive toxicity will be expected to have a threshold below which adverse effects would not be demonstrated.* ***Even when clear effects have been demonstrated in animal studies the relevance for humans may be doubtful*** *because of the doses administered, for example, where effects have been demonstrated only at high doses, or where marked toxicokinetic differences exist, or the route of administration is inappropriate.* ***For these or similar reasons it may be that classification in category 3, or even no classification, will be warranted****."*

With the new epoxiconazole data **a "secondary […] consequence of other toxic effects",** i.e. a maternally-mediated mechanism for the induction of post-implantation loss / late fetal resorptions was demonstrated (i.e., depletion of maternal estradiol via aromatase inhibition resulting in dose-dependent placental damage and late fetal resorptions; estradiol co-treatment prevented late fetal resorptions and significantly reduced the placental damage). This mechanism of maternal toxicity, however, was not at all "unspecific" but in fact "species-specific". The fundamental differences between humans and murid rodents in the hormonal regulation of pregnancy are well known. In combination with the provided clear experimental evidence from epoxiconazole studies in pregnant rats and guinea pigs, plus in-vitro evidence for a considerably higher sensitivity of rats for inhibition of ovarian aromatase compared to humans, the overall data available for epoxiconazole leaves little doubt that the observed post-implantation loss and late fetal resorptions in rat studies are not relevant for humans.

Therefore, the perturbation of the rodent-specific hormonal regulation via aromatase inhibition observed in rat developmental toxicity studies with epoxiconazole that led to placental damage and post-implantation loss / late fetal resorptions is not considered to be predictive for developmental toxicity of epoxiconazole in humans. In guinea pigs, no evidence of treatment-related developmental toxicity was observable. Hence no classification of epoxiconazole is required for developmental toxicity according to the criteria of the Dangerous Substance Directive DSD.

2) Malformations as cleft palates

The following DSD criteria are considered applicable to support the current legal classification of epoxiconazole in Repr.Cat. 3; R63

- *"In common with most other types of toxic effect, substances demonstrating reproductive toxicity will be expected to have a threshold below which adverse effects would not be demonstrated.* ***Even when clear effects have been demonstrated in animal studies the relevance for humans may be doubtful*** *because of the doses administered, for example, where* ***effects have been demonstrated only at high doses****, or where marked toxicokinetic differences exist, or the route of administration is inappropriate.* ***For these or similar reasons it may be that classification in category 3, or even no classification, will be warranted****."*

Moreover the DSD contains the following statement:

- *"The object of classification is to identify all the physico-chemical, toxicological and ecotoxicological properties of substances and preparations which may constitute a risk during normal handling or use. Having identified any hazardous properties, the substance or preparation must then be labelled to indicate the hazard(s) in order to protect the user, the general public and the environment."*

  The current harmonized legal classification of epoxiconazole for developmental toxicity in Repr.Cat.3; R63 as published in the 29th ATP of the DSD (and then adopted for the CLP Regulation 1272/2008) resulted from the data evaluation by the TC C&L of the ECB in 2002/2003. The discussions of the TC C&L that ultimately resulted in the current DSD classification of epoxiconazole were very much focused on the relevance of cleft palates.

  The additional epoxiconazole data generated since the last detailed evaluation by the TC C&L comprise the published investigations by Taxvig et al. (2007, 2008), where no malformations were reported to have occurred, plus the investigations by BASF that are summarized in this Additional Report. These BASF studies provide further confirmatory evidence that cleft palates are induced by epoxiconazole in rats only above a high threshold dose level in the presence of marked maternal toxicity. Moreover, mechanistic investigations in rats suggest that rat-specific perturbed hormonal regulation resulted in marked reductions of estradiol and progesterone and in massive placental damage, which is likely to have significantly contributed to the observed formation of cleft palates in rats. Since aromatase inhibitors are known to interfere with rat-specific hormonal regulation of pregnancy and parturition, the results obtained with epoxiconazole in rat prenatal and rat reproduction toxicity studies are considered to be of limited relevance for human risk assessment. In prenatal developmental toxicity studies in guinea pigs, which are considered to be the animal model of choice for predicting the human developmental toxicity of aromatase inhibitors, no evidence of cleft palate formation was found up to dose levels of 90 mg/kg bw/d, administered from GD 6-63.

Hence, the current classification of epoxiconazole classification in Repr.Cat. 3; R63 can be justified against the DSD classification criteria, based on reasonable doubt about the relevance of the observed rat finding "cleft palates" for humans, and because cleft palates occurred only at high dose levels which are not relevant for humans under normal conditions of handling and use.

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