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TRITICONAZOLE

Volume 3 – B.6 (AS)

Rapporteur Member State: Austria
Co-Rapporteur Member State: United Kingdom

Version History

When	What
September 2003	Initial DAR, first version
September 2004	Addendum 1
January 2005	Addendum rev. 2
July 2018	DRAR

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In the last evaluation table (Doc. 16800/EPCO/BVL/04 rev. 1-1 (27.05.2005)) of first approval of active substance triticonazole, for which BASF was the only notifier with the complete dossier, no open points for mammalian toxicity remained.
In the EFSA Scientific Report (2005) 33, 1-69, Conclusion on the peer review of triticonazole, following remaining toxicological issues are however mentioned:

Particular conditions proposed to be taken into account to manage the risk(s) identified:

- Appropriate PPE (i.e. coverall and gloves) is considered in the estimations of operator exposure in order to be below the AOEL

Critical areas of concern:

- Operator exposure assessments were performed based on Seed TROPEX model covering ”large scale users” in seed treatment facilities. With respect to seed treatment systems operating on farm level (small scale users) risk assessment has to be evaluated on Member State level taking into account the different methods and conditions, which are usually applied in the different regions.
- For operators adequate PPP (i.e. coverall and gloves) has to be considered.
- Given the packaging description originally submitted (1 L, 5L, 200 L and 1000 L containers) the small sizes (1 L and 5 L) cannot be supported since only professional use is intended.

For the renewal of approval of triticonazole, the notifier BASF submitted complete dossiers by the deadline of end of October, 2015. The dossier for the renewal contain additional new studies (based on e.g. new data requirements or literature search), but the major part of the dossier is the same as it was for the first approval of triticonazole (*Commission Directive 2006/39/EC of 12 April 2006 amending Council Directive 91/414/EEC to include triticonazole as an active substance*).

Regarding the old studies, originally evaluated in the DAR (RMS Austria), mostly re-wording was conducted and additional information was included in DRAR where considered necessary for better overview. Finally, the validity of studies in view of updated OECD guidelines was proven.

Regarding the originally derived reference values the RMS followed the original outcome (Review report for the active substance triticonazole, SANCO/10442/2005 final rev 1, 12 March 2010) regarding the AOEL and ADI (both 0.025 mg/kg bw per day) as well as ARfD (0.05 mg/kg bw). Additionally, RMS derived also an AAOEL, same as ARfD (0.05 mg/kg bw) in case of other application regimes than seed treatment.

The RMS also paid special attention to new criteria for classification and labelling according to Regulation (EC) 1272/2008. The outcome of the Meeting of the Commission Working Group on the Classification and Labelling of Dangerous Substances Pesticides, ECB Ispra, 22 August 2007 (ECBI/90/06 Rev. 8), that no classification and labelling for triticonazole is necessary for human health, could be partially supported. During the renewal of triticonazole RMS concluded that STOT RE 2 should additionally be considered for triticonazole.

In all of the toxicity studies conducted with triticonazole for the first approval the content of batches is known. The confirmation of the representativness of toxicological batches for the specification and toxicological conclusion on impurities is included in Volume 4 while the toxicological studies with impurities are included in B.6.8.1.
Methanol is considered to be relevant impurity. No toxicological concern is raised by starting material, theoretical impurities or other specified impurities.

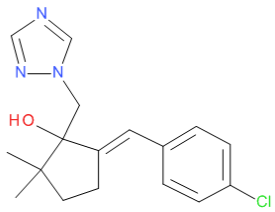
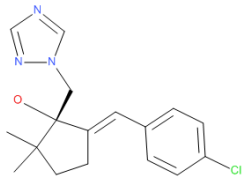
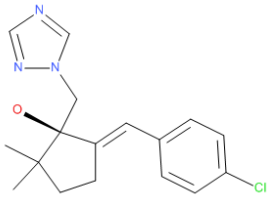
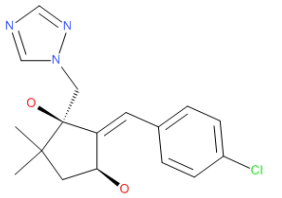
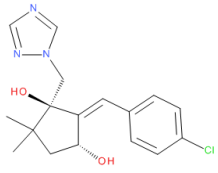
Analytical methods used in the toxicological studies

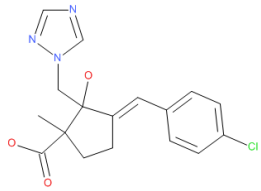
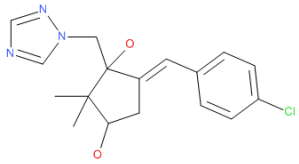
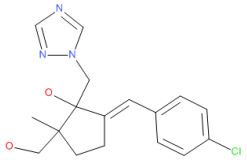
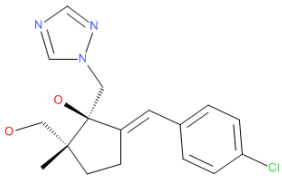
Analytical methods used in toxicological studies are evaluated in Vol 3, B5. It could be confirmed that analytical methods used in key studies (one year dog study, used for derivation of ADI and AOEL, and rabbit developmental study, used for derivation of ARfD) are valid and therefore these studies are considered fit for purpose.

B.6. TOXICOLOGY AND METABOLISM DATA

B.6.1. ABSORPTION, DISTRIBUTION, METABOLISM AND EXCRETION IN MAMMALS

For better understanding the RMS included below the structure, codes and synonyms of the active substance and the metabolites identified in rat metabolism studies.

Code Number		Description		Remark	Structure
Manufacturing code	**Reg No.**	**Chemical name**	**CAS-No.**		
BAS 595 F Triticonazole former BAS 9318 F RPA 400727 M595F000	43785 13	(*RS*)-(*E*)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1*H*-1,2,4-triazol-1-ylmethyl)cyclopentanol	131983-72-7	Rat	
Triticonazole **Isomer (+)-(S)** RPA 407026 M595F000S	50793 61	(1S,5E)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol	950830-94-1	Rat	
Triticonazole **Isomer (-)-(R)** RPA 407027 M595F000R	50793 85	(1R,5E)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol	950830-92-9	Rat	
cis-diol RPA 404766 M595F001 R2	50792 85	(1RS,2E,3SR)-2-(4-chlorobenzylidene)-5,5-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)-1,3-cyclopentanediol	none	Rat (detected and identified by LC/MS analysis in urine at levels below the sensitivity of the radioactivity detector used for quantification of metabolites)	AND Enantiomer 
trans-diol RPA 406341 AE 0540093 M595F002 (isomer von 404766)	50591 44	(1RS,2E,3RS)-2-(4-chlorobenzylidene)-5,5-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)-1,3-cyclopentanediol	none	Rat (detected and identified by LC/MS analysis in urine at levels below the sensitivity of the radioactivity detector used for	AND Enantiomer 

Code Number		Description		Remark	Structure
Manufacturing code	Reg No.	Chemical name	CAS-No.		
				quantification of metabolites)	
RPA 406972 M595F006	5079450	(3E)-3-(4-chlorobenzylidene)-2-hydroxy-2-(1H-1,2,4-triazol-1-ylmethyl)-1-methylcyclopentanecarboxylic acid	none	Rat	
RPA 406780 M595F007 R5	5079286	E-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentane-1,3-diol	none	Rat (detected and identified by LC/MS analysis in urine at levels below the sensitivity of the radioactivity detector used for quantification of metabolites)	
RPA 405826	5079360	(5E)-5-(4-chlorobenzylidene)-2-hydroxymethyl-2-methyl-1-(1H-1,2,4-triazol-1-ylmethyl)-cyclopentanol	none	Rat	
RPA 404886 M595F005 R4	5079247	(1RS,5E,2RS)-5-(4-chlorobenzylidene)-2-hydroxymethyl-2-methyl-1-(1H-1,2,4-triazol-1-ylmethyl)cyclopentanol	none	Rat	AND Enantiomer 

B.6.1.1. Absorption, distribution, metabolism and excretion by oral route

Previous evaluation: DRAR (2016)	DAR (2003) Only some additional information in the materials and methods and results added. Conclusion of the original assessment not changed

Reference:	RPA 400727: Preliminary ADME study in the rat
Author(s), year:	[redacted] 1992
Report/Doc. number:	C018956 / -
Guideline(s):	OECD 417 (1984); US EPA Pesticide Assessment Guidelines, Subdivision F, No. 85-1
GLP:	Yes
Deviations from OECD 417 (2010):	Minor (partially) reporting bias: - age of animals not given (however, based on the body weight information (170 – 185 g), the animals were between 6 and 12 weeks old, which is compliant to the OECD 417 (2010)
	Limitations of the study:

	- only two animals per sex and dose used (in total four animals) - most of the metabolites not identified
Acceptability:	Yes as preliminary/supplementary study

The study was conducted in compliance with the US EPA Pesticide Assessment Guidelines, Subdivision F, No. 85-1 and performed in accordance with GLP principles. Because of some limitations in this preliminary study (e.g. low number of animals), the study is regarded only as supplementary information.

Material and methods

Two rats per sex (strain: Sprague Dawley CRL:CD(SD)BR; source: [redacted]) were given a single oral dose of 500 mg/kg bw [^{14}C] triticonazole (universally phenylring labelled; batch no. CCM 2061; radiochemical purity 99.6 %; specific activity 32 mCi/mmole; chemical purity of non-labelled material 96.8 % (batch no DA 646) suspended in 0.5 % w/v methylcellulose in distilled water) by gavage. The active ingredient concentration (HPLC) and radioactivity content (LSC) were assayed both before and after the dosing.

The excreta (urine and faeces) of the rats were collected at 24 hours interval up to 168 hours after administration of the substance. The expired air was collected into an absorbing solution (ethanolamine in 2-ethoxyethanol 33 % v/v) for the first 48 hours post dose. The cages were washed 168 hours post dose. 7 days after dosing, the animals were sacrificed and selected tissues/organs (liver, kidneys, heart, lungs, brain, muscle, abdominal fat, spleen, adrenals, gonads, uterus, GI tract plus contents, bone and marrow and the skin and fur) were collected for determination of the distribution of the total residual radioactivity. The total radioactive residues (TRR) in the tissues and organs were determined by either direct liquid scintillation counting (urine, cage wash, carbon dioxide trap fluid and plasma) or following a combustion technique (faces and tissues). No exact value for the limit of quantification (LOQ) is given in the study report.

For metabolism investigations, pooled urine and faecal samples (collected for 0 to 24 hours, 24 to 48 hours and 48 to 72 hours post dosing) were prepared for chromatographic analysis using sequences of alcoholic solvent extraction and analyses performed by using HPLC and TLC techniques.

Results

Rate and extent of excretion: The total mean recovery of the administered radioactivity was 97.3 % with a range of 95.6 % - 100.8 %.

Results showed that the major route of excretion in both sexes was via faeces with the majority of the elimination occurring during the first 48 hours. Elimination of radioactivity via urine was a minor route of excretion. The mean values of radioactivity found in the excreta (as % of radioactive dose administered) are given in table B.6.1.1-1. Rates and routes of excretion were similar in male and female rats. At 168 hours post-dose, there was only low retention of total radioactivity in the body for both sexes. No significant levels of radiolabelled carbon dioxide were detected in the trap fluids collected up to 24 hours after dosing for either males and females (mean for the 4 animals < 0.1 % of administered radioactivity).

Table B.6.1.1-1: Excretion of radiolabelled material (mean % of radioactive dose) after single oral application of 500 mg/kg [^{14}C] triticonazole to rats

sample	Collection intervall (h)	♂	♀
urine	0 – 24 h	1.92	5.97
	0 – 48 h	4.16	7.98
	0 – 72 h	4.98	8.72
	total (0 – 168 h)	**5.25**	**9.01**
faeces	0 – 24 h	62.73	79.64
	0 – 48 h	82.95	88.51
	0 – 72 h	89.28	89.25
	total (0 – 168 h)	**90.77**	**89.56**
cage wash	after 168 hours	**0.15**	**0.18**
Tissues/carcass	after 168 hours	**0.23**	**0.06**
Total recovery	after 168 hours	**96.40**	**98.83**

Tissue residues: 168 hours after application of the radiolabelled dose, the concentrations of radioactivity (µg equivalents/g) found in the females ranged between 0.03 (brain) – 1.10 (skin and fur) µg/g with the results being consistent between the two animals. The results obtained for the two males were less consistent between the two animals with one rat displaying concentrations approx. double to those found for the other male rat. Moreover the concentrations found in both male rats were consistently higher than those found for the female animals with total radioactive residues found in the male tissues ranging between values of 0.10 (brain) – 4.55 (skin and fur) µg/g.
Individual total radioactive residues (µg equivalents/g) in the different tissues/organs of the four rats are given in table B.6.1.1-2.

Table B.6.1.1-2: Individual radioactivity in the tissues of male and female rats 168 hours after single oral application of 500 mg/kg [^{14}C] triticonazole (µg equivalents/g)

Tissue	♂		♀		Tissue	♂		♀	
Adrenals	2.92	0.68	0.41	0.32	Kidneys	1.30	0.60	0.38	0.23
Blood	2.83	1.35	0.43	0.27	Liver	1.40	0.69	0.32	0.23
Bone & Marrow	0.55	0.17	0.10	0.07	Lung	1.35	0.55	0.27	0.20
Brain	0.21	0.10	0.05	0.03	Muscle	0.43	0.19	0.06	0.06
fat (abdominal)	0.78	0.55	0.30	0.23	Plasma	3.71	1.62	0.40	0.29
G.I.T. + contents	0.87	0.58	0.21	0.16	Residual carcass	0.56	0.32	0.10	0.09
Gonads	0.48	0.22	0.22	0.17	Skin & Fur	4.55	1.79	1.10	0.87
Heart	1.18	0.41	0.23	0.13	Uterus	-	-	0.19	0.13

The highest mean concentration of total radioactivity was found in skin and fur for both sexes (3.17 µg/g in males, 0.99 µg/g in females). The three other tissues presenting higher mean concentrations were adrenals (1.80 µg/g in males, 0.37 µg/g in females), plasma (2.67 µg/g in males, 0.35 µg/g in females) and blood (2.09 µg/g in males, 0.35 µg/g in females). Lowest mean concentrations were found in the brain (0.16 µg/g in males, 0.04 µg/g in females) and muscle (0.31 µg/g in males, 0.0.06 µg/g in females), resp.

The tissue/plasma ratios were all less than 1 (indicating that the substance rather remained in the plasma) with the exception of the adrenals for the females (1.07) and the skin and fur samples for all four animals (1.16 [♂] and 2.89 [♀]). The mean blood/plasma ratios were 0.8 for males and 1.01 for females, indicating that the substance was almost uniformly distributed between the blood components but had a small preference for the plasma.

Metabolism: In faecal extracts six radiolabelled components were observed using both the HPLC and TLC. Comparison of retention times and Rf (retardation time) values, resp. with analytical standards indicated that the major component in the faeces (54.99 – 57.92 % in males and 78.01 – 79.11 % in females, resp. of administered dose) was the parent compound followed by RPA 405826, the hydroxylated parent compound (9.6 – 13.11 % in males and 4.07 – 4.53 % in females, resp., of administered dose).
The analysis of the urine metabolites was only successful using the TLC technique which revealed 10 radiolabelled polar components. Urine samples from females contained 2 metabolite fractions at levels greater than 1 % (fraction E: 2.23% and fraction H: 5.5%) of total radioactivity administered while urine samples from males contained only fraction H above 1% (2.1%). However, none of these components corresponded to the parent compound or available analytical standards. It was stated in the report that these metabolites are probably conjugates formed by phase II metabolic transformation.

Conclusion

Following a single oral dose of 500 mg [^{14}C] radiolabelled triticonazole/kg bw, the radioactivity was rapidly and almost quantitatively excreted within 168 hours by both male and female rats with faecal excretion predominated (approx. 90 % in both sexes). Most of the remainder of the dose was found in the urine (5.25 % in males and 9.01 % in females, resp.). The majority of the elimination occurred during the first 72 hours. No significant levels of expired radiolabelled carbondioxid were detected in the trap fluids collected up to 24 hours after dosing for either males or females.
The organs/tissues contained in sum between 0.06 and 0.23 % of the administered radioactivity with highest concentrations of total radioactivity found in skin & fur; adrenals, plasma and blood. Lowest mean concentrations were found in the brain and muscle, resp. The tissue/plasma ratios were all less than 1 with the exception of the adrenals for the females and the skin and fur samples for all four animals.
Investigation of metabolic fate revealed the presence of five metabolite components plus the parent compound in the faeces.

Previous evaluation:	DAR (2003)
DRAR (2016)	Only some additional information in the materials and methods and results added. Conclusion of the original assessment not changed

Reference:	RPA 400727: ADME study in the rat (1993) Triticonazole: Choice of radiolabel used in the rat ADME study (2002) Triticonazole: Quantification of faecal metabolites in the rat ADME report - Addendum to Report R013078 (2002)
Author(s), year:	[redacted]; 2002
Report/Doc. number:	R013078 / -
Guideline(s):	OECD 417 (1984); US EPA Pesticide Assessment Guidelines, Subdivision F, No. 85-1
GLP:	Yes
Deviations from OECD 417 (2010):	Minor (partially) reporting bias: - age of animals not given (however, based on the body weight information, the animals were between 6 and 12 weeks old, which is compliant to the OECD 417 (2010)
Acceptability:	Yes

Material and methods

For this ADME study, groups of 5 male and 5 female rats (strain Sprague Dawley (CD); source [redacted]) received single oral doses of either 5 or 500 mg/kg bw triticonazole (universally phenylring labelled; batch no. CMM 2061; radiochemical purity > 99 %; specific activity 32 mCi/mmole; chemical purity of non-labelled material 96.8 % (batch no DA 646) suspended in 0.75 % w/v aqueous methylcellulose). A further group of 5 rats/sex were given oral doses of non-radiolabelled triticonazole (batch no. DA 646, purity 96.8 %) at a rate of 5 mg/kg bw per day by gavage for 14 days, followed by one radiolabelled oral dose of the test substance. Additionally, 5 animals/sex were given single oral doses of 5 and 500 mg/kg bw in order to investigate the blood and plasma pharmacokinetic properties of triticonazole.

In the ADME experiments following the radiolabelled dose, urine and faeces of the rats were collected for analysis at 24 hour intervals. Exhaled carbon dioxide was not collected (not necessary based on the results from the preliminary study). 168 hours after dosing, all animals were sacrificed and blood, tissues and organs (liver, kidneys, heart, lungs, brain, spleen, muscle, abdominal fat, adrenals, gonads, uterus, GI tract plus contents, bone and marrow and the skin and fur) were sampled for determination of the distribution of the residual radioactivity. Cage washes were taken after the animals had been removed from the cages. The total radioactive residues in the tissues were determined by either direct liquid scintillation counting (urine, cage wash, plasma), or by combustion technique (faeces and organs).

For the pharmacokinetic experiment, duplicate blood samples (from one of which plasma was separated) were taken prior to dosing and at approx. 0.5, 1, 2, 3, 4, 6, 8, and 24 hours post-dosing and at 24 hour intervals thereafter until 168 hours post-dosing.

For metabolism investigations, samples of urine and faeces were initially analysed individually up to 48 or 72 hours post-dosing in order to ensure that there were no qualitative differences between animals of the same sex. Further samples were then pooled according to sex and time interval to create pooled samples (urine: pooled samples for each sex for the intervals 0 – 24 hours and 24 – 48 hours for all three experiments; faeces: pooled samples for each sex for the time periods 0 – 24 hours, 24 – 48 hours and 48 – 72 hours for the single high dose and the repeated dose experiment, and 0 – 24 hours and 24 – 48 hours for the single low dose experiment). Analyses were performed by HPLC, TLC, after enzyme deconjugation with ß-glucuronidase and sulfatase, resp. and mass spectrometry.

Results

Pharmacokinetic investigations (blood and plasma residues after single high and low dose application): The male and female animals in both dose groups displayed very similar blood and plasma radioactivity concentrations/ time

profiles. The levels of triticonazol-related radioactivity in the whole blood reached a maximum (C_{max}) of 1.58 ± 0.6 µg equivalents/g in males and 0.58 ± 0.6 µg/g in females after 0.5 to 1 hour post-dose at the low dose level, and 25.38 ± 4.9 µg equivalents/g in males and 19.28 ± 6.1 µg/g in females after 1 – 2 hours post-dosing at the high dose level, resp. Thereafter at both dose levels, radioactivity in the whole blood declined rapidly over 24 – 48 hours (initial phase half-life approximately 4 – 6 hours) followed by a more protracted elimination phase with terminal elimination half-lives of 119.4 hours for the males and 116.4 hours for the females at the low dose level and 95.62 hours for the males and 106.3 hours for the females at the high dose level, resp.

Plasma results were very similar to the whole blood results with C_{max} values 2.37 ± 0.9 µg equivalents/g in males and 0.86 ± 0.9 µg/g in females between 0.5 to 1 hour post-dose at the low dose level, and 33.24 ± 6.9 µg equivalents/g in males and 23.52 ± 8.4 µg/g in females after 1 – 3 hours post-dosing at the high dose level, resp. The terminal elimination half-lives of radioactivity from the plasma were calculated to be 109.7 (males) to 113.0 (females) hours for low dosed animals and 82.7 (males) to 99.9 (females) hours for the high dose level.

Table B.6.1.1-3: C_{max}, T_{max} and $t_{0.5}$ values in male and female rats after single oral application of 500 mg/kg [14C] triticonazole (TOPFIT programme, version 1.1)

Whole blood	**C_{max} (µg/g)**		**T_{max} (h)**		**$t_{0.5}$ (h)**	
	Mean	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
Males	25.38	4.9	2.0	0.0	95.6	4.6
Females	19.28	6.1	1.8	0.5	106.3	6.2
Plasma						
Males	33.24	6.9	2.0	0.7	82.7	11.0
Females	23.52	8.4	1.6	0.6	99.9	14.9

Table B.6.1.1-4: C_{max}, T_{max} and $t_{0.5}$ values in male and female rats after single oral application of 5 mg/kg [14C] triticonazole (TOPFIT programme, version 1.1)

Whole blood	**C_{max} (µg/g)**		**T_{max} (h)**		**$t_{0.5}$ (h)**	
	Mean	**S.D.**	**Mean**	**S.D.**	**Mean**	**S.D.**
Males	1.58	0.6	0.6	0.2	119.4	13.0
Females	0.58	0.6	0.6	0.2	116.4	8.3
Plasma						
Males	2.37	0.9	0.6	0.2	109.7	6.2
Females	0.86	0.9	0.6	0.2	113.0	20.8

Rate and extent of excretion: After oral administration, the majority of the administered radioactivity was eliminated within the first 48 hours following dosing in all three dose groups. In all groups elimination via faeces was greater than that via urine. In males, a tendency towards higher levels of radioactivity in the faeces compared to females was observed with statistical significance for the repeated dose group. The excreted radioactivity via urine displayed

some significant differences between the sexes for the two lower dose groups but not for the high dose group. The observed routes and rates of excretion for the two low dose groups (single and repeated application) were similar, indicating that there was no time-dependent change in the pharmacokinetics of triticonazole (e.g. enzyme induction).

Table 6.1.1-5: Mean radioactivity recovered in urine, faeces, cage wash and tissues/carcass at different time points (mean % of radioactive dose, cumulative) after single and repeated oral application of [^{14}C] triticonazole to rats

sample	Collection intervall (h)	500 mg/kg bw ♂ (single)	♀	5 mg/kg bw ♂ (single)	♀	5 mg/kg bw per day ♂ (repeated)	♀
urine	0 – 24	1.41	3.01	9.96	27.17	7.98	11.35
	0 – 48	2.77	4.21	12.79	30.87	12.18	20.08
	0 – 72	3.15	4.53	13.40	31.57	13.74	23.85
	0 – 168	**3.31**	**4.70**	**13.72**	**32.21**	**14.68**	**25.60**
faeces	0 – 24	76.36	85.41	52.75	51.78	30.48	15.67
	0 – 48	92.16	93.35	77.91	63.44	63.76	47.69
	0 – 72	95.42	95.19	82.38	64.95	75.43	65.20
	0 – 168	**96.16**	**95.69**	**83.39**	**65.26**	**80.96**	**71.05**
cage wash	at 168 h	**0.07**	**0.10**	**0.19**	**0.44**	**0.35**	**1.20**
tissues/carcass	at 168 h	**0.08**	**0.04**	**0.41**	**0.12**	**0.57**	**0.22**
total recovery	at 168 h	**99.62**	**100.53**	**97.72**	**98.02**	**96.56**	**98.07**

Tissue residue distribution: The mean levels of radioactivity in all tissues/organs 168 hours after dosing with 500 mg/kg bw were very low (representing 0.08 % of the dose administered in males and 0.04 % in females) and low in the two low dose groups (single and repeated). However, the increase of concentrations in tissues observed for the high dose group was not proportional to the dose applied.

At the high dose level, highest mean values of radioactivity (expressed in µg equivalents/g) were found in skin & fur and liver at both sexes, and also in blood and plasma in males only. Generally, the levels of radioactivity in tissues in males tended to be slightly higher than in females. The mean tissue/plasma ratios were less than 1 for most tissues except skin & fur in both sexes (1.01 in males and 4.53 in females) and liver (1.36), gastrointestinal tract (1.29) and whole blood (1.09) in females.

After single and repeated low dose application the mean levels of radioactivity in all tissues at 168 hours were very low in both groups (< 0.2 µg equivalents/g), being below the limit of detection in many tissues. Levels of radioactivity in tissues in males again tended to be slightly higher than in females. Mean tissue/plasma ratios were less than 1 for most tissues with the exception of skin & fur (1.47) and adrenals (4.01) in females after single dose, the whole blood (1.60) in males after repeated dose and the fat (2.42), adrenals (2.60) and the whole blood (1.69) for the repeated low dose females.

The mean values of radioactivity (expressed as µg equivalents/g tissue) observed in the organs/tissues of rats after single high and low dose application, and repeated low dose application, resp. are given in table 6.1.1-6.

Table 6.1.1-6: Radioactivity in the tissues (mean values, expressed as µg equivalents/g) 168 hours after single and repeated oral application of [^{14}C] triticonazole to rats

Tissue	500 mg/kg bw ♂ single) ♀		5 mg/kg bw ♂ (single) ♀		5 mg/kg bw ♂ (repeated) ♀	
Adrenals	n.d.	n.d.	0.06	0.04	0.07	0.07
Blood	0.71	0.18	0.05	0.01	0.18	0.05
Bone & Marrow	0.08	n.d.	0.01	n.d.	0.01	n.d.
Brain	0.03	n.d.	n.d.	n.d.	0.01	n.d.
Fat	0.32	0.18	0.02	0.01	0.05	0.06
G.I. tract + contents	0.38	0.19	0.01	n.d.	0.04	0.02
Gonads	0.17	n.d.	0.01	0.01	0.02	0.02
Heart	0.33	n.d.	0.02	n.d.	0.03	0.01
Kidney	0.47	0.15	0.02	0.01	0.05	0.02
Liver	0.65	0.23	0.03	0.01	0.05	0.02
Lung	0.40	0.09	0.03	0.01	0.04	0.01
Muscle	0.06	n.d.	0.01	n.d.	0.01	n.d.
Plasma	0.87	0.16	0.07	0.01	0.11	0.03
Residual carcass	0.11	n.d.	0.01	n.d.	0.02	0.01
Skin & fur	0.76	0.81	0.03	0.01	0.03	0.02
Spleen	0.06	n.d.	0.01	n.d.	0.02	0.01
Uterus	-	0.14	-	0.01	-	0.02

n.d. = not detected

Metabolites in faeces: In the 0 – 72 (48) hours pooled faecal data (extracts plus residues remaining in faeces) (representing 99.2 % [♂] and 99.5 % [♀], 93.4 % [♂] and 97.2 % [♀], and 93.4 % [♂] and 92 % [♀] of the total faecal elimination of radioactivity for the single high dose group, single low dose group and repeated dose group, respectively) using the HPLC system, ten radiolabelled components were observed. These components were ascribed numbers in the format FMET1 to 10 in order of their appearance in the chromatogram, i.e. FMET10 was the least polar component and had the longest retention time. Of these 10 components, seven were observed in the high dose group, eight in the single low dose group and ten in the repeated dose group (table 6.1.1-7).

Table 6.1.1-7: Percentage (%) of administered dose represented by radioactive components (Faecal METabolites) in the faeces from male and female rats

FMET	1	2	3	4	5	6	7	8	9	10
Males										
	Single high dose (500 mg/kg bw)									
0-24 h	n.d.	n.d.	0.22	n.d.	0.31	n.d.	1.42	0.57	1.76	72.09
24-48 h	n.d.	n.d.	0.25	n.d.	0.84	n.d.	3.72	0.44	2.99	7.57

FMET	1	2	3	4	5	6	7	8	9	10
48-72 h	n.d.	n.d.	0.07	n.d.	0.38	0.19	1.44	0.17	0.91	0.08
Sum	**n.d.**	**n.d.**	**0.54**	**n.d.**	**1.53**	**0.19**	**6.58**	**1.18**	**5.66**	**79.74**
	Single low dose (5 mg/kg bw)									
0-24 h	n.d.	0.33	1.06	n.d.	7.06	2.47	21.25	5.00	14.25	1.34
24-48 h	n.d.	0.27	0.48	n.d.	3.58	1.89	12.55	1.02	5.36	n.d.
Sum	**n.d.**	**0.6**	**1.54**	**n.d.**	**10.64**	**4.36**	**33.80**	**6.02**	**19.61**	**1.34**
	Repeated low dose (5 mg/kg bw per day)									
0-24 h	0.08	0.50	0.56	0.51	5.17	1.21	12.20	2.47	7.55	0.23
24-48 h	0.09	0.60	0.77	0.61	7.27	1.89	15.44	0.86	5.75	n.d.
48-72 h	0.05	0.19	0.25	0.24	1.44	0.81	6.52	0.33	1.84	n.d.
Sum	**0.22**	**1.29**	**1.58**	**1.36**	**13.88**	**3.91**	**34.16**	**3.66**	**15.14**	**0.23**
Females										
	Single high dose (500 mg/kg bw)									
0-24 h	n.d.	n.d.	0.94	n.d.	n.d.	n.d.	0.81	n.d.	2.84	80.82
24-48 h	n.d.	n.d.	0.63	n.d.	0.06	0.17	0.96	n.d.	1.83	4.29
48-72 h	n.d.	n.d.	0.04	n.d.	0.20	0.08	0.81	0.11	0.53	0.07
Sum	**n.d.**	**n.d.**	**1.61**	**n.d.**	**0.26**	**0.25**	**2.58**	**0.11**	**5.20**	**85.18**
	Single low dose (5 mg/kg bw)									
0-24 h	n.d.	0.24	6.85	n.d.	1.46	3.29	13.65	1.57	22.88	1.85
24-48 h	n.d.	0.28	1.52	n.d.	0.54	1.71	3.96	0.31	3.33	n.d.
Sum	**n.d.**	**0.52**	**8.37**	**n.d.**	**2.00**	**5.00**	**17.61**	**1.88**	**26.21**	**1.85**
	Repeated low dose (5 mg/kg bw per day)									
0-24 h	n.d.	0.29	0.71	n.d.	0.35	0.90	4.07	0.45	8.72	0.18
24-48 h	n.d.	1.39	3.66	n.d.	0.96	3.08	10.65	0.59	11.08	n.d.
48-72 h	0.05	0.88	2.27	n.d.	0.74	2.76	6.87	0.23	4.31	n.d.
Sum	**0.05**	**2.56**	**6.64**	**n.d.**	**2.05**	**6.74**	**21.59**	**1.27**	**24.11**	**0.18**

n.d. = not detected

The metabolite profiles obtained for males and females were qualitatively very similar and differed rather in quantitative terms: In the high dose experiment, most of the radioactivity was associated with component FMET10 (60 – 70 % of the administered dose) 24 hours after dosing but decreased rapidly to < 0.1 % of the dose by 72 hours post-dosing. Comparison of the retention times with standard substances indicated that this component was likely the parent compound (RPA 400727). It was suggested in the report that these high levels of parent compound in the high dose experiment indicated that the rats capacity for absorption/metabolism is exhausted. All other compounds were represented in quantities less than 6.6 % of the administered dose up to 72 hours post-dosing.

Only low levels (< 1.5 % of the dose) of triticonazole were detected in faeces 24 hours after dosing for the two low dose experiments. The major components observed in faecal extracts were FMET9 (RPA 405826/404886) and

FMET7 (RPA 406972), and three other more polar components FMET6 (females only), FMET5 (males only) and FMET3 (females only) were present in higher quantities. Detailed structure of these metabolites is given in table 6.1.1-9.

The findings of the analysis by TLC, which was only successful for metabolites in faecal extracts showed reasonable correspondence between the major peaks observed by HPLC and TLC.

Metabolites in urine: In the 0 – 48 hours pooled urine samples (representing 84 % [♂] and 90 % [♀], 93 % [♂] and 96 % [♀], and 83 % [♂] and 78 % [♀] of the total urinary elimination for the high dose group, single low dose group and repeated dose group, respectively) a total of 12 components were observed using the HPLC systeme (table 6.1.1-8). These components were ascribed numbers in the format UMET1 to 12 in order of their appearance in the chromatogram, i.e. UMET12 had the longest retention time.

In the high dose experiment, only one component (UMET11) represented > 1% of the administered dose; all other components were at lower level. After single low dose application four metabolites were at > 1% of the administered dose in males (UMET6, 9, 11/12) and four metabolites were at > 1 % of the dose in females (UMET5, 9, 11/12) as well. One of these metabolites (UMET11) was present at 23 % in females, but only at 2.5 % in males. A similar pattern was seen after repeated dosing differing only in the total reduced level of radioactivity found in the female samples. No parent material was detected in the urine of all three dose groups. As was observed for the faecal samples the quantities of the metabolites decreased rapidly after dosing exhibiting the highest levels in the 0 – 24 hours samples.

Table 6.1.1-8: Percentage (%) of administered dose represented by radioactive components (Urine METabolites) in the urine from male and female rats

UMET	1	2	3	4	5	6	7	8	9	10	11	12	Sum
Males													
	Single high dose (500 mg/kg bw)												
0-24 h	0.01	0.11	0.05	0.04	0.04	0.26	0.05	0.03	0.12	0.04	0.43	0.23	
24-48 h	n.d.	0.06	0.05	0.03	0.04	0.36	0.04	0.05	0.11	0.05	0.35	0.23	
Sum	**0.01**	**0.17**	**0.10**	**0.07**	**0.08**	**0.62**	**0.09**	**0.08**	**0.23**	**0.09**	**0.78**	**0.46**	**2.78**
	Single low dose (5 mg/kg bw)												
0-24 h	0.08	0.51	0.43	0.29	0.24	3.02	0.26	0.38	0.82	0.30	1.78	1.85	
24-48 h	0.03	0.14	0.11	0.11	0.18	0.63	0.08	0.17	0.26	0.11	0.77	0.24	
Sum	**0.11**	**0.65**	**0.54**	**0.40**	**0.42**	**3.65**	**0.34**	**0.55**	**1.08**	**0.41**	**2.55**	**2.09**	**12.79**
	Repeated low dose (5 mg/kg bw per day)												
0-24 h	0.11	0.76	0.51	0.26	0.30	2.91	0.20	0.40	0.68	0.34	0.62	0.89	
24-48 h	0.19	0.32	0.38	0.26	0.18	1.11	0.15	0.22	0.39	0.34	0.47	0.20	
Sum	**0.30**	**1.08**	**0.89**	**0.52**	**0.48**	**4.02**	**0.35**	**0.62**	**1.07**	**0.68**	**1.09**	**1.09**	**12.18**

UMET	1	2	3	4	5	6	7	8	9	10	11	12	Sum
Females													
	Single high dose (500 mg/kg bw)												
0-24 h	0.02	0.06	0.07	0.05	0.48	0.11	0.03	0.02	0.16	0.02	1.79	0.20	
24-48 h	0.01	0.02	0.05	n.d.	0.21	0.02	0.02	0.01	0.08	0.02	0.72	0.04	
Sum	**0.03**	**0.08**	**0.12**	**0.05**	**0.69**	**0.13**	**0.05**	**0.03**	**0.24**	**0.04**	**2.51**	**0.24**	**4.21**
	Single low dose (5 mg/kg bw)												
0-24 h	0.02	0.07	0.58	0.13	2.05	0.38	0.11	0.52	1.08	0.07	20.45	1.73	
24-48 h	0.02	0.13	0.06	0.13	0.08	0.10	0.05	0.14	0.26	0.08	2.28	0.39	
Sum	**0.04**	**0.20**	**0.64**	**0.26**	**2.13**	**0.48**	**0.15**	**0.65**	**1.34**	**0.15**	**22.72**	**2.12**	**30.88**
	Repeated low dose (5 mg/kg bw per day)												
0-24 h	0.02	0.17	0.57	0.13	2.33	0.26	0.07	0.25	0.99	0.03	4.56	1.98	
24-48 h	0.04	0.22	0.89	0.10	1.09	0.23	0.05	0.43	0.88	0.09	3.91	0.78	
Sum	**0.06**	**0.39**	**1.46**	**0.23**	**3.42**	**0.49**	**0.12**	**0.68**	**1.87**	**0.12**	**8.47**	**2.76**	**20.08**

n.d. = not detected

Representative samples of pooled urine (high dose group, 0 – 24 hours) and faecal extracts (single low dose group, 24 – 48 hours) were subjected to deconjugation conditions using ß-glucuronidase or sulfatase. Comparison of the metabolic profile did not indicate the presence of conjugates susceptible to hydrolysis by these enzymes.

Analysis by LC/MS enabled identification of most of the major metabolites (some of which were diastereoisomers). These were identified as different hydroxylated metabolites of the parent compound, mostly involving hydroxylation of the cyclopentane ring, or one of its 5-methyl side chains. Further hydroxylation of the phenylring and oxidation of the hydroxyl group at the 5-position of the cyclopentane ring to form a carboxylic acid side chain was also identified.

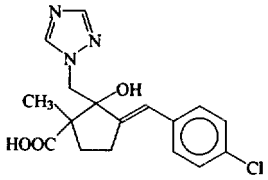
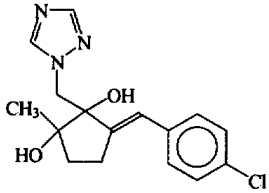
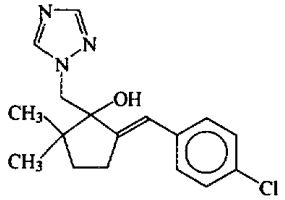
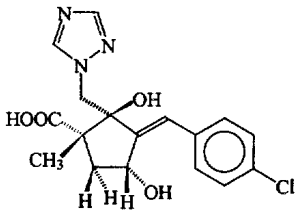
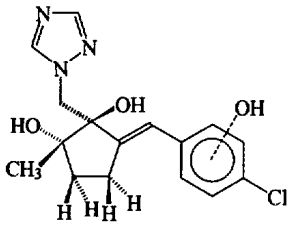
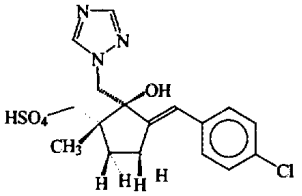
In the faeces, following molecules were identified: FMET10 as parent material (RPA 400727), FMET9 as RPA 405826/404886 (diastereoisomers of the parent compound hydroxylated on the 5-methyl side chain), FMET8 and FMET6 as dihydroxylated parent compounds, FMET7 as RPA 406972 (carboxylic acid metabolite) and FMET5 as hydroxylated RPA 406972.

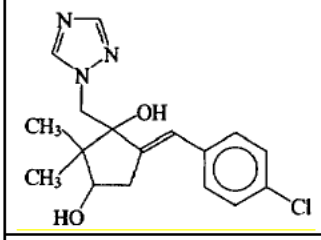
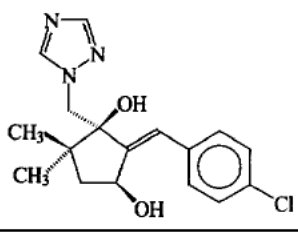
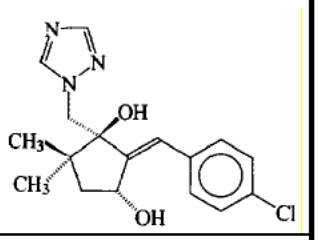
In the urine samples UMET11/12 were identified as diastereoisomers of RPA 406972 and UMET6 as a hydroxylated analogue of RPA 406972. UMET9 and UMET2 could be hydroxylated parent compound with the hydroxylation occurring on the phenyl or triazole rings. In addition, mass spectroscopic analysis indicated the presence of small quantities of "conjugated" parent compound. The components RPA 404766, RPA 406780 and RPA 406341 were also detected and identified by LC/MS analyses as hydroxylated parent compounds (at the cyclopentane ring) at levels below the sensitivity of the radioactivity detectors used for the quantification of the metabolites. It was also assumed that the metabolites UMET4 and FMET3 could be the sulphate conjugates of the parent compound but no exact identification for both components was performed in the study.

Based on the characterisation of the faecal and urinary metabolites by mass spectrometry, a total of 97 – 98 %, 86 – 89 % and 75 – 79 % of the administered radioactivity was assigned structures for the single high dose group, single low dose group and repeated low dose group, resp.

Structural formulas of the identified metabolites and their respective percentages of total radioactive dose found in the 0 – 72 (48) hours pooled urine and faecal extracts after single high and low dose, and repeated low dose are given in table 6.1.1-9.

Table 6.1.1-9: Structural formulas of identified metabolites and their respective percentages of administered dose (%) found in the excreta after single high (SHD) and low dose (SLD), and repeated low dose (RLD) application

Structure		UMET 11 & 12, FMET 7 RPA 406972 		FMET 9 RPA 405826/404886 		FMET 10 Triticonazole 	
		males	females	males	females	males	females
SHD	urine	1.24	2.75	n.d.	n.d.	n.d.	n.d.
	faeces	6.58	2.58	5.66	5.20	79.74	85.18
	total	**7.82**	**5.33**	**5.66**	**5.20**	**79.74**	**85.18**
SLD	urine	4.64	24.84	n.d.	n.d.	n.d.	n.d.
	faeces	33.80	17.61	19.61	26.21	1.34	1.85
	total	**38.44**	**42.45**	**19.61**	**26.21**	**1.34**	**1.85**
RLD	urine	2.18	11.23	n.d.	n.d.	n.d.	n.d.
	faeces	34.16	21.59	15.14	24.11	0.23	0.18
	total	**36.34**	**32.82**	**15.14**	**24.11**	**0.23**	**0.18**
Structure		UMET 6, FMET 5 		FMET 6 & 8 		UMET 4, FMET 3 	
		males	females	males	females	males	females
SHD	urine	0.62	0.13	n.d.	n.d.	0.07	0.05
	faeces	1.53	0.26	1.37	0.36	0.54	1.61
	total	**2.15**	**0.39**	**1.37**	**0.36**	**0.61**	**1.66**
SLD	urine	3.65	0.48	n.d.	n.d.	0.40	0.26
	faeces	10.64	2.00	10.38	6.88	1.54	8.37
	total	**14.29**	**2.48**	**10.38**	**6.88**	**1.94**	**8.63**
RLD	urine	4.02	0.49	n.d.	n.d.	0.52	0.23
	faeces	13.88	2.05	7.57	8.01	1.58	6.64

	total	**17.90**	**2.54**	**7.57**	**8.01**	**2.10**	**6.87**
Structure		RPA 406780 		RPA 404766 		RPA 406341 	
		These components were detected and identified by LC/MS analysis in urine at levels below the sensitivity of the radioactivity detector used for quantification of metabolites					

Conclusion

Following a single oral administration of 5 or 500 mg [phenyl-^{14}C] radiolabelled triticonazole/kg bw, the radioactivity was rapidly and almost completely excreted by both male and female rats with the majority of the administered radioactivity (> 90 %) eliminated within the first 48 hours following dosing. The major route of elimination in both sexes was the faeces (65.26 – 96.16 % of total radioactivity) and the remainder of the dose applied was found in the urine (3.31 – 32.21 %). The pattern of elimination of radioactivity was similar in animals receiving repeated doses of 5 mg/kg bw per day compared with animals receiving the single low dose. For both low dose groups, a sex difference in excretion pattern was observed as the mean percentages of radioactivity in the urine were found to be only 14 % of the dose in males but around 26 - 32 % in females.

The pharmacokinetic investigations in both the single oral low and high dose groups exhibited very similar blood and plasma radioactivity profiles with no significant differences between males and females. The maximum concentrations in blood were obtained at the low dose between 0.5 to 1 hour and between 1 to 2 hours at the high dose; terminal elimination half-lives from blood were calculated to be 95.62 and 119.4 hours at the low and high dose level, resp.

The levels of radioactivity in the tissues at 168 hours after application were low in the high dose group (< 0.9 µg/g tissue) and very low (< 0.1 µg/g tissue) in the two low dose groups. Highest levels were detected in skin & fur, liver, blood and plasma with levels in males tended to be slightly higher than in females. Mean tissue/plasma ratios were less than 1 for most tissues with the exception of skin and fur, liver, fat, adrenals and whole blood. Comparison of the tissue radioactivity levels between the high and low dose groups showed that the increase in concentrations observed for the high dose group was not proportional to the dose.

Metabolism of triticonazole was found to be rapid and extensive at the low dose level (single and repeated application) with low amounts of parent material found in the faeces 24 hours after dosing only. At the high dose level, triticonazole was identified the major compound in the faeces indicating limited absorption/metabolism. Analysis by HPLC revealed a total of 10 and 12 components in faecal and urinary extracts, resp. The metabolite profiles obtained for males and females were qualitatively very similar and differed rather in quantitative terms. Based on the identified metabolites found in urine and faeces by LC/MS, a metabolic pathway was proposed (Figure 6.1.1-1) which involved hydroxylation at different positions of the molecule.

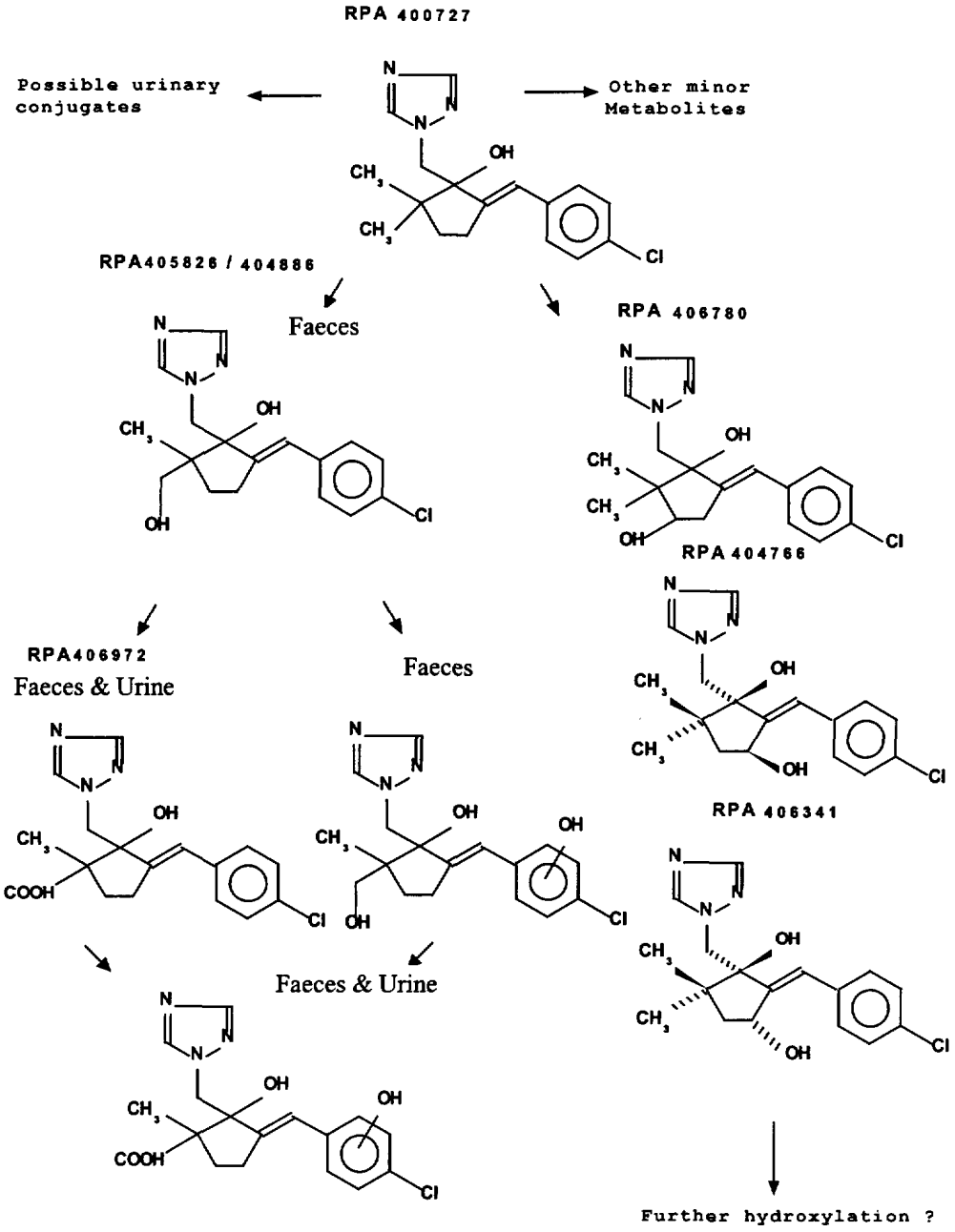


Figure 6.1.1-1: Proposed metabolic pathway of triticonazole in the rat

Previous evaluation: DRAR (2016)	DAR (2003) Only some minor information in the materials and methods and results added. Conclusion of the original assessment not changed

Reference:	Triticonazole: Rat bile excretion study
Author(s), year:	[redacted] 2000
Report/Doc. number:	R012111 / -
Guideline(s):	OECD 417 (1984); US EPA Health Effect Test Guidelines OPPTS 870.7485 (1998)
GLP:	Yes
Deviations from OECD 417 (2010):	- age of animals not given (however, based on the body weight information, the animals were between 6 and 12 weeks old, which is compliant to the OECD 417 (2010)
Acceptability:	Yes

Material and methods

Four rats per sex and dose (strain Sprague Dawley CD; source [redacted]) were given single oral doses of either 5 or 500 mg [^{14}C] triticonazole (universally phenylring labelled, batch no. CFQ11862; radiochemical purity 99.2 %, specific activity 34 mCi/mmole; chemical purity of non-labelled material 99.9 % (batch no YG 2262/I) suspended in 0.75 % w/v aqueous methylcellulose)/kg bw by gavage.

Surgery of the rats (cannulation of bile ducts) was performed 24 hours before administration of the substance. Urine and bile were collected at 0 – 6 hours, 6 – 24 hours and 24 – 48 hours; faeces and cage wash were collected at 24 hour intervals following dosing. 48 hours after dosing, all animals were sacrificed and the amounts of radioactivity were determined in selected tissues/organs (blood and plasma, intestinal tract, intestinal tract contents, stomach, stomach contents, residual carcass) by liquid scintillation counting directly (liquid samples) or following a combustion technique (solid samples). The limit of quantification was reported at twice the measured background rate in blank samples.

Results

Rate and extent of excretion: At the low dose group, elimination of radioactivity via bile was higher than that via urine which was higher than that via the faeces in both sexes. However, lower levels of radioactivity were present in the urine (3.14 % [♂] and 11.95 % [♀] of the administered dose at 48 hours) than those observed in the previous ADME study (approx. 13 % [♂] and 31 % [♀] at 48 hours post-dosing) although they were within the same order of magnitude.

In contrast, after administration of the high dose, amounts of radioactivity eliminated via faeces were higher than that via bile which was higher than that via urine in both sexes indicating a limited absorption rate most likely due to dissolution effects of triticonazole in the gut/intestinal fluids. Results from the previous ADME study support this hypothesis in that the faeces from the high dosed animals were found to contain much higher levels of parent material.

The mean values of radioactivity found in the excreta and in tissues after low and high dose administration (in % of dose administered) are given in table 6.1.1-10.

Table 6.1.1-10: Recovery of radioactivity in excreta and tissues (mean % of radioactive dose) after single oral application of 5 and 500 mg/kg [^{14}C] triticonazole to rats

Sample	Time period	5 mg/kg bw ♂	5 mg/kg bw ♀	500 mg/kg bw ♂	500 mg/kg bw ♀
Urine	0 – 6 h	1.39	4.51	0.14	0.51
	6 – 24 h	1.56	6.95	0.85	5.66
	24 – 48 h	0.19	0.49	0.29	2.05
	total (0 – 48 h)	**3.14**	**11.95**	**1.28**	**8.22**
Bile	0 – 6 h	82.61	68.59	6.57	5.03
	6 – 24 h	12.50	18.75	18.42	10.54
	24 – 48 h	0.14	0.16	4.91	6.81
	total (0 – 48 h)	**95.24**	**87.50**	**29.89**	**22.38**
Faeces	0 – 24 h	0.94	0.33	39.83	26.72
	24 – 48 h	0.09	0.21	19.16	19.62
	total (0 – 48 h)	**1.02**	**0.54**	**58.99**	**46.34**
Cage wash	0 – 48 h	**0.17**	**0.52**	**0.10**	**2.08**
Tissues	at 48 h	**0.31**	**0.54**	**7.44**	**22.63**
Total recovery		**99.88**	**101.04**	**97.70**	**101.65**

Tissue residue distribution: Animals from the low dose group exhibited low levels of radioactivity in tissues. Mean measurable levels of radioactivity were found to range between 0.0002 µg equivalents/g (stomach content in males) to 0.0993 µg equivalents/g (plasma of males). Concentration observed in blood and plasma of males was higher than in females whereas levels in the carcass samples were the same.

The concentrations found in rats given the high dose were higher ranging between 1.9 µg equivalents/g (blood of females) to 895.8 µg equivalents/g (intestinal contents in females). No differences in concentrations were observed in the blood and plasma between the sexes. However, there were higher concentrations of radioactivity in the stomach, carcass and intestine samples from the females compared to the males.

Table 6.1.1-11: Mean concentration of radioactivity in tissues (µg equivalents/g), and in brackets expressed as % of total dose administered 48 hours after single oral application of 5 and 500 mg/kg [^{14}C] triticonazole to rats

Sample	5 mg/kg bw ♂	5 mg/kg bw ♀	500 mg/kg bw ♂	500 mg/kg bw ♀
Blood	0.0582 (0.0194 %)	0.0108 (0.0029 %)	2.221 (0.0064 %)	1.8498 (0.0054 %)
Plasma	0.0993 (0.0097 %)	0.0145 (0.0011 %)	4.2727 (0.0032 %)	3.2163 (0.0024 %)
Stomach	0.0359 (0.0040 %)	0.0330 (0.0036 %)	56.069 (0.0605 %)	64.2710 (0.0936 %)

Sample	5 mg/kg bw ♂	5 mg/kg bw ♀	500 mg/kg bw ♂	500 mg/kg bw ♀
Stomach contents	0.0002 (0.0001 %)	0.0007 (0.0005 %)	22.098 (0.1820 %)	322.250 (1.4349 %)
Intestine	0.0183 (0.0108 %)	0.0275 (0.0197 %)	13.076 (0.0812 %)	73.7590 (0.4931 %)
Intestine contents	0.0194 (0.0349 %)	0.0617 (0.2662 %)	303.73 (6.7663 %)	895.810 (18.1205 %)
Carcass	0.0140 (0.2292 %)	0.0140 (0.2292%)	2.0069 (0.3394 %)	13.4870 (2.4768 %)

The levels found in the high dose male blood and plasma samples increased by 41 times the concentrations achieved at 5 mg /kg but the same tissue in the females increased by 197 times. A similar picture could be seen with the carcass results where the males increased by 143 times and the females increased by 963 times.

The absorption rates from the intestinal tract were estimated from the recoveries obtained in urine (plus cage wash), bile and tissues (excluding the intestinal and stomach content). Based on the recovered amounts obtained, the calculated absorption rates were 98.8 % in males and 100.2 % in females at the low dose, and 31.8 % in males and 35.8 % in females after high dose application.

Conclusion:

Based on the recoveries of radioactivity obtained in the urine (plus cage wash), bile and tissues (excluding the intestinal and stomach contents), the enteral absorption rate of triticonazol in the rat can be calculated as > 98 % after single low dose application (both sexes) and 31.8 % (♂) – 35.8 % (♀) after single high dose application. This significant difference in the absorption rate can be explained by dissolution effects of triticonazole in the intestinal fluids influencing this absorption rate.

B.6.1.2. Absorption, distribution, metabolism and excretion by other routes

No ADME study by a route other than oral has been submitted. The study by intravenous exposure route is not considered necessary since the information on oral absorption and bioavailability is gained from bile cannulation study.

B.6.1.3. Other ADME studies

The notifier provided a comparative *in vitro* metabolism study with triticonazole. Additionally, literature data were identified addressing comparability of rat and human metabolites.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[14C]BAS 595 F – In Vitro Comparative Metabolism in Human, Rat, Dog and Rabbit Liver Microsomes
Author(s), year:	Thibaut, R., 2016

Report/Doc. number::	-/ 2016/1052691
Guideline(s):	No
GLP:	Yes
Deviations:	-
Acceptability:	Yes

A comparative *in vitro* metabolism study was performed with Triticonazole (BAS 595 F, Reg. No. 4378513). The objective of this study was to compare the *in vitro* metabolism in liver microsomes of animal species used in toxicological testing of this substance to the metabolism in human liver microsomes and to determine whether metabolic profiles are similar and whether unique human metabolites occur.

Material and methods

Test Material:	
Description:	Triticonazole (BAS 595 F)
Batch # / purity:	(Phenyl-U-^{14}C) triticonazole: 866-1601 99.1%
Stability of test compound	Stable during testing
Test system**:**	Microsomes Positive control: microsomes were incubated with testosterone instead of the active substance to prove the metabolic activity of the microsomes
Test animals:	Mammals
Species:	Rat, dog, rabbit and human
Strain:	Male and female Sprague Dawley rats; Male and female Beagle dog; Male and female New Zealand rabbit

Test substance preparation

^{14}C-Triticonazole stock solution was an acetonitrile solution at a concentration of 28.0 MBq/g. For the preparation of the application solution for experiments with 10 µM Triticonazole, stock solution was diluted with acetonitrile. The obtained isotope ratio was approximately 1/1 (^{14}C/^{12}C) and further confirmed by MS analysis. The actual concentrations of the radiolabelled Triticonazole in the application solutions were determined by LSC. The specific radioactivity of the application solution accounted for 119,325,000 dpm/µmol and its purity was confirmed by HPLC analysis of a diluted aliquot.

Testosterone stock solution was prepared by dissolving testosterone in methanol at a concentration of 36.05 mg/mL. ^{14}C-testosterone Stock solution was an ethanol solution at a concentration of 0.1 mCi/mL. Application solution for experiments with 250 µM testosterone was prepared by mixing stock solutions of ^{14}C-testosterone with testosterone. The solvents were concentrated to dryness under a nitrogen stream and the residue was dissolved in 500 µL of acetonitrile.

7-Ethoxycoumarin stock solution was prepared by dissolving 7-Ethoxycoumarin in methanol at a concentration of 47.55 mg/mL. ^{14}C-7-Ethoxycoumarin stock solution was prepared by dissolving 50 µCi of ^{14}C-7-Ethoxycoumarin in 555 µL of methanol, in order to reach the concentration of 0.09 mCi/mL. Application solution was prepared by mixing stock solutions of ^{14}C-7-Ethoxycoumarin with 7-Ethoxycoumarin. The solvent was concentrated to dryness under a nitrogen stream and the residue was dissolved in 500 µL of acetonitrile.

Microsomal preparations

Pool of mixed gender (ratio of 1:1) liver microsomes from human (100 male and 100 female), rat (from > 100 male and > 100 female), dog (from 8 male and 12 female) and rabbit (from 8 male and 3 female) were used.

Microsomes were thawed quickly by placing them in a water bath at room temperature for 2-5 min. After thawing, dilution with a solution of sucrose to a concentration of 10 mg protein/mL was done.

In vitro assays

Purity assessment

The purity of the application solution for the relevant in-vitro assays and the retention time of triticonazole, testosterone and 7-Ethoxycoumarin were determined by HPLC analysis. Purity checks of the application solution at beginning and end of the study were done.

On each incubation day, aliquots of the application solution were analysed by LSC to calculate the amounts of applied radioactivity per tube and of total applied Triticonazole per assay (representing 100 % AR). Microsomal incubations were performed at a final target concentration of 10 µM Triticonazole with human, rat, dog or rabbit liver microsomes. For Testosterone and 7-Ethoxycoumarin, the incubations were performed at a final target concentration of 250 µM and 500 µM, respectively.

In vitro metabolism assays

Human, rat, dog and rabbit liver microsomes were preincubated with triticonazole or marker substrates (testosterone and 7-Ethoxycoumarin) in 100 mM potassium phosphate buffer (pH 7.4; 3 mM $MgCl_2$) for 3-5 minutes in a shaking water bath at 37°C. Incubations were initiated by the addition of NADPH-regenerating system and the final volume was 1 mL. The samples with triticonazole were then incubated for 10, 30, 60 and 180 minutes. The samples with testosterone and 7-Ethoxycoumarin were incubated for 20 minutes.

Two negative controls (stability in the incubation buffer system without microsomes and with heat-inactivated microsomes), two positive controls (testosterone and 7-Ethoxycoumarin) and a time zero control incubation were performed for each analysed species.

In the negative controls no metabolism should occur. For the “stability control without microsomes”, the application solution was mixed with incubation buffer and NADPH-regenerating system. For the “stability control with heat-inactivated microsomes”, the application solution was mixed with incubation buffer, heat-inactivated microsomes and NADPH-regenerating system. Microsomes were inactivated by heating at 95°C for 10 minutes. For the “time zero control incubation”, application solution, microsomes, buffer system and NADPH-regenerating system were added to an equal volume of ice-cold solvent in order to stop the reaction immediately.

In the positive control, testosterone, instead of the test substance, was incubated with human liver microsomes to indicate the metabolic activity of the microsomes. 7-Ethoxycoumarin, instead of the test substance, was incubated with rat, dog and rabbit liver microsomes to indicate the metabolic activity of the microsomes. To prove the metabolic activity of the microsomes, at least 80% of the enzymatic rate reported by the supplier should be

observed. In human microsomes, a rate of at least 2584 pmol/min/mg protein should be observed for 6β-hydroxytestosterone formation. In rat, dog and rabbit liver microsomes, rates of at least 1083, 2748 and 2516 pmol/min/mg protein should be observed for 7-hydroxycoumarin formation, respectively.

In each experimental setup, the incubation of the substrates as well as all control assays was performed in triplicates.

Results

Radio-HPLC of **human microsomes samples** incubated with triticonazole allowed the assignment of three peaks accounting for more than 5% AR (see table below)

Apart from the active substance triticonazole (P16), only two peaks accounting for more than 5 % AR, namely P10 and P18, were found in the **human microsome samples** incubated with 10 µM Triticonazole. The mean % AR attributed to P10 increased from 0.3 % at 0 min to 1.9 % after 10 min, to 4.2 % after 30 min, to 6.7 % after 60 min and to 12.8 % after 180 min. The mean % AR attributed to P18 increased from 0.1 % at 0 min to 2.0 % after 10 min, to 4.3 % after 30 min, to 7.5 % after 60 min and to 14.0 % after 180 min.

In **rat microsome samples** the relevant peaks P10 and P18 from the incubation with human liver microsomes were detected in addition to the active substance Triticonazole. P10 accounted for 0.1 % AR at 0 min and increased to 9.2 % after 10 min, to 28.3 % after 30 min, to 38.6 % after 60 min and to 69.5 % after 180 min. P18 which was not detected at 0 min increased to 0.9 % after 10 min, to 3.2 % after 30 min, to 4.1 % after 60 min and to 5.6 % after 180 min. Amount of P10 in rat was much higher compared to human samples, reaching approximately five times the level observed in human microsomes after 180 min of incubation. On the contrary, P18 amount in rat microsomes was lower than the amount observed in human microsome samples.

In **dog microsome samples** the relevant peaks P10 and P18 from the incubation with human liver microsomes were detected in addition to the active substance Triticonazole. P10 accounted for 0.5 % AR at 0 min and increased to 1.6 % after 10 min, to 3.7 % after 30 min, to 7.4 % after 60 min and to 15.1 % after 180 min. P18 which was not detected at 0 min increased to 0.9 % after 10 min, to 2.8 % after 30 min, to 5.3 % after 60 min and to 9.3 % after 180 min. Amounts of P10 were similar in dog and human microsome samples. Levels of P18 were slightly lower in dog compared to human liver microsomes.

In **rabbit microsome samples** the relevant peaks P10 and P18 from the incubation with human liver microsomes were detected in addition to the active substance Triticonazole. P10 accounted for 0.5 % AR at 0 min and increased to 14.7 % after 10 min, to 28.6 % after 30 min and to 35.2 % after 60 min. P10 levels decreased to 30.5 % after 180 min. P18 which was not detected at 0 min increased to 7.6 % after 10 min, to 16.4 % after 30 min and to 20.2 % after 60 min. P18 slightly decreased to 18.0 % after 180 min. Amounts of P10 were approximately three times higher in rabbit compared to human microsome samples after 180 min. Levels of P18 were slightly higher in rabbit compared to human liver microsomes after 180 min of incubation.

Two metabolites P10 and P18 remained identified. No unique human metabolite was estimated in the study.

Table 6.1.3-1: Comparison of relevant metabolites of triticonazole after incubation with human, rat, dog or rabbit liver microsomes

Relevant Peak[1]		**P10**	**P16 (Parent)**	**P18**
		[% AR]		
0 min	Human	*0.3*	**92.9**	*0.1*
	Rat	*0.1*	**94.2**	-
	Dog	*0.5*	**93.1**	-
	Rabbit	*0.5*	**93.1**	-
10 min	Human	*1.9*	**88.4**	*2.0*
	Rat	9.2	**83.8**	*0.9*
	Dog	*1.6*	**88.6**	*0.9*
	Rabbit	14.7	**67.6**	7.6
30 min	Human	*4.2*	**83.4**	*4.3*
	Rat	28.3	**59.7**	*3.2*
	Dog	*3.7*	**81.9**	*2.8*
	Rabbit	28.6	**39.2**	16.4
60 min	Human	6.7	**77.0**	7.5
	Rat	38.6	**47.8**	*4.1*
	Dog	7.4	**71.6**	5.3
	Rabbit	**35.2**	20.5	20.2
180 min	Human	12.8	**61.4**	14.0
	Rat	**69.5**	10.1	5.6
	Dog	15.1	**48.9**	9.3
	Rabbit	**30.5**	*4.4*	18.0

[1] Relevant peak: > 5 % AR (supernatant) on at least one time point in a human sample

Conclusion

After the incubation of human liver microsomes with the active substance, three ^{14}C peaks were detected that represented more than 5 % of applied radioactivity (AR) on at least one time point. One of these signals represented the unchanged active substance triticonazole (m/z value of 318.14). The other peaks (P10 and P18) corresponded to metabolites of triticonazole (peaks at 20.0 min and 28.0 min) with m/z values of 334.13 and 316.12, respectively. Those metabolites and the active substance were also detected in rat, dog and rabbit liver microsome samples.

No unique human metabolites were found and it is concluded that the metabolic pattern of triticonazole in human, rat, dog and rabbit liver microsomes is qualitatively comparable.

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Cross-Species Comparison of Conazole fungicide metabolites using rat and rainbow trout

	(Onchorhynchus mykiss) hepatic microsomes and purified human CYP 3A4
Author(s), year:	Mazur C.S.,Kenneke J.F., 2007
Report/Doc. number::	2008/1103236/ -
Guideline(s):	No
GLP:	No
Deviations:	-
Acceptability:	Yes, as supplementary information

Material and methods

In the present study, in vitro metabolic profiles were determined for thirteen conazole fungicides (among them triticonazole) using rat and rainbow trout (Oncorhynchus mykiss) liver microsomes and purified human CYP 3A4. In the following, only the results obtained with rat microsomes and the human protein are briefly summarized.

Frozen hepatic microsomes from male Sprague-Dawley rats at a protein concentration of 20 mg microsome protein/ml were purchased from In Vitro Technologies (Baltimore, MD). Human recombinant CYP 3A4 was purchased from In Vitro Technologies (Baltimore, MD). The screening assays (20 – 40 µM) were conducted using 10 pmol of recombinant CYP3A4. Triticonazole, as all other investigated conazoles, was obtained from the U.S.EPA National Pesticide Standard Repository (Fort Meade, MD).

Analysis of all conazole samples was performed using LC/MS/MS analytical methods (with the retention times and the mass peak given).

Results

For triticonazole, parent was identified in rat microsomes and human CYP 3A4 at a retention time of 20.72 and 20.15 min (mass peak of 318 m/z). One metabolite, Triti-M1, was identified at a retention time of 11.89 in rat microsomes and 11.60 in human CYP 3A4. The molecular mass of this metabolite is 334 m/z. No structure analysis was performed.

Conclusion

In general, the conclusion of the study was that nearly every metabolite detected in the rat and trout microsomal assay was detected within the human CYP 3A4 assays. Regarding triticonazole, there was no indication for a difference in rat and human Phase I metabolism.

B.6.2. ACUTE TOXICITY

B.6.2.1. Oral

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Acute oral toxicity study in the rat
Author(s), year:	[redacted] 1990
Report/Doc. number::	R013003 / -
Guideline(s):	OECD 401 (1987); US EPA Pesticide Assessment Guidelines, Subdivision F, No 81-1
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and methods

Groups of 5 rats/sex (strain: albino CD Sprague-Dawley; source: [redacted] weighing between 99 and 118 g (5-week old) received a single dose of 0 (vehicle control) and 2000 mg/kg bw triticonazole (batch no. BD 1074; purity 99.3 %, suspended in 0.5 % w/v methylcellulose in distilled water) by oral gavage. After administration all animals were kept under observation for 14 days. Clinical observations were made daily. Body weights were recorded on the day before dosing and on days 1, 8 and 15. At termination all surviving rats were examined at necropsy for macroscopic abnormalities.

Results

Clinical signs and mortality: There were no treatment-related deaths. Signs of reaction to treatment were confined to decreased motor activity and ataxia in one male and all 5 females on day 1 occurring within half an hour after dosing. There was complete recovery in all rats by day 2. No treatment related effects on body weights/weight gain were observed.

Pathology: No gross pathological findings were noted in the rats at the final sacrifice on day 15.

Conclusion

Under the conditions of the study and based on the information given in the study report, oral LD_{50} of triticonazole in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute oral toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.2.2. Dermal

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Acute percutaneous toxicity study in the rat
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013017 / -
Guideline(s):	OECD 402 (1987)
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and methods

Groups of five male and five female rats (strain: albino CD, remote Sprague Dawley origin; source: [redacted] weighing between 216 and 244 g received a topical application of triticonazole technical (batch no. DA646; purity 97.1 %; moistened with 0.2 ml distilled water) at a dose level of 2000 mg/kg bw, which was placed on a gauze patch (5 x 5 cm), applied on the clipped dorsum and occluded with aluminium foil and a waterproof adhesive bandage. After the 24-hour exposure period, the bandage and foil were removed and the treated skin was washed with water. Within the 14 days observation period, clinical signs were recorded twice per day, body weights of the animals were recorded on the day before treatment and on days 1, 8 and 15. At termination, all animals were subjected to a macroscopic post mortem examination.

Results

There were no mortalities or signs of systemic toxicity observed during the 14-day study period. Local signs of irritation (very slight to well defined erythema and eschar formation) at the site of administration were observed in 2 females from day 3 to 10 after treatment. One of these two rats also showed loss of skin flexibility (days 3 – 6) and sloughing (days 7 - 10). The other rat affected demonstrated slight exfoliation. Body weight gains were normal for all animals and there were no abnormal necropsy findings at termination of the study.

Conclusion

Under the conditions of the study and based on the information given in the study report, dermal LD_{50} of triticonazole in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute dermal toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.2.3. Inhalation

B.6.2.3.1. First study

Previous evaluation:	DAR (2003)
DRAR (2016)	Some information on batch used added. No further remarks on the original assessment

Reference:	RPA 400727: Acute inhalation toxicity study in the rat
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013028 / -
Guideline(s):	OECD 403 (1981)
GLP:	Yes
Deviations from OECD 403 (2009):	-MMAD not indicated in the report
Acceptability:	Yes

Material and methods

A group of five male and five female rats (strain: albino CD-Sprague Dawley; source: [redacted].) weighing between 180 and 220 g were exposed for four hours (nose-only) to an atmosphere containing triticonazole (batch no YG2156/1 (in the study report mistakenly stated to be DA 646; purity 954 g/kg,) at a concentration of 1.4 mg/l air (measured gravimetrically and stated to be the maximum practicable concentration). The nominal chamber concentration was 10.68 mg/l. The MMAD was not indicated in the report. However, the mean proportion of particles of inhalable size derived from cascade impactor samples and measured once during each hour of the exposure period was 47.4 % < 6 µm and 29.8 % of particles < 3.5 µm. After exposure, the rats were kept under observation for 15 days. Body weights were recorded before treatment and then daily until the end of the observation period. At termination, all animals were subjected to a detailed macroscopic examination and the organ weights of lungs, liver and kidneys were recorded.

Results

Clinical signs and mortality: There were no deaths as a result of exposure. During exposure, wet fur and excessive salivation was observed in two males and one female. These findings were not present at observations performed 30

minutes after completion of the exposure. No further clinical signs were noted throughout the remainder of the observation period.

Body weight: Slight body weight loss/reduced body weight gain was recorded for 3 males and 3 females on the day following exposure only.

Pathology: At termination of the study, there were no abnormal findings at necropsy. The weights of lungs, liver and kidneys were unaffected by exposure.

Conclusion

Under the conditions of the study and based on the information given in the study report, inhalative LC_{50} in male and female rats was above 1.4 mg/L when exposed to triticonazole for four hours nose-only. Therefore, no classification for acute inhalation toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.2.3.2. Second study

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Triticonazole: Acute inhalation toxicity study in rats
Author(s), year:	[redacted] 1998
Report/Doc. number::	C014044 / -
Guideline(s):	OECD 403 (1981)
GLP:	Yes
Deviations from OECD 403 (2009):	No
Acceptability:	Yes

Material and methods

Groups of 5 males and 5 females rats (strain: albino HSD:Sprague-Dawley; source: [redacted]) weighing between 189 and 347 g were exposed nose-only for 4 hours to an atmosphere containing triticonazole (batch no. OP9750215; purity: 90.76 %). The concentration of test material (fine powder) in the test atmosphere was measured gravimetrically. The nominal chamber concentration was 6.96 mg/l and the achieved concentration was 2.63 mg/l air with an average MMAD of 2.4 µm. The particle size, taken from the breathing zone of the animals, was derived from cascade impactor samples and reported as 50 % of particles $\leq$ 3.2 µm.

Observations (including individual body weight measurements on days 0 before treatment, 7 and 14) were made for 14 days following exposure, after which the rats were sacrificed and examined at necropsy for abnormal tissues or organs.

Results

Clinical signs and mortality: No treatment-related deaths were observed throughout the study. Clinical signs were confined to wet fur, piloerection and decreased activity in both sexes. All animals had recovered by day 3. Body weight gain was unaffected by the administration of the test article.

Pathology: At termination of the study, no significant gross necropsy findings were noted.

Conclusion

Under the conditions of the study and based on the information given in the study report, inhalative LC_{50} in male and female rats was above 2.63 mg/L when exposed to triticonazole for four hours nose-only. Therefore, no classification for acute inhalation toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.2.3.3. Third study

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Triticonazole: Acute inhalation toxicity study in rats
Author(s), year:	[redacted] 1998
Report/Doc. number::	C014043 / -
Guideline(s):	OECD 403 (1981)
GLP:	Yes
Deviations from OECD 403 (2009):	No
Acceptability:	Yes

Material and methods

Five male and five female Sprague-Dawley rats (strain albino HSD; source: [redacted]) weighing between 183 and 289 g were exposed for 4 hours via nose-only inhalation to a dust aerosol (dynamic conditions) of triticonazole (batch no. OP9750215; purity: 90.76 %) at an exposure (gravimetric) concentration of 5.61 mg/l air (nominal concentration 19.7 mg/l). The mass median aerodynamic diameter (MMAD) was reported to be 7 µm with a geometric standard deviation of 9.4 µm after 2¼ hours of exposure and 4.7 µm with a geometric standard deviation of 8.7 after 3¼ hours of exposure. Analyses taken from the breathing zone of the animals after 3¼ hours of exposure showed that 50 % of the particles were 4.7 µm or less. After exposure, the rats were kept under observation for 14 days. Body weights were recorded before treatment and on days 7 and 14. At termination, all animals were subjected to a detailed macroscopic examination.

Results

Clinical signs and mortality: There were no mortalities observed during the 14-day study period. Body weight gain was unaffected by treatment. Clinical signs, already occurring during exposure comprised of the fur coated with urine and/or faeces, decreased activity and piloerection and were seen in all animals. In one female, sensitivity to touch on day 1 after exposure was noted. All animals had completely recovered by day 6.

Pathology: At termination of the study, no macroscopic necropsy findings were noted.

Conclusion

Under the conditions of the study and based on the information given in the study report, inhalative LC_{50} in male and female rats was above 5.61 mg/L when exposed to triticonazole for four hours nose-only. Therefore, no classification for acute inhalation toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.2.4. Skin irritation

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Acute dermal irritation/corrosin test in the rabbit
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013022 / -
Guideline(s):	OECD 404 (1981)
GLP:	Yes
Deviations from OECD (2015):	No deviations in study design: OECD 404 (2015) describes how to integrate and use existing testing and non-testing data for the assessment of the skin irritation and skin corrosion potentials of chemicals and proposes an approach when further testing is needed
Acceptability:	Yes

Material and methods

Triticonazole (batch no. DA646; purity 97.1 %; 0.5 g a.i. moistened with 0.2 ml of distilled water) was applied to a 6 cm x 6 cm area of the clipped dorsal region of 3 male New Zealand White rabbits (source: [redacted] under a semi-occlusive dressing for a 4-hour exposure period. Following the exposure period, the test sites were washed with warm water to remove any remaining test material and dried. The skin sites were examined 1, 24, 48 and 72 hours after treatment and reactions were assessed according to the criteria of Draize (1959).

Results

Clinical signs: Throughout the study period, all treated sites appeared normal; no oedema or erythema were noted. In addition, no signs of systemic toxicity were observed. Triticonazole showed a primary irritation score of 0.00 when applied for 4 hours (semi-occluded) to intact rabbit skin.

Conclusion

Under the conditions of the study and based on the information given in the study report, rabbits exposed dermally to Triticonazole for four hours did not develop any sign of skin irritation. Therefore, no classification for skin irritation according to Regulation (EC) 1272/2008 is necessary.

B.6.2.5. Eye irritation

B.6.2.5.1. First study

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Acute eye irritation test in the rabbit
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013018 / -
Guideline(s):	OECD 405 (1987)
GLP:	Yes
Deviations from OECD (2012):	No deviations in study design: OECD 405 (2012). A preferred sequential testing strategy, which includes the performance of validated in vitro or ex vivo eye corrosion/irritation tests, is included as a Supplement to this Guideline (2012). It is recommended that this testing strategy be followed prior to undertaking in vivo testing.
Acceptability:	Yes

Material and methods

Groups of 6 adult male New Zealand white rabbits (source: [redacted]) received a single application of 100 mg of triticonazole (batch no. DA646, purity 97.1 %) into the conjunctival sac of the right eye.

The eyelids were held together for one second following instillation. The left eyes remained untreated and served as a control. The eyes were examined for ocular reactions 1 hour after the instillation, and then 1, 2, and 3 days thereafter. In addition to standard assessments, pain response, area of cornea affected and conjunctival discharge were also investigated. As a result of seeing a severe response in one of the 3 animals initially used, a further 3 rabbits were tested and ocular reactions were examined and scored. Grading and scoring of the ocular lesions were performed using the numerical scoring system according to the relevant guidelines.

Results

After instillation, a slight initial pain response was observed in three animals, the other three rabbits being unaffected. During the first 24 hours following treatment, slight injection of the conjunctival vessels was observed in all rabbits. In addition, a very slight discharge was evident in four rabbits at the 1 hour examination. No corneal effects or conjunctival chemosis was noted in any rabbit. Very slight iritis was also observed in two rabbits at the one and 24 hour examinations. The eyes of five rabbits were apparently normal within 48 hours after treatment.

In one rabbit, iritis was evident from the 24 hour observation through to 14 days with a crescent shaped lesion in the pupil at 7 and 14 days, which was considered to be iris tissue, adhered to the lens as a result of the iridial congestion. The same animal showed moderate conjunctival redness from 1 hour to day 14 and also pannus (abnormal layer of fibrovascular tissue or granulation tissue) formation at the day 14 examination.

Mean scores (24 – 72 hours) in the 5 animals not showing a severe response were 0.0 (conjunctival chemosis), 0.1 (conjunctival redness), 0.0 (corneal opacity), and 0.1 (iridial lesions), resp. Mean scores for the severly affected rabbit were 0.0 for corneal opacity and conjunctival chemosis, 1.0 for iridial lesions and 1.7 for redness of the conjunctiva. Since the findings were considered to be an irreversible lesion of the eye, the animal was subsequently killed. This unexpected reaction in one animal only was considered likely to be idiosyncratic in nature by the study author.

Conclusion

The results of five animals showed no evidence of distinct eye irritation induced by triticonazole, but the presence of irreversible lesions in one animal is of serious concern. Normally, the results of the study would lead to consideration of classification; however it has to be noted that a second eye irritation study is available and evaluated hereinafter.

B.6.2.5.2. Second study

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Triticonazole: Acute eye irritation test in the rabbit
Author(s), year:	[redacted] 1997
Report/Doc. number::	R012105 / -
Guideline(s):	OECD 405 (1987); Directive EEC 92/69 Method B5 (1992)
GLP:	Yes
Deviations from OECD (2012):	No
Acceptability:	Yes

Material and methods

Approximately 100 mg of triticonazole (batch no. 013951; purity 97.2 %) were placed into the conjunctival sac of the left eye of 6 female New Zealand White rabbits (source: [redacted]). The eyelids were gently held together for one second following application. The collateral eyes remained untreated and served as a control.

The animals were observed twice daily for clinical signs and mortality during a 72-hour period. The local eye irritation was assessed 1 hour after the instillation, and then 24, 48 and 72 hours thereafter. Grading and scoring of the ocular lesions were performed using the numerical scoring system given in the mentioned OECD guidelines.

Results

All six animals showed a slight redness of the conjunctiva one hour after application which was accompanied by slight discharge in one case. However, the redness had disappeared on day 1. According to the classification system, the overall mean scores from the 24, 48, and 72 hour observations for redness, chemosis, corneal opacity and iritis were 0.0, each.

Conclusion

Under the conditions of the study and based on the information given in the study report, triticonazole is not an eye irritant. Therefore, no classification for eye irritation toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.2.6. Skin sensitization

Three skin sensitisation studies were included in the dossier; two of them (Magnuson and Kligman maximisation test and Modified Buehler test) were already evaluated for the first approval of triticonazole. Since in none of the previous two tests positive control was included to prove the sensitivity of the system, the notifier provided a third skin sensitisation study (3 inductions Buehler assay) including positive controls for purpose of renewal. All three tests were negative, although each of them had limitations (stated for each test under the point “deviations”). In the LLNA with the representative formulation (available since 2008 for national registrations and provided for the renewal in 2015) no skin sensitisation has been observed. As a weight of evidence it is concluded that triticonazole does not have skin sensitising properties under the tests conditions.

Remark to Buehler test, 3 inductions: Buehler test (3 inductions) is a validated OECD test (OECD 406). Based on the application regimen (only topical application) it is considered to be a realistic model for exposure in field. However, it is known that several European Member States consider the Buehler 3 induction test as less sensitive and require either the Magnusson and Kligmann test or Modified Buehler test (9 inductions). The notifier provide a publication[1] (not evaluated in the DRAR) where six mild to moderate sensitisers were all in parallel tested in a 3 and a 9-induction Buehler test. The results of the investigation failed to show any significant difference in the ability of Buehler 3-inductions and Buehler 9-inductions test to detect the skin sensitisation potential of these reference substances.

[1] Botham, P et al., 2005: A comparative study of the sensitivity of the 3-induction and 9-induction Buehler test procedure for assessing skin sensitisation potential. Food and Chemical Toxicology 43 (2005) 65-75.

B.6.2.6.1. Magnuson and Kligman Maximisation test

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Delayed contact hypersensitivity study in Guinea pigs
Author(s), year:	[redacted] 1993
Report/Doc. number::	R013081 / -
Guideline(s):	OECD 406 (1981)
GLP:	Yes
Deviations from OECD (1992):	- No positive control included; no information on any positive control data from that time period
Acceptability:	Yes; limited reliability since no information on positive controls

Material and methods

20 guinea pigs (10 males, 10 females; strain: albino Dunkin Hartley; source: [redacted]) were given triticonazole (batch no DA 646; purity 96.4 %) intradermally and topically. Additionally 10 male and 10 female guinea pigs were used as negative control group. The concentrations used for the treatment in this study were based on the results of a preliminary screening study.

In the main study, intradermal induction (two sites per dose, 0.1 ml/injection) was performed with (i) Freunds Complete Adjuvant (FCA), (ii) 5 % w/v triticonazole in propylene glycol and (iii) 5 % w/v triticonazole in a mixture of propylene glycol and FCA by intradermal injections into the dermis on either side of the dorsal median line parallel to the spinal column at the scapular region. Control animals received similar injections except triticonazole was replaced with vehicle. The day of intradermal induction was designated day 1.

On day 7, 10 % (w/v) sodium lauryl sulphate in petrolatum was applied to the clipped dorsal skin of all animals. Topical induction (for 48 hours under occlusive dressing) was carried out on day 8 using a concentration of 50 % triticonazole in propylene glycol (0.6 ml/animal) for test animals and propylene glycol only for control animals.

On day 22, the challenge phase was performed on all guinea pigs (controls and test animals) by applying 50 % triticonazole in propylene glycol dermally for 24 hours under occlusive dressing on the right flank (5 x 5 cm area) while the left flank received the vehicle only. A separate site on the right flank was challenged with 10 % triticonazole in propylene glycol. The dressings were removed 24 hours later and skin reactions were quantified 24 and 48 hours thereafter.

Results

In the preliminary study, signs of skin irritation (slight to moderate erythema) were observed 24 und 48 hours after intradermal application of 0.1, 0.3, 0.5, 1, 3, or 5 % w/v triticonazole in propylene glycol. It was stated in the report that 5 % was the maximum concentration that would pass through a hypodermic needle. Topical application of triticonazole in propylene glycol at 5, 10, 30 or 50 % w/v induced no dermal response 24 and 48 hours after removal of the dressing.

In the main study, mild to moderate skin reactions were observed in most animals (test and controls) following intradermal injection. Also after topical induction, mild skin irritation and exfoliation was evident in almost all animals (test and control animals). Following topical challenge with 50 % test material in propylene glycol, erythema was noted in 3 males and 3 females in the control groups (barely perceptible or slight) and in 2 males and 2 females in the test groups (barely perceptible). In addition, exfoliation was evident in 3 males and 5 females in the

controls compared with 5 males and 8 females in the test group. Following topical challenge with 10 % triticonazole in propylene glycol, a barely perceptible erythema was noted in one female in the test group only. In addition, exfoliation was noted in 3 control females and one male and one female from the test groups. Topical challenge with propylene glycol alone produced no skin reactions.

Conclusion

It was concluded that under the conditions of the Maximization test, triticonazole did not cause delayed contact hypersensitivity in guinea pigs. The results do not indicate classification of the test material for skin sensitization according to Regulation (EC) 1272/2008, although the reliability of the study cannot be completely confirmed (no positive control included or information on positive historical controls available).

B.6.2.6.2. Modified Buehler test

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Delayed contact hypersensitivity study in Guinea pigs
Author(s), year:	[redacted] 1992
Report/Doc. number::	R013063 / -
Guideline(s):	OECD 406 (1981)
GLP:	Yes
Deviations from OECD (1992):	- No positive control included; no information on any positive control data from that time period
Acceptability:	Yes; limited reliability since no information on positive controls

Material and methods

10 male and 10 female Dunkin/Hartley guinea pigs (source: [redacted]) received topical applications of triticonazole (batch no. DA 646, purity 97.0 %) in propylene glycol (50 % w/v, 0.25 ml/patch) on their shaven left flanks (5 x 5 cm) for 6 hours under occlusive dressing. The induction phase consisted of nine applications each, on days 1, 3, 5, 8, 10, 12, 15, 17, and 19 of the study period. The concentration tested was based on a preliminary test with the concentration of 50 % being considered to be “sub-irritant”. 5 male and 5 females serving as controls were treated in the same way, except that the vehicle only was used in the induction phase.

On day 29, all test and control animals were challenged by a 6 hours occluded topical application of 50 % w/v triticonazole in propylene glycol (0.25 ml) to one site, 10 % w/v to the second site and propylene glycol alone to the third on their shaven right flanks. Dermal reactions were assessed on the morning following each induction application, and at 24 and 48 hours after removal of the occlusive dressings.

Results

In the preliminary test, very faint erythema was evident in one (of four) animals 24 and 48 hours after patch removal following testing of 50 % and 10 % w/v triticonazole in propylene glycol. During the induction phase of the main study, very faint erythema at the application site was observed in one male during the first week of induction and at the majority of animals during the second and third week of induction with 50 % w/v triticonazole in propylene glycol. No reaction was observed amongst the control animals. After challenge application with 50 % w/v, no dermal reactions were observed in treated or control animals. Challenge application of 10 % w/v produced very faint

erythema in one female of the test group animals and no reactions amongst controls. Challenge with propylene glycol alone did not cause any dermal reactions.

Conclusion

It was concluded that under the conditions of this study, repeated occluded dermal application of triticonazole did not cause delayed contact hypersensitivity in guinea pigs. The results do not indicate classification of the test material for skin sensitization according to Regulation (EC) 1272/2008, although the reliability of the study cannot be completely confirmed (no positive control included or information on positive historical controls available).

B.6.2.6.3. Buehler test

Previous evaluation:	No
DRAR (2016)	New study; the notifier provided this study as the additional information to confirm non-sensitising potential of triticonazole since only in this study positive (historical) control data were available to show the sensitivity of the system

Reference:	BAS 595 F - BUEHLER test in guinea pigs
Author(s), year:	[redacted] 2006
Report/Doc. number::	2006/1001981
Guideline(s):	OECD 406 (1992)
GLP:	Yes
Deviations:	No
Acceptability:	Limited new information since: - Vehicle used in the test (propylene glycol) is not the same vehicle as the one used for regular check of system sensitivity in the laboratory (Lutrol E 400, polyethylene glycol) - Positive control data only available for Magnuson & Kligmann und Modified Buehler assay, not for Buehler assay with 3 inductions - Since the test is conducted in a different laboratory (BASF) it cannot prove/confirm the sensitivity of the first two test systems where the studies were conducted by Rhone-Poulenc Agrochimie

Materials and methods

Test Material:	BAS 595 F
Description:	not specified
Lot/Batch #:	COD-000601
Purity/content:	90.3%
Stability of test compound:	The stability of the formulation under storage conditions over the study period was guaranteed.
Vehicle	Propylene glycol
Positive control:	Not concurrently tested; α-HCA tested regularly in the laboratory for the sensitivity of the system
Test animals:	
Species:	Guinea pigs
Strain:	Dunkin Hartley, HsdPoc: DH
Sex:	female

Age:	6 - 7 weeks
Weight on day 0:	318 - 394 g
Source:	[redacted]
Acclimation period:	7 days
Diet:	Kliba-Labordiät (Kanninchen & Meerschweinchenhaltung "GLP"), Provimi Kliba SA, Kaiseraugst, Basel, Switzerland, ad libitum
Water:	Tap water ad libitum
Housing:	Groups of 5 animals were housed in stainless steel wire mesh cages with plastic-coated grating (floor area: min. 2000 cm²)

Environmental conditions	
Temperature:	20 - 24°C
Humidity:	30 - 70%
Air changes:	Central air-conditioning system
Photo period:	Alternating 12-hour light and dark cycles, artificial light from 6 am to 6 pm

Clipping of the test animals was conducted at least 15 hours before each test substance application at the appropriate application sites, if necessary, additionally at least 2 hours before evaluation of the skin reactions. A check for any dead or moribund animal was made twice each workday and once on weekends or public holidays.
Evaluations of the skin reactions were performed according to the grading scale of Magnusson and Kligman (The Identification of Contact Allergens by Animal Assay. The Guinea Pig Maximization Test. J. Invest. Dermatol. 52, 268 - 276 (1969)).

Preliminary tests

2 x 2 cm gauze patches (6 layers surgical gauze Ph. Eur. From Lohmann GmbH Co. KG) containing 0.5 mL of the test substance were applied to the skin of the left and right flanks under an occlusive dressing for 6 hours. The dressing consisted of rubberized linen patches (4 x 4 cm, Russka), patches of Idealbinde (5 x 5 cm from Pfälzische Verbandstoff-Fabrik) and Fixomul®Stretch adhesive fleece (Beiersdorf AG). The test was performed on 3 animals per test concentration.
The evaluation of the skin reactions was performed 1, 24 and 48 h after removal of the patch. In accordance with the test guideline a slightly irritating concentration should be used in the main test for induction, whereas the maximum non-irritant concentration should be applied for challenge.
In the pre-test, concentrations of 25 and 50% test substance preparations in propylene glycol were analysed. Since no skin irritation was noticed 24 and 48 hours after removal of the patch, both test item concentrations were assessed as suitable for the main test.

Main study

Induction

Inductions were performed on days 0, 7 and 14 on the same application area using 50% test item suspension in

propylene glycol.

2 x 2 cm gauze patches (6 layers surgical gauze Ph. Eur. Lohmann GmbH & Co. KG) containing the 0.5 mL of the test substance were applied for 6 hours to the skin of the flank under an occlusive dressing. The dressing consisted of rubberized linen patches (4x4 cm, Ruska), patches of Idealbinde (5 x 5 cm from Pfälzische Verbandstoff-Fabrik) and Fixomul®stretch adhesive fleece (Beiersdorf AG).

The evaluation of the skin reactions was performed 24 hours after removal of the patch.

The control group animals were treated with the vehicle propylene glycol in the same way as the test group animals.

Challenge

The challenge was carried out 14 days after the last induction using 0, 25 and 50% test item preparations in propylene glycol.

For this 0.5 mL of the test substance preparation was applied under occlusive conditions to the intact flank (right flank anterior and posterior: test substance formulations and left flank posterior: vehicle propylene glycol) of the animals for 6 hours. The patches were prepared as described above for the epidermal pre-test. Skin reactions were determined 24 and 48 hours after removal of the patches.

Positive controls

A positive control (reliability check) with a known sensitiser was not performed in this study. However, separate positive control studies were performed twice a year in the laboratory. The positive control with α-hexylcinnamaldehyde (HCA) technical 85% showed that the test system was able to detect sensitizing compounds under the laboratory conditions chosen (in Maximisation Test and Modified Buehler Test).

Evaluation of results

The number of animals with skin findings at 24 and/or 48 hours after removal of the patch was taken into account for the determination of the sensitisation rate. The evaluation "sensitising" results if at least 15% of the test animals exhibit skin reactions (grade ≥ 1) in this test.

Analysis of treatment solutions

A concentration control analysis of both test substance preparations (50% and 25%) used for the challenge application was performed. The stability and homogeneity of the test substance in the vehicle was determined indirectly by the concentration control analysis. The homogeneity of the test substance preparation during application was provided by stirring with a spatula (50%) or with a magnetic stirrer (25%).

Statistics

Not performed in this study

Results

Pre-test

After application of 50% and 25% test substance preparations in propylene glycol no skin irritation was noticed 24 and 48 hours after removal of the patch.

Table 6.2.6.3-1: Skin irritation scores – preliminary test

Animal No.	Weight [g]	Findings 1 hour after removal of the patch		Findings 24 hours after removal of the patch		Findings 24 hours after removal of the patch	
Application site		right flank	left flank	right flank	left flank	right flank	left flank
Concentration in propylene glycol		50%	25%	50%	25%	50%	25%
4	748	1	2	0	0	0	0
5	711	1	1	0	0	0	0
6	788	1	2	0	0	0	0

Based on the data from the pre-test, a test concentration of 50% BAS 595 F was used for the epidermal inductions and both concentrations of 25 and 50% BAS 595 F were used for the challenge.

Induction

After the first induction no skin irritation was observed in all animals of the control and test group. The second and third induction caused discrete or patchy to moderate and confluent erythema in several animals of the control and test group, each.

Table 6.2.6.3-2: Skin reactions in animals treated with 50% BAS 595 F or vehicle

Description	Findings 24 hours after beginning of application					
	Propylene glycol			50% BAS 595 F		
	1st	2nd	3rd	1st	2nd	3rd
Grade 0	10/10	8/10	7/10	20/20	14/20	15/20
Grade 1	-	2/10	2/10	-	5/20	5/20
Grade 2	-	-	1/10	-	1/20	-

x/y: number of animals with findings / number of animals tested

Challenge

After the challenge discrete or patchy or moderate and confluent erythema was noticed in the application sites of the 50% test substance preparation of one animal of the control group and two animals of the test group. No skin reactions were noticed in all application sites of the 25% test substance preparation and the vehicle propylene glycol. Thus, 10% of guinea pigs revealed skin reaction after challenge which is below the value of 15% that would trigger a classification.

Table 6.2.6.3-3: Challenge skin reaction scores 24 and 48 hours in the control group

Skin findings	Propylene glycol		25% BAS 595 F		50% BAS 595 F	
	24 h	48 h	24 h	48 h	24 h	48 h
Grade 0	10/10	10/10	10/10	10/10	9/10	9/10
Grade 1	-	-	-	-	-	-
Grade 2	-	-	-	-	1/10	1/10

x/y: number of animals with findings / number of animals tested

Table 6.2.6.3-4: Challenge skin reaction scores 24 and 48 hours in the test group

Skin findings	Propylene glycol		25% BAS 595 F		50% BAS 595 F	
	24 h	48 h	24 h	48 h	24 h	48 h
Grade 0	20/20	20/20	20/20	20/20	18/20	18/20
Grade 1	-	-	-	-	1/20	2/20

Skin findings	Propylene glycol		25% BAS 595 F		50% BAS 595 F	
	24 h	48 h	24 h	48 h	24 h	48 h
Grade 2	-	-	-	-	1/20	-

x/y: number of animals with findings / number of animals tested

No clinical signs of systemic toxicity or mortality were observed.

The expected body weight gain was generally observed in the course of the study.

Results with HCA are summarized in Table 6.2.6.3-5. The positive control HCA technical 85% showed that the guinea pig strain used was sufficiently sensitive for detection of skin sensitizing compounds in the Buehler test. However, positive control data are only available for Maximisation test and Modified Buehler Test.

Table 6.2.6.3-5: Results of positive control substance test (HCA) in the Modified Buehler test

	Challenge					
	Positive control 15% HCA in Lutrol® E 400			Vehicle control: Lutrol® E 400		
	24 h	48 h	Total	24 h	48 h	Total
Control group*	0/10	0/10	0/10	0/10	0/10	0/10
Test group	19/20	16/20	19/20	0/20	0/20	0/20

* Induction (epicutaneous): HCA 20% in Lutrol® E 400

x/y: number of positive reactions/number of animals tested (reading at 24 h and/or 48 h after removal of the patch)

Conclusion

It was concluded that under the conditions of this study, repeated occluded dermal application of triticonazole did not cause delayed contact hypersensitivity in guinea pigs in the 3-inductions Buehler assay. The results do not indicate classification of the test material for skin sensitization according to Regulation (EC) 1272/2008, although the sensitivity of the 3-inductions Buehler assay is limited.

B.6.2.7. Phototoxicity

According to new data requirements for active substances (Regulation (EU) No 283/2012) an *in vitro* phototoxicity study is required where the active substance absorbs electromagnetic radiation in the range 290- 700 nm and is liable to reach the eyes or light-exposed areas of skin, either by direct contact or through systemic distribution. If the Ultraviolet/visible molar extinction/absorption coefficient of the active substance is less than 10 L x mol^{-1} x cm^{-1}, no toxicity testing is required.

Triticonazole absorbs electromagnetic radiation mostly below 290 nm and at 313 nm the extinction coefficient is zero. However, at 290 nm the extinction coefficient of triticonazole is still > 1000 L x mol^{-1} x cm^{-1}, which is above the trigger of 10 L x mol^{-1} x cm^{-1}. Therefore, testing triticonazole for phototoxicity is justified, according to legal requirements.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	In vitro 3T3 NRU phototoxicity test
Author(s), year:	Cetto V.,Landsiedel R., 2015

Doc. number:	2013/1089154
Guideline(s):	OECD 432 (2004)
GLP:	Yes
Deviations from OECD 432 (2004):	No
Acceptability:	Yes

Material and methods

Test Material	Triticonazole (BAS 595 F)
Description:	Solid; white
Lot/Batch #:	COD-001440
Purity:	91.3% (tolerance ± 1%)
Stability of test compound:	The stability of the test substance under storage conditions over the test period was guaranteed until 11 Feb 2014 .The homogeneity of the test substance was ensured by mixing prior to preparation of test substance solutions.
Solvent used:	DMSO

Control Materials:

Vehicle control:	The vehicle control cultures with and without irradiation only contained the vehicle used for the test substance/positive control at the same concentration and volume as used for the test substance and the positive control.
Solvent/final concentration:	DMSO 1% (v/v)
Positive control compounds:	Chlorpromazine (CPU) was dissolved in DMSO

A complete 96-well plate containing 8 concentrations was performed in parallel to demonstrate sensitivity of the test method.

Without irradiation	1.9-3.8-7.5-15-30-60-90-180 µg/mL
With irradiation	0.03-0.05-0.1-0.2-0.4-0.8-1.6-3.2 µg/mL

Test organisms:	The BaIb/c 3T3, clone A31, cell line was isolated from the muscle tissue of mouse embryo. This fibroblast cell line has a high proliferation rate (doubling time 16 - 20 hours) and a high plating efficiency (>70%)) of untreated cells both necessary for the appropriate performance of the study. The Balb/c 3T3 cell line which was used in this experiment was obtained from the “European Collection of Cell Cultures” Salisbury, Wiltshire SP4 OJG, UK (date 09 Aug 2006) and is stored at -196°C (liquid nitrogen).

Culture media and reagents

Culture medium:	Dulbecco's Modified Eagle's Medium (DMEM) supplemented with - 10% (v/v) newborn calf serum (NCBS) - 4 mM L-glutamine - 100 IU penicillin

- 100 µg/mL streptomycin

Neutral Red solution:
- 0.4 g Neutral Red powder (NR; Sigma N4638)
- 100 mL deionized water

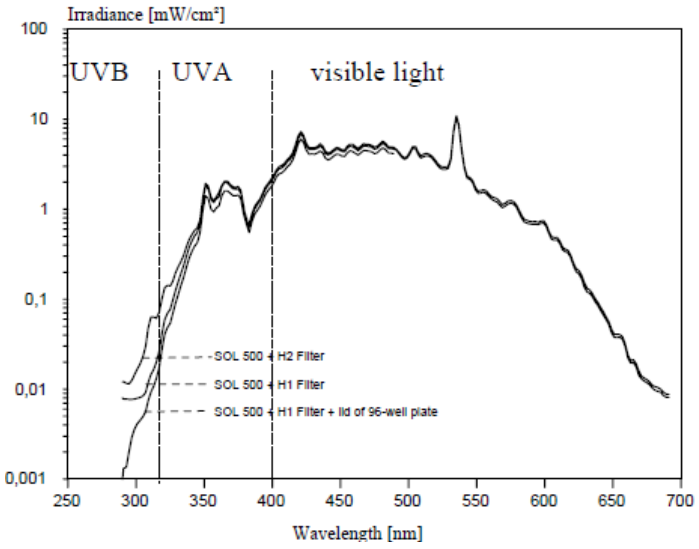
Neutral Red medium:
- 1 mL Neutral Red solution
- 79 mL culture medium (DMEM incl. supplements)

Incubated overnight at 37° C with 5% CO_2 and filtered with a 0.22 µm filter prior to use.

Other solutions and reagents:
- phosphate buffered saline (PBS) without Ca/Mg
- trypsin/EDTA solution (0.05%; 0.02%)
- Neutral Red desorb solution (1 mL acetic acid, 50 mL ethanol, 49 mL deionized water)

Irradiation source: The Sol 500 solar simulator (Dr. Hönle AG, 82166 Gräfelfing, Germany) used with filter H1. This irradiation source is recommended in the Annex 3 of OECD TG 432.

Figure 6.2.7-1: Spectral power distribution of a filtered solar simulator



As shown in Figure 6.2.7-1 the chosen solar simulator mainly emits light in the UVA and visible range, (which is usually associated with high direct cytotoxicity) and to a lesser extent in the UVB range (which is associated with high cytotoxicity and regarded to be of less relevance in the context of substance induced phototoxicity (OECD TG 432). However, the experimental setting was shown to be sufficient to detect phototoxic effects also fo chemicals typically absorbing in the UVB range, e.g. the concurrent positive control chlorpromazine (absorption peak at 309 nm) or Amiodarone (absorption peak 242 nm and 300 nm) (shoulder). The exposure rates were determined with UV-meter RM-21 (Dr. Gröbel GmbH, 76275 Ettlingen, Germany).

Test concentrations

Pretest:	Up to 1100 µg/mL with and without irradiation.
NRU test conditions:	An appropriate amount of test article substance was taken up in the vehicle, shaken thoroughly and diluted in accordance with the planned doses under light protection conditions immediately before administration.

The experiment was performed in 96 well plates in one experiments (6 replicates per concentration with and without irradiation; two plates per substance (test substance or positive control) were prepared.) The test substance concentrations were:

Without:	4.6; 10.00; 21.5; 46.4; 100.0, 215.4, 464.2, 1100.0 µg/mL
With:	4.6; 10.00; 21.5; 46.4; 100.0, 215.4, 464.2, 1100.0 µg/mL

Two 96 well-plates per substance (test substance or positive control) were used for cultivation of cells (1.5 x 10^5 cells/well). After an attachment period of about 24 hours the cells were washed once with 100 µL PBS and subsequently treated with the respective substance (8 concentrations each with 6 replicates of the test substance or the positive control) and the vehicle control in parallel for 1 hour in the dark (5% (v/v) CO_2, ≥90% humidity; 37° C). Then, one microplate per substance was irradiated for 50 minutes with UV/VIS (UV intensity underneath the lid 1.5 - 2.1 mW/cm^2 = 5 J/cm²) whereas the respective reference plate was kept in the dark for the same period. After test substance removal and washing step (100 µL PBS) the cells were incubated in culture medium overnight. The medium was removed after 24 hours, the cells washed again, 100 µL medium containing 50 µg/mL Neutral red was added and the plates were incubated for another 3 hours. Each step was performed under light protected conditions in the lab to prevent uncontrolled photo activation. Afterwards, the cells were washed and the dye was extracted by Neutral Red desorb solution. Cytotoxicity was determined by measuring the Neutral Red Uptake using a microplate reader (Perkin Elmer, Waltham, Massachusetts, US; Wallac 1420 multilabel counter) equipped with a 550 nm filter to read the absorption of the extracted dye. The absorption shows a linear relationship with the number of surviving cells.

For the assessment of the phototoxic potential of a compound two prediction models are currently available:

The Photo-Irritancy-Factor Prediction model for substances which allow the comparison of two equi-effective concentrations (EC_{50}) in the concurrently performed experiments in the presence and absence of light. This model includes the special case of absence of cytotoxicity in the presence and absence of light for substances obviously showing no phototoxic potential (see below).

The Mean Photo Effect prediction model which is used if no equi-effective concentrations (EC_{50}) are obtained in the absence and presence of UV light. This special case does not apply to this study.

Cytotoxicity

The mean absorbance values obtained for each test group of every plate were used to calculate the percentage of cell viability relative to the respective vehicle control, which is arbitrarily set at 100 %.

$$Cytotoxicity\,[\%] = \frac{\text{Absorbance}_{mean}\ \text{of the test group}}{\text{Absorbance}_{mean}\ \text{of the vehicle control}} \times 100$$

In case of cytotoxicity, an EC_{50} value (Inhibition concentration 50% relative to the respective vehicle control) was calculated by a linear interpolation method (linear dose-response curve).

Photo-Irritancy-Factor

If no cytotoxicity occurs to determine an EC_{50} value in the concurrently performed experiments in the absence and presence of UV light up to the highest applied test concentration it has to be considered that the test substance has no phototoxic potential.

In this case, a formal PIF = *1 is used to characterize the result:

$$PIF = *1 = \frac{C\max(-UVA)}{C\max(+UVA)}$$ resulting in the following classification rule:

PIF = *1	no phototoxic potential predicted

Other parameters

pH:

The pH was measured at least for the two top doses and for the vehicle controls with and without irradiation.

Osmolarity:

Osmolarity was measured at least for the two top doses and for the vehicle controls with and without irradiation.

Solubility:

Test substance precipitation was checked immediately after treatment and at the end of treatment.

Cell morphology:

Test cultures of all test groups were examined microscopically before staining with NRU, which allows conclusions to be drawn about attachment of the cells.

Statistics

No special statistical tests were performed.

Mean absorbance values and standard deviations were calculated from the single values using calculation software (e.g. MS Excel). The calculations were made using the unedited values. For the report the values were rounded, therefore there may be deviations in the given relative values. If technical errors occurred in single wells (outlier) at least 4 single values per test group were sufficient for calculating reliable mean values. Outliers are defined as values that have half or double the value of the respective mean.

Acceptance criteria

The assay has to be considered valid if the following criteria are met:

- The vehicle control needs to fulfill the following criteria:
  - The mean OD540 value (with and without UV/VIS irradiation) should be ≥ 0.4.
  - Cell viability after irradiation should be at least 80% of the concurrent non-irradiated vehicle control.
- The positive control chlorpromazine needs to fulfill the following criteria:
  - the EC_{50} value should be in the ranges:

    With irradiation (+UV/VIS): 0.1 - 2.0 µg/mL

    Without irradiation (-UV/VIS): 7.0 - 90.0 µg/mL
  - and the PIF ≥ 6.

Results

Osmolarity and pH values were not influenced by test substance treatment. In this study, in the absence and the presence of UV/VIS irradiation precipitation in culture medium was observed at test substance concentrations of 100 µg/mL and above. In addition, no changes in cell morphology were observed at the end of exposure period with and without irradiation.

After treatment with the test substance, no cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed in the main experiment in the absence and the presence of UV/VIS irradiation. Therefore, no EC_{50} values could be calculated.

Based on these observations a formal PIF = *1 has to be used to characterize the result.

Table 6.2.7-1: Mean relative cytotoxicity of BAS 595 F with and without UV/VIS irradiation in Balb 3T3 cells

Test group	UV/VIS irradiation*	Precipitation**	Mean $OD_{corr.}$ ***	Cytotoxicity [% of control]
Vehicle control (1% DMSO)	-	-	0.405	100.0
BAS 595 F				
4.6 µg/mL	-	-	0.345	85.1
10.0 µg/mL	-	-	0.350	86.3
21.5 µg/mL	-	-	0.361	89.0
46.4 µg/mL	-	-	0.369	91.1
100.0 µg/mL	-	+	0.412	101.7
215.4 µg/mL	-	+	0.394	97.2
464.2 µg/mL	-	+	0.428	105.7
1100.0 µg/mL	-	+	0.324	79.9
Vehicle control (1% DMSO)	+	-	0.399	100.0
BAS 595 F				
4.6 µg/mL	+	-	0.375	94.0
10.0 µg/mL	+	-	0.385	96.5
21.5 µg/mL	+	-	0.388	97.3
46.4 µg/mL	+	-	0.388	97.2
100.0 µg/mL	+	+	0.395	99.0
215.4 µg/mL	+	+	0.428	107.3
464.2 µg/mL	+	+	0.436	109.2
1100.0 µg/mL	+	+	0.436	109.3

*: Irradiation with Sol 500 solar simulator for 50 minutes (approx. 5 J/cm²)
**: Precipitation in PBS at the end of exposure period
***: Mean OD corrected: mean absorbance (test group) minus mean absorbance (blank)

After treatment with the positive control chlorpromazine clear cytotoxic effects indicated by Neutral Red absorbance values of below 50% of control were observed in the absence and the presence of UV/VIS irradiation at least in the highest applied concentrations.

Without UV/VIS irradiation, there was a decrease in the cell number from 30.0 µg/mL (EC_{50}: 24.3 µg/mL) onward. The cell densities were distinctly reduced. With UV/VIS irradiation, there was a decrease in the cell number at 0.8 µg/mL (EC_{50}: 0.7 µg/mL) and above. Based on the EC_{50} values a PIF of 36.7 (phototoxic potential) was obtained (see Table 6.2.7-2).

Table 6.2.7-2: Mean relative cytotoxicity of Chlorpromazine with and without UV/VIS irradiation in Balb/c 3T3 cells

Test group	UV/VIS irradiation	Mean OD *	Mean $OD_{corr.}$ **	Relative Cytotoxicity [% of control]	Standard deviation [%]
Blank	-	0.035	-	-	-
Vehicle control 1	-	0.411	0.375	-	5.8
Vehicle control 2	-	0.407	0.372	-	4.1
Vehicle control mean (1% DMSO)	-	0.409	0.374	100.0	4.9
Chlorpromazine					
1.9 µg/mL	-	0.446	0.411	109.8	5.4
3.8 µg/mL	-	0.457	0.422	112.8	3.4
7.5 µg/mL	-	0.468	0.433	115.9	5.7
15.0 µg/mL	-	0.413	0.378	101.2	4.2
30.0 µg/mL	-	0.103	0.068	18.3	4.0
60.0 µg/mL	-	0.035	0.000	-0.1	0.1
90.0 µg/mL	-	0.036	0.001	0.4	0.8
180.0 µg/mL	-	0.035	0.000	0.1	0.1
Blank	+	0.036	-	-	-
Vehicle control 1	+	0.343	0.307	-	4.5
Vehicle control 2	+	0.392	0.356	-	3.4
Vehicle control mean (1% DMSO)	+	0.367	0.332	100.0	8.6
Chlorpromazine					
0.03 µg/mL	+	0.348	0.313	94.2	4.2
0.05 µg/mL	+	0.348	0.313	94.2	2.9
0.10 µg/mL	+	0.347	0.312	94.0	3.0
0.20 µg/mL	+	0.339	0.304	91.6	4.0
0.40 µg/mL	+	0.308	0.273	82.2	2.6
0.80 µg/mL	+	0.144	0.109	32.7	6.8
1.60 µg/mL	+	0.037	0.001	0.3	0.6
3.20 µg/mL	+	0.039	0.004	1.2	0.3

*: Mean absorbance at 540 nm of 6 wells, in general
**: Mean absorbance (test group) minus mean absorbance (blank)

Conclusion

Triticonazole did not have any phototoxic effects on BALB/c 3T3 cells when being irradiated with wavelengths mainly in UVA spectrum.

The RMS agrees that triticonazole was not phototoxic to BALB/c 3T3 cells under conditions of the study.

However, the suitability of the validated electromagnetic spectrum in case of triticonazole is questioned. Since triticonazole absorbs electromagnetic radiation at 290 nm wavelength (UVB) above the trigger of 10 L x mol^{-1} x cm^{-1} (and also above 1000 L x mol^{-1} x cm^{-1}) but shows no absorption already at 310 nm, the results of the study do not allow a definite conclusion on the suitability of the test in the spectral range relevant for triticonazole (mainly below 310 nm) even if the solar simulator with H1 filter allows UVB to some extent. On the other hand, the positive control chlorpromazine has the absorption peak near to 310 nm (309 nm) and showed clear positive result in the test. Additionally it should be noted that the OECD GD 432 (2004) states that "*The choice of an appropriate light source and filters is a crucial factor in phototoxicity testing. Light of the UVA and visible regions is usually associated with phototoxic reactions whereas generally UVB is of less relevance but is highly cytotoxic; the cytotoxicity increases 1000-fold as the wavelength goes from 313 to 280 nm.*" Triticonazole absorbs light only below 313 nm, in the highly cytotoxic area.

In the absence of any other validated method (reconstructed human skin model is not yet validated for phototoxicity), better suitable for testing photoxicity of substances being UVB absorbers (like triticonazole), RMS concluded that the notifier fulfilled his duty to address phototoxicity of triticonazole.

B.6.3. SHORT-TERM TOXICITY

B.6.3.1. Oral 28-day study

B.6.3.1.1. Rat

Previous evaluation: DRAR (2016)	DAR (2003) Some additional information added in the results; conclusion so far changed as a NOAEL for males and females has been derived (the conclusion 2003 stated that no NOAEL can be set).

Reference:	RPA 400727: Preliminary toxicity study by dietary administration to F-344 rats for 4 weeks
Author(s), year:	[redacted], 1991
Report/Doc. number::	R013012 / -
Guideline(s):	OECD 407 (1981)
GLP:	Yes
Deviations from OECD 407 (2008):	- Not all organs from OECD 407 (2008) investigated
Acceptability:	Yes; range finding test

Material and methods

Groups of 5 male and 5 female rats (strain: F-344; source: [redacted].) received diet containing 0, 500, 1500, 5000, 15000 or 50000 ppm triticonazole (batch no. YG 2156/1; purity 98.9 %) equivalent to 0, 50.1, 152.3, 513.2, 1494 and 4802 mg/kg bw per day in males and 0, 52.4, 151.3, 489.4, 1476 and 4945 mg/kg bw per day in females, resp. for 4 weeks. Diets were prepared weekly; concentrations of triticonazole in the diet, and stability and homogeneity of the test substance were confirmed by analysis.

Animals were observed twice daily for clinical signs or reaction to treatment. Food consumption was measured weekly, body weights were assessed twice weekly. Ophthalmoscopic examinations were performed prior to treatment and on day 25 of treatment. On day 27 of treatment, blood samples were taken from all animals for haematological (haematocrit, haemoglobin, RBC, Reticulocyte count, WBC differential, MCHC, MCV, MCH, platelets count, prothrombin time) and clinical chemistry (AP, ALT, AST, Creatine phosphokinase, urea, creatinine, glucose, total bilirubin, total cholesterol, total protein, sodium, potassium, chloride, calcium, inorganic phosphorus) investigations. Urinalysis (appearance, volume, pH, specific gravity, protein, glucose, ketones, bilirubin, urobilin, nitrite, blood, sediment) was performed in urine samples collected on day 24 to 25 of treatment from all animals.

At necropsy, each animal was subjected to a detailed gross pathological examination and the weights of selected organs (adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroids, uterus) were recorded for each animal. Histopathological examinations were performed on adrenals, brain, heart, kidneys, liver, ovaries, prostate, stomach, testes, thyroid, urinary bladder and uterus from control and high dosed rats (50000 ppm). In addition, the liver and uterus from rats of all other dose groups were also examined.

Results

General observations: There were no deaths during the study period related to treatment. Clinical signs (marked hair loss on the head and dorsal surface, piloerection, hunched posture) were noted throughout the entire treatment period for all animals of both sexes receiving 50000 ppm.

Body weight gains of males receiving 5000 ppm and above and females receiving 50000 ppm were significantly lower than those of the respective control values from day 4 to 28. For females receiving 5000 and 15000 ppm, body weight gains were also lower during the first four days of treatment, but unaffected thereafter. Animals of both sexes receiving 1500 ppm showed only slightly lower weight gain and no dose response was observed at this dose (table 6.3.1.1-1). Mean body weights and weight gain of animals receiving 500 ppm were similar to that of the controls over the treatment period. Terminal body weight was in males recorded to be lower (above 10% comparing to control) from 5000 ppm while in females this was the case first for the highest dose group.

Table 6.3.1.1-1: 4 weeks dietary repeat dose study in rats group mean body weight and body weight gain (g)

	Dose group level (ppm)											
	Males						**Females**					
	0	**500**	**1500**	**5000**	**15000**	**50000**	**0**	**500**	**1500**	**5000**	**15000**	**50000**
body weight on day 0 (g)	100	100	98	99	99	100	89	88	90	90	90	89
terminal body weight (g) (% control)	225	217 (96.4)	219 (97.3)	195 (86.7)	181 (80.4)	156 (69.3)	144	142 (98.6)	138 (95.8)	139 (96.5)	136 (94.4)	115 (79.9)
weight gain (g) day 0 – 4 (% control)	19	16* (84.2)	19 (0)	14*** (73.7)	4*** (21.1)	-9*** (-47.4)	12	11 (91.7)	10 (83.3)	7*** (58.3)	2*** (16.7)	-8*** (-66.7)
weight gain (g) day 4 – 28 (% control)	106	100 (94.3)	102 (96.2)	82*** (77.4)	79*** (74.5)	64*** (60.4)	42	43 (102.4)	37 (88.1)	42 (0)	45 (107.1)	34** (80.9)

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's t-test)

There was also a dose-related decrease in food consumption in animals of both sexes treated with 5000 (90 – 93 %), 15000 (84 – 88 %) and 50000 ppm (72 – 76 %) compared with the respective control values.

Ophthalmoscopic examinations revealed no evidence of treatment-related changes at any dose group.

Evaluation of haematological parameters after 3 weeks of treatment revealed dose-related effects in animals receiving 5000 ppm and higher, consisting of lower haematocrit values, lower haemoglobin concentration, lower red blood cells, lower total leucocyte counts (both neutrophils and lymphocytes) and lower platelet counts (table 6.3.1.1-2).

Table 6.3.1.1-2: 4 weeks dietary repeat dose study in rats; relevant haematological findings (group mean values) after 3 weeks of treatment

Parameter	Dose group level (ppm)											
	Males						Females					
	0	500	1500	5000	15000	50000	0	500	1500	5000	15000	50000
Haematocrit (%)	46	46	46	46	45[a]	44[c]	44	45	46[a]	44	43	42[a]
Haemoglobin (g%)	15.9	15.8	15.8	15.5[a]	15.5[a]	15.1[c]	15.5	15.8	16.1[b]	15.4	15.2	14.4[c c]
RBC (mil/cmm)	8.40	8.48	8.49	8.34	8.31	8.27	8.22	8.45	8.60[a]	8.20	8.12	8.07
White blood cells (1000/cm^3)	8.1	9.0	7.5	7.7	7.3	5.1[c]	9.3	8.0	8.7	8.9	6.1[c]	7.3[a]
Platelet count (1000/cm^3)	813	789	785	773[a]	766[a]	642[c]	747	759	723	761	663[c]	555[c]

a ($p \leq 0.05$); b ($p \leq 0.01$); c ($p \leq 0.001$) significantly different from controls (Student's t-test)

Concerning clinical chemistry, treatment-related findings were limited to a dose-related hypoglycaemia among rats receiving ≥5000 ppm (table 6.3.1.1-3). Higher ALT activities in both sexes were noted at 50000 ppm; higher AST activities in males at 5000 and 50000 ppm; higher AP activities in females at 15000 and 50000 ppm, low creatinine levels among males at 5000 ppm or more; higher cholesterol concentrations in males at 5000, 15000 and 50000 ppm and in females receiving 1500, 5000 and 15000 ppm. However, it was stated in the study report that the majority of the individual values in all of these respects were within the range of expectancy for young F-344 rats at this laboratory.

Table 6.3.1.1-3: 4 weeks dietary repeat dose study in rats; relevant clinical chemistry findings (group mean values) after 3 weeks of treatment

Parameter	Dose group level (ppm)											
	Males						Females					
	0	500	1500	5000	15000	50000	0	500	1500	5000	15000	50000
ALT (iU/l)	33	30	28[a]	21[c]	24[c]	40	30	28	25[a]	28	28	35[a]
AST (iU/l)	77	80	85	94[b]	84	97[c]	88	80	79	82	83	95
AP (iU/l)	212	209	240	212	248	230	141	156	144	155	188[c]	196[c]
Glucose (mg%)	149	151	158	133[b]	120[c]	93[c]	129	126	118	115	102[b]	77[c]
Cholesterol (mg%)	45	46	48	58[c]	59[c]	50[a]	66	71	83[b]	93[c]	90[c]	68

a ($p \leq 0.05$); b ($p \leq 0.01$); c ($p \leq 0.001$) significantly different from controls (Student's t-test)

Urinalysis: When compared with control animals (pH 6.2), significant lower urinary pH values were noted in males treated at 15000 (pH 6.0; $p<0.05$) and 50000 (pH 5.8; $p<0.001$) ppm. In addition, slightly lower urinary protein content was noted in males treated with 5000 ppm and above than in controls. A marked ketonuria was evident in animals of both sexes of the top dose.

Organ weight analysis after 4 weeks of treatment revealed dose-related increase in absolute and relative liver weights among females receiving 5000 ppm and above, showing that the observed findings in liver weight in females were indeed effects and not a consequence of the decrease in body weight gain. In males, the relative liver weight was increased from 15000 ppm, but no dose-response and no effects were observed for absolute liver weight. This is in line with body weight (and gain) results where it was obvious that males were affected at lower doses than females.

The absolute prostate weights of males receiving 5000 ppm or more were lower than those of their respective controls; however the difference was significant for relative weight only in the highest dose tested. Lower absolute uterus weights were also noted among all treated females when compared with controls; the relative uterus weight was statistically significantly decreased only in animals of the highest dose. In addition, absolute testes weights were significantly lower in males which received 50000 ppm. The results are presented in table 6.3.1.1-4.

Table 6.3.1.1-4: 4 weeks dietary repeat dose study in rats; organ weight changes (mean group values)

	Dose group level (ppm)											
	Males						Females					
	0	**500**	**1500**	**5000**	**15000**	**50000**	**0**	**500**	**1500**	**5000**	**15000**	**50000**
Liver												
absolute weight (g)	10.9	9.5[a]	10.2	9.6[a]	11.2	10.6	5.7	5.6	5.7	7.6[b]	9.9[b]	9.5[b]
Relative weight (%)	5.02	4.63	4.84	5.1	6.27[b]	6.65[b]	4.07	4.09	4.29	5.63 [b]	7.43[b]	8.46[b]
(% control)		(92.2)	(96.4)	(101.6)	(124.9)	(132.5)		(100.5)	(105.4)	(138.3)	(182.6)	(207.8)
Uterus												
absolute weight (g)	-	-	-	-	-	-	0.23	0.18	0.16[a]	0.16[a]	0.16[a]	0.07[b]
Relative weight (%)	-	-	-	-	-	-	0.162	0.131	0.122	0.114[a]	0.121	0.07[b]
Prostate												
absolute weight (g)	0.131	0.110	0.115	0.08[a]	0.07[a]	0.02[b]	-	-	-	-	-	-
Relative weight (%)	0.06	0.054	0.054	0.012	0.04	0.02[b]	-	-	-	-	-	-
Testes (l&r)												
absolute weight (g)	2.54	2.56	2.58	2.53	2.49	2.07[b]	-	-	-	-	-	-

a ($p \leq 0.05$); b ($p \leq 0.01$); c ($p \leq 0.001$) significantly different from controls (Student's t-test)

Macroscopic examination of animals killed after 4 weeks of treatment revealed abnormal (pale) caecal contents among animals which received 50000 ppm.

Histopathological examinations identified the liver and the uterus as the main target organs. In the liver, atypical fatty centriacinar and microvesicular panacinar vacuolation was seen in males treated with 50000 ppm and in females which received 15000 and 50000 ppm. Necrosis of individual hepatocytes was noted in males at 15000 and 50000 ppm and in one female treated with 15000 ppm. Also hepatocyte hypertrophy was evident in four females and in one male at the top dose level. In the uterus, reduction of the endometrial stroma was observed among females receiving 15000 and 50000 ppm giving the organ a rather atrophic appearance. No treatment-related changes were observed at 5000 ppm and below.

Incidences of the relevant findings in the liver and the uterus are given in table 6.3.1.1-5.

Table 6.3.1.1-5: 4 weeks dietary repeat dose study in rats; group incidences of histopathological changes in the liver and the uterus

	Dose group level (ppm)											
	Males						Females					
	0	500	1500	5000	15000	50000	0	500	1500	5000	15000	50000
Liver panacinar microvacuolation	0/5	0/5	0/5	0/5	1/5	5/5	0/5	0/5	0/5	0/5	5/5	3/5
centriacinar fatty vacuolation	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	3/5	1/5
hepatocyte hypertrophy	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	4/5
necrosis of hepatocytes	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5	4/5
Uterus reduced endometrial stroma	-	-	-	-	-	-	0/5	0/5	0/5	0/5	2/5	5/5

Conclusion

In the DAR 2003, it was stated that *clear evidence of toxicity was noted at concentrations of 5000 ppm triticonazole when administered to F-344 rats for three weeks via diet; the liver and uterus were identified as the major target organs. Since effects on the uterus weight were recorded at all dose levels, a NOAEL could not be determined.*

The RMS (2016) is of the opinion that a NOAEL can be set for males at 1500 ppm (152.3 mg/kg bw per day), based on the decrease in body weight gain (-22.6%) as a sole finding observed at 5000 ppm. In females, RMS (2016) would propose the NOAEL at 500 ppm (52.4 mg/kg bw per day), since at 1500 ppm the only observed effect was statistically significant decrease in absolute uterus weight (without any accompanied histopathological findings). This NOAEL in females is considered conservative but since no explanation for atrophy of uterus in relatively young females is given, this is considered to be precautionary decision.

It was concluded in the study report that the highest concentration of triticonazole which would be suitable for prolonged dietary administration to rats is between 5000 and 15000 ppm.

B.6.3.1.2. Mouse

Previous evaluation:	DAR (2003)
DRAR (2016)	Some additional information added in the results and conclusion; no substantial changes compared to the original assessment

Reference:	RPA 400727: Preliminary toxicity study by dietary administration to CD-1 mice for 6 weeks
Author(s), year:	[redacted] 1991
Report/Doc. number::	C019001 / -
Guideline(s):	OECD 407 (1981)
GLP:	Yes
Deviations from OECD 407 (2008):	- Not all organs from OECD 407 (2008) investigated - Not for all doses and both sexes the histopathology investigated - Neither haematology nor clinical chemistry parameters investigated
Acceptability:	Yes (limited information); range finding test

Material and methods

Groups of 12 male and 12 female mice (strain: CD-1; source: [redacted].) were fed diet containing 0, 500, 1500, 5000, 15000 or 50000 ppm triticonazole (batch no. YG 2156/1; purity 98.9 %), equivalent to 0, 77, 233, 851 and 3270 mg/kg bw per day in males and 0, 98.8, 286, 982 and 4091 mg/kg bw per day in females, resp. (data for mice treated at 50000 ppm are not given) for 6 weeks. Diets were prepared weekly; concentrations of triticonazole in the diet, and stability and homogenicity of the test substance were confirmed by analysis.

Animals were observed twice daily for clinical signs. Food consumption was measured weekly, body weights were assessed twice weekly. Neither haematology nor clinical chemistry parameters were investigated.

At necropsy, each animal was subjected to a gross pathological examination and the weights of selected organs (brain, heart, kidneys, liver, lungs, spleen, testes, uterus) were recorded. The histopathological examinations were performed on adrenals, brain, heart, kidneys, liver, stomach, testes, thyroid with parathyroid and urinary bladder from control and mice treated with 5000 ppm and 15000 ppm (males only). In addition, the liver from all mice dosed with 500 and 1500 ppm, and all animals with macroscopic abnormalities were also examined.

Results

General observations: All animals treated with 50000 ppm and also one male and ten females receiving 15000 ppm died or were killed in extremis within the first week of treatment. Before death, the mice displayed signs of ill-health including hunched posture, piloerection, flaccid muscle tone, hypoactivity, hypothermia and marked body weight loss and emaciation.

Body weight: During the first three days of dosing, marked dose-related decreases in body weights were reported in animals receiving 5000 ppm and above. Thereafter the body weight gain of males treated with 5000 ppm and of females treated with 5000 and 15000 ppm was superior to that of the respective control values. Body weights of males receiving 15000 ppm remained more or less static during the study period (table 6.3.1.2-1).

Bodyweight gains of animals receiving 500 and 1500 ppm were unaffected by treatment.

Table 6.3.1.2-1: 6 weeks dietary repeat dose study in mice; Group mean body weight and body weight gain (g)

	Dose group level (ppm)									
	Males					Females				
	0	**500**	**1500**	**5000**	**15000**	**0**	**500**	**1500**	**5000**	**15000**
body weight on day 0 (g)	24.7	24.2	24.5	24.1	24.4	20.9	19.8	21.0	20.5	20.5
terminal body weight (g) (% of controls)	35.7	34.5 (96)	34.5 (96)	32.4 (90)	19.1 (53)	26.1	24.6 (94)	26.1 (100)	25.0 (95)	19.0 (72)
weight gain (g) day 0 – 3	2.3	1.8	2.1	-1.8***	-5.3***	0.2	0.7	0.8	-0.9**	-4.8**
weight gain (g) day 0 – 42 (% of control)	11.8	11.4 (97)	11.0 (93)	9.8* (83)	-4.2* (-35.6)	5.8	5.2 (90)	6.2 (107)	4.9 (84)	1.0*** (17)

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's test)

Food consumption of mice receiving 50000 ppm was negligible and of mice treated with 15000 ppm was marked lower than that of controls during the first week of treatment. Thereafter, food intake in this dose group (15000 ppm)

was only slightly lower than that of controls. Food consumption of animals receiving ≤ 5000 ppm was unaffected by treatment. However, feed conversion ratio was lower than that of controls for males receiving 5000 ppm.

Organ weight analysis from animals killed after 6 weeks of treatment revealed clear dose-related increase in absolute and relative liver weights in all treated groups when compared with controls (table 6.3.2-2). Higher liver weights were also noted for some of the mice killed or dying during the treatment period. All other inter-group differences occasionally attaining statistical significance were considered not to be of toxicological relevance.

Table 6.3.1.2-2: 6 weeks dietary repeat dose study in mice; Terminal absolute and relative liver weights, group mean values

	Dose group level (ppm)									
	Males					Females				
	0	**500**	**1500**	**5000**	**15000**	**0**	**500**	**1500**	**5000**	**15000**
absolute weight (g) (% control)	1.89	2.08 (110.1)	2.76[b] (146.0)	4.16[b] (220.1)	3.21[b] (169.8)	1.43	1.47 (102.8)	1.80[b] (125.8)	2.82[b] (197.2)	3.08[a] (215.4)
relative weight (%) (% control)	5.29	6.02[b] (113.8)	7.99[b] (151.0)	12.86[b] (243.1)	16.85[b] (318.5)	5.46	6.02[b] (110.3)	6.90[b] (126.4)	11.22[b] (205.5)	16.19[b] (296.5)

a ($p \leq 0.05$); b ($p \leq 0.01$); significantly different from controls (Dunett's test)

Pathology: Macroscopic examination after six weeks of treatment revealed liver enlargement in animals receiving 5000 ppm and more. There was also a range of histopathological findings related to treatment. The major finding was hypertrophy of periacinar hepatocytes in males and females at ≥ 1500 ppm. In males which received 15000 ppm and in females which received 5000 ppm, this finding was frequently associated with fatty vacuolation. In addition, increased ploïdy of hepatocytes was noted at these dose levels. Proliferation of bile duct cells, increased incidences of inflammatory cells in the portal area and focal mineralisation were also evident in males receiving 5000 and 15000 ppm. Incidences of the findings are given in table 6.3.1.2-3.

Table 6.3.1.2-3: 6 weeks dietary repeat dose study in mice; Group incidences of pathological changes in the liver

Finding description	Dose group level (ppm)									
	Males					Females				
	0	**500**	**1500**	**5000**	**15000**	**0**	**500**	**1500**	**5000**	**15000**
enlarged liver	0/12	0/12	1/12	5/12[a]	7/11[b]	0/12	0/12	0/12	4/12	2/2
hepatocytic hypertrophy	0/12	0/12	0/12	12/12[c]	11/11[c]	0/12	0/12	2/12	10/12[c]	0/2
fatty vacuolation	1/12	1/12	1/12	2/12	11/11[c]	0/12	0/12	0/12	6/12[a]	0/2
increased ploïdy	0/12	0/12	0/12	2/12	11/11[c]	0/12	0/12	0/12	9/12[c]	0/2
inflammatory cells	0/12	2/12	4/12	3/12	5/11[a]	0/12	1/12	0/12	0/12	0/2
focal mineralisation	0/12	0/12	0/12	2/12	4/11[a]	0/12	0/12	0/12	0/12	0/2

a ($p \leq 0.05$); b ($p \leq 0.01$); c ($p \leq 0.001$) significantly different from controls (Fisher's exact test)

Conclusion

As already concluded in DAR 2003, no NOAEL could be determined as treatment-related findings (liver weight > 10% increase (males and females), liver histopathology (increased inflammatory cells in males), decreased body weight gain in females) were observed already at 500 ppm. It was concluded in the report, that the maximum tolerated dose for repeated dietary administration of triticonazole in mice lies between 1500 and 5000 ppm.

B.6.3.1.3. Dog

Previous evaluation:	DAR (2003)
DRAR (2016)	Some additional information added in the results and conclusion; no substantial changes compared to the original assessment

Reference:	RPA 400727: Preliminary toxicity study by oral (capsule) administration to Beagle dogs for four weeks
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013014 / -
Guideline(s):	No OECD guideline for 28 days studies with non-rodents available
GLP:	Yes
Deviations from OECD:	No OECD guideline for 28 days studies with non-rodents available but the number of animals was anyhow too low for any other information but estimation of approximate dose for further testing
Acceptability:	Yes (limited information); range finding test

Material and methods

Triticonazole (batch no. YG 2156/1; purity 95.4 %) was given to groups of 2 male and 2 female beagle dogs (source [redacted]) at dose levels of 0 (empty capsule), 10, 30, 100 and 300 mg/kg bw per day in gelatine capsules for 28 consecutive days. The individual daily doses of the test substance for each dog were weighed directly into the capsules based on the most recently recorded bodyweight. Daily dosing was made after feeding.

The animals were observed at regular intervals throughout the working day for clinical signs and mortalities. Body weights were recorded prior to treatment commenced and then weekly, whereas food consumptions were calculated weekly based on the weight of food supplied to each dog. Ophthalmoscopic, veterinary and neurological examinations were performed prior to the first dosing and after 24 days of treatment.

Blood samples for biochemical investigations (total protein, albumin, globulin, calcium, phosphate, sodium, potassium, urea, creatinine, glucose, cholesterol, bilirubin, chloride, AST, ALT, AP) and haematological investigations (haematocrit, haemoglobin, RBC, reticulocyte count, MCHC, MCV, MCH, WBC total, WBC differential, platelets count, prothrombin time, thromboplastin time) were collected prior to treatment and after 25 days of treatment. Urine samples were taken before commencement of treatment and after 22 days of treatment, and following parameters were investigated: colour, volume, pH, specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, nitrite, blood, sediment.

At termination of the study, all animals were subjected to detailed macroscopic necropsy and the following organs were weighed: adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid, and uterus. Histopathological examinations were performed on adrenals, aorta, bone marrow smear, brain, bronchi, caecum, colon, duodenum, epididymides, eyes and optic nerves, gall bladder, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, mammary gland, oesophagus, ovaries, pancreas, pituitary, prostate with urethra sample, rectum, salivary glands, sciatic nerve, skeletal muscle, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus.

Results

General observations: No treatment-related mortalities occurred at any dose level. Signs of reaction to treatment were confined to only one male dog receiving 300 mg/kg bw and consisted of ataxia, abnormal gait, hypoactivity, circling and head shaking. These signs were observed approx. 5 – 6 hours after dosing on days 3 to 5 of treatment and there was a complete recovery by the following morning. Subsequently, no signs of reaction to treatment were seen.

Body weight: Decreases in overall body weight gains were observed in animals of both sexes receiving 300 mg/kg when compared with control animals. This effect was less evident in males (63 % of controls) than in females (40 % of controls) and was considered to arise from initial body weight loss during the first 3 days of treatment. The body weight gains of all other treated animals were unaffected by treatment. There was also a decrease in food consumption in females receiving 300 mg/kg bw per day (92 % of controls).

Neurological and veterinary examinations and ophthalmoscopy revealed no treatment-related changes in any dose group.

Haematological investigations exhibited higher haematocrit value, haemoglobin concentration and erythrocyte count in one female receiving 300 mg/kg after 25 days of treatment. The study author contributed these findings to the low food intake and poor weight gain of this animal.

Clinical chemistry analysis revealed significant treatment-related elevated activities of alkaline phosphatase in male dogs receiving 100 (two-fold increase) and 300 mg/kg (three-fold increase), and in female dogs receiving 300 mg/kg (three-fold increase) compared to control values. There were no other haematological or biochemical changes in the plasma that could be attributed to treatment. In addition, no treatment-related changes were seen in animals of the other dose-groups.

Urinalysis: The composition of the urine was unaffected by treatment with triticonazole at all dose levels.

Organ weight analysis: When compared with control animals, treatment-related findings were limited to slight increase in relative liver weights in males receiving 300 mg/kg (+18.9% compared to control) and in females receiving 100 (+9.7%) and 300 (+8.5%) mg/kg bw per day. Absolute liver weight was only higher in males of the top dose (+17.9%). In addition, absolute and relative thymus weights of animals receiving 100 (males absolute: -20.9%, males relative: -23.1%; females absolute: -8.1%, females relative: -12.5%) and 300 mg/kg (males absolute: -40.4%, males relative: -40.6%; females absolute: -20.1%, females relative: -15.2%) were lower than in controls.

Pathology: There were no macroscopic changes after four weeks which could be attributed to treatment with triticonazole. Upon histopathological examination, treatment-related findings were limited to periacinar hypertrophy of hepatocytes with associated fatty vacuolation in both males receiving 300 mg/kg. There were no other findings considered to be related to treatment. In 2015 the notifier provided an external pathologist (*Millar, P.M.,* 2015) with

the old histological slides for reassessment/confirmation of study results. It was confirmed that no treatment-related pathology findings were seen in the adrenal glands.

Conclusion

As already concluded in DAR 2003, following continuous oral administration of triticonazole to Beagle dogs for four weeks, systemic toxicity (reduced body weight gain, biochemical and haematological changes, organ weight effects and histopathological findings) was observed at the dose level of 300 mg/kg bw per day. Based on increased activity of the alkaline phosphatase (males), slightly increased liver weight (females), and decrease in relative and absolute thymus weight (males and females) evident at 100 mg/kg bw per day, the NOAEL for this study was confirmed at 30 mg/kg bw per day as already concluded in DAR 2003.

B.6.3.2. Oral 90- day study

B.6.3.2.1. Rat

Previous evaluation:	DAR (2003)
DRAR (2016)	Some additional information and re-assessment of histological slides (Millar, 2015) added in the results; conclusion of the study from DAR 2003 not changed

Reference:	RPA 400727: Toxicity study by dietary administration to CD rats for 13 weeks
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013029 / -
Guideline(s):	OECD 408 (1981) and US EPA guideline 82-1
GLP:	Yes
Deviations from OECD 408 (1998):	- no functional observations conducted
Acceptability:	Yes

Material and methods

Groups of 10 male and 10 female CD rat (source: [redacted].) received triticonazole (batch no. YG 2160/1, purity 98.2 % used for the first nine weeks; batch no. YG 2156/1, purity 98.9% used subsequently) with their diet at dose levels of 0, 25, 250, 12500 and 25000 ppm for 13 weeks (equivalent to 0, 2.0, 19.8, 1117.0 and 2309.3 mg/kg bw per day in males and 0, 2.2, 22.3, 1183.5 and 2368.8 mg/kg bw per day in females). Test diets were prepared freshly each week and stability, homogeneity and concentration of the test material was determined periodically during the study. (It was noted that the achieved concentration in the 25 ppm group in week 13 was higher than intended [147 %]).

Animals were observed daily for clinical signs and mortality. Body weights and food consumption were recorded weekly throughout the study. Ophthalmoscopic examinations were performed in all animals of the control and high dose groups prior to to the commencement of treatment and after 12 weeks of treatment. Blood samples for haematology (haematocrit, haemoglobin concentration, RBC, reticulocyte count, MCHC, MCV, MCH, WBC total, WBC differential, platelet count, prothrombin time) and clinical chemistry analysis (AP, ALT, AST, creatine phosphokinase, urea, creatinine, glucose, bilirubin, cholesterol, total protein, albumin, globulin, calcium, phosphate, sodium, potassium, chloride) were taken after 12 weeks of treatment; urinalysis (colour, volume, pH, specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, blood cells and sediment) was performed after 11 weeks of treatment. At necropsy, each animal was subjected to a gross pathology examination and selected organs

(adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid, uterus) were weighed. Microscopic examinations were performed on a full range of tissues/organs (adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eye and optic nerve, femoral bone, heart, ileum, jejunum, kidneys, liver, lungs, mammary gland (caudal), mandibular and mesenteric lymph nodes, oesophagus, ovaries, pancreas, pituitary, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum plus marrow, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus with cervix, vagina) from all animals in the control and high dose group, and all tissues from animals in any group which were identified as abnormal at macroscopic examination. In addition, the kidneys, liver, uterus and adrenals were examined from all animals in the remaining dose groups. Additional sections of the liver from 5 males and 5 females from each dose group were stained with Oil-Red-O.

Results

General observations: No treatment-related mortalities occurred at any dose level. One female receiving 250 ppm was killed in extremis during week 13 of the study following a renal failure (not attributed to treatment).

Clinical signs were limited to a generalised hair loss (predominantly affected on head, ventral and dorsal body surface) that persisted until week 9 in animals treated at 12500 ppm and throughout the study in all animals treated with 25000 ppm.

Body weight: During the first 5 weeks of dosing, marked significant decreases in body weight gains were reported in rats treated at 12500 and 25000 ppm. Thereafter, overall body weight gains were comparable with controls. The overall body weight gains of males receiving 12500 and 25000 ppm were 80 % and 70 % of control values, resp., whereas overall body weight gains in females receiving 12500 and 25000 ppm were 75 % and 70 % of control values, resp. Animals receiving 25 or 250 ppm were unaffected by treatment.

Food consumption was depressed in animals at 25000 ppm, especially during weeks 1 – 9. Food consumption of males and females receiving 12500 ppm was also low until weeks 5 and 7 respectively. The overall efficiency of food conversion at both high dose levels was also reduced compared with controls (week 1 to 13: males 12500 ppm: .87.6%, males 25000: 80%; females 12500: 83.5%, females 25000: 80.7%). Animals receiving 25 or 250 ppm were unaffected by treatment.

Table 6.3.2.1-1: 13 weeks dietary repeat dose study in rats group mean body weight and body weight gain (g)

	Dose group level (ppm)									
	Males					**Females**				
	0	**25**	**250**	**12500**	**25000**	**0**	**25**	**250**	**12500**	**25000**
terminal body weight (g)	574	586	563	480	435	344	332	348	289	275
(% control)		(102.1)	(98.1)	(83.6)	(75.8)		(96.5)	(101.2)	(84.0)	(79.9)

	Dose group level (ppm)									
	Males					Females				
	0	**25**	**250**	**12500**	**25000**	**0**	**25**	**250**	**12500**	**25000**
weight gain (g) week 0-13 (% control)	451	465 (103.1)	440 (97.6)	362** (80.3)	314** (69.6)	227	217 (95.6)	233 (102.6)	171** (75.3)	160** (70.5)

** ($p \leq 0.01$) (Student's t-test)

Ophthalmoscopic examinations revealed no treatment-related effects at any dose level.

Urinalysis: When compared with control animals, treatment-related findings consisted of lower protein content of the urine in males at 12500 and 25000 ppm and slightly lower urinary pH in males at 25000 ppm and in females at all dose levels. However, the slightly decreased pH noted in females at 25 ppm was considered incidental. The lower protein content in urine is not considered an adverse finding.

Haematological investigations indicated a number of treatment-related effects in the two higher dose groups. When compared with controls values, lower packed cell volumes (males and females at 25000 ppm), lower haemoglobin concentrations (males at 25000 ppm and females at 12500 and 25000 ppm), lower erythrocyte counts (males at 12500 and 25000 ppm), lower MCV and MCH (females at 12500 and 25000 ppm) and higher total leucocyte and lymphocyte counts (females at 25000 ppm) were observed (table 6.3.2.1-2). The decrease of erythrocytes, haematocrit and haemoglobin was within 10% of difference to control animals and might be considered of low adversity.

In addition, examination of blood smears revealed more spherocytes (sphere-shaped erythrocytes) and a greater prevalence of anisocytosis (erythrocytes of unequal size) in males treated at 25000 ppm and in females at $\geq$ 12500 ppm. This corresponds to changes in MCV (approximately 5% change to control) observed in females in 12500 and 25000 group also indicating morphological abnormality of erythrocytes. However, based on still low severity in haematological parameters, treatment-related anaemia is not assumed.

There were no haematological changes that were considered treatment-related at 25 and 250 ppm.

Table 6.3.2.1-2: 13 weeks dietary repeated dose study in rats; Haematological findings; group mean values

	Dose group level (ppm)									
	Males					Females				
	0	**25**	**250**	**12500**	**25000**	**0**	**25**	**250**	**12500**	**25000**
Haematocrit (%) (% control)	45	46	46	45	43** (95.6)	45	46	46	44	43** (95.6)
Haemoglobin (g%) (% control)	15.8	16.2	16.1	15.8	15.2** (96.2)	15.9	15.9	16.0	15.1* (94.9)	15.0** (94.3)
Erythrocytes (10^{-12}/l) (% control)	8.95	8.85	8.94	8.5* (94.9)	8.47** (94.6)	8.4	8.59	8.59	8.4	8.28
MCV (cμ)	51	52	52	53**	51	54	53	54	52**	51***

	Dose group level (ppm)									
	Males					Females				
	0	**25**	**250**	**12500**	**25000**	**0**	**25**	**250**	**12500**	**25000**
									(96.3)	(94.4)
MCH (pg)	18	18	18	19**	18	19	19	19	18***	18**
White blood cells (10^{-9}/l)	13.7	17.4**	14.6	13.9	13.3	8.8	7.4	7.2	10.2	12.7***

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's test)

Clinical chemistry analysis in plasma revealed also changes relative to controls in animals treated at 12500 and 25000 ppm which were considered treatment-related: slightly higher (30% increase) plasma alkaline phosphatase (males at 25000 ppm), higher cholesterol concentrations (males and females at 12500 and 25000 ppm), reduced glucose levels (males and females at 25000 ppm), higher plasma protein concentrations (females at 12500 and 25000 ppm) associated with some changes in the different protein fractions (lower albumin concentration and higher α1-globulin concentration with a concomitant reduction in the albumin to globulin ratio at 12500 ppm; increased β-globulin concentration at 12500 and 25000 ppm). Other intergroup differences attaining statistical significance at some dose levels when compared with controls were considered as biological variability with no toxicological relevance because they were not dose-related or were within the normal range of controls. Relevant clinical chemistry findings are given in table 6.3.2.1-3.

Table 6.3.2.1-3: 13 weeks dietary repeat dose study in rats; Clinical chemistry findings; group mean values

	Dose group level (ppm)									
	Males					Females				
	0	**25**	**250**	**12500**	**25000**	**0**	**25**	**250**	**12500**	**25000**
Alkaline Phosphatase (iu/l) (% control)	90	93	104*	97	120*** (133.3)	51	61	55	59	57
Cholesterol (mg%) (% control)	54	59	52	77*** (142.6)	72*** (133.3)	77	86	80	142*** (184.4)	157*** (203.9)
Glucose (mg%))	138	155*	150	133	121*	115	122	123	115	98**
total protein (g%)	6.8	7.0*	6.7	6.9	6.8	7.3	7.2	7.3	7.6	7.7***

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's test)

Pathology: Macroscopic examination of animals at the terminal sacrifice exhibited enlarged livers in females at 25000 ppm and also enlarged ovaries in one female of each group receiving ≥ 250 ppm.

Analysis of organ weights revealed a number of differences between groups which were considered to be secondary to the reduction of bodyweight. The critical effects which were considered significant and treatment-related were increased absolute and relative liver weights in animals treated at ≥ 12500 ppm, and increased absolute and relative (organ to body ratio) ovary weights at the same dose levels. Markedly high absolute ovary weights were also noted for one female which received 250 ppm (Since no histopathological changes were observed, the weight changes of

the ovaries were considered to be of doubtful toxicological significance by the study author). Since organ to brain ratio is considered to be more relevant for ovaries[2], RMS has included it in the DRAR 2016 (no statistical analysis conducted). Ovaries to brain ratio showed less pronounced effects than ovaries to body ratio but still there was an increase in relative ovaries weight at 12500 and 25000 ppm.

Microscopic examination revealed treatment-related changes in the liver and the adrenals. In the liver significant increased incidences of minimal to slight periacinar hypertrophy and minimal to slight centriacinar hepatocytic fatty vacuolation were evident in animals at 12500 and 25000 ppm. Marginal hepatocyte hypertrophy was also recorded in two males that had received 250 ppm.

In the adrenal cortex, degeneration of the *zona reticularis* in females at 12500 and 25000 ppm, and fatty vacuolation of the *zona fasciculata* (and also of the *zona reticularis* in more severe cases) was evident in males at all dose levels and in females at ≥ 12500 ppm.

In order to further evaluate the incidence and severity of the changes observed in the adrenals, a blind re-examination of all adrenal sections from male rats was performed [redacted] *2000)* and then peer-reviewed by a consultant pathologist ([redacted] *2000)*. According to this peer-review, a significantly higher incidence of vacuolation of the adrenal cortex was confirmed in animals treated at 12500 and 25000 ppm. However, in contrast to the first diagnosis, findings observed at 25 and 250 ppm were not considered treatment-related. The slight vacuolation evident in males at both lower dose levels was considered to be typical of spontaneous changes commonly seen in untreated animals (the distribution and severity of the vacuolation in the *zona fasciculata* was similar between rats treated at ≤ 250 ppm and controls). The vacuolation considered to be treatment-related at higher dose levels showed a different pattern of distribution across the *zona fasciculata* (more concentrated in the inner area instead of across the complete zone). In 2015 the notifier provided again an external pathologist (*Millar, P.M.,* 2015) with the old histological slides (all sections from males and all sections from the control group and females given 250 ppm and selected females given 12500 or 25000 ppm) for reassessment/confirmation of evaluation from 2000. The re-examination confirmed the incidence and severity of the findings reported for males in the reports from [redacted]*, 2000 and* [redacted] *2000*, and the diagnostic criteria employed. In addition to the findings noted in the reports from 2000, the zona glomerulosa appeared thickened due to the presence of diffuse microvacuolation of the corticocytes in males given 12500 or 25000 ppm. In females, re-examination confirmed the presence of minimal to marked degenerative changes in the adrenal glands of animals given 12500 or 25000 ppm and the absence of treatment-related findings in females given 250 ppm. The appearance of the treatment-related lesions in females differed significantly from that seen in treated males. There was no diffuse cortical vacuolation as seen in males; cells of the reticularis, and to a variable extent the fasciculata, were hypertrophic with lipid depletion and dense, eosinophilic cytoplasm. At the junction between the zona reticularis and zona fasciculata, a band of variable size was easily observed at low power due to separation of corticocytes by pink fluid, vacuolation, increased prominence of the stroma and blood vessels and an impression of reduced corticocyte number. At higher power degenerate corticocytes were observed. They displayed a variety of degenerative changes including: vacuolation, balloon degeneration, granular, eosinophilic cytoplasm with cell shrinkage, angularity of the cell outline, nuclear pyknosis or loss of nucleus. Associated with these findings were minimal or mild mononuclear inflammatory cell infiltrates with

[2] Bailey at al., 2004: Relationships Between OrganWeight and Body/BrainWeight in the Rat: What Is the Best Analytical Endpoint. Toxicologic Pathology, 32:448–466, 2004

an occasional macrophage giant cell, early fibrosis or collagen deposition and an increase in cells containing yellow/brown pigment. Severity grading of the degenerative changes ranged from minimal to marked. The external pathologist concluded that the morphological appearance observed in males is usually associated with impaired stereogenesis resulting in excess storage of unmetabolised steroid precursors. With regard to possible mechanism of the lesion seen in females the external pathologist concluded that they most closely resembled vacuolar and granular degeneration of the zona reticularis and zona fasciculata due to mitochondrial vacuolation and dilatation of the smooth endoplasmic reticulum.

There were no other histopathological findings considered to be related to treatment with triticonazole.

Table 6.3.2.1-4: 13 weeks dietary repeat dose study in rats; relevant necropsy findings

	Dose group level (ppm)									
	Males					**Females**				
	0	**25**	**250**	**12500**	**25000**	**0**	**25**	**250**	**12500**	**25000**
Organ weight changes (mean group values)										
Liver absolute weight (g) (% control)	17.4	17.7	17.3	19.1	18.6	11.2	12.3	12.0	15.4[b] (137.5)	17.3[b] (154.5)
Relative weight (%) (% control)	3.19	3.16	3.22	4.23[b] (132.6)	4.5[b] (141.1)	3.29	3.79[b] (115.2)	3.57	5.51[b] (167.5)	6.36[b] (193.3)
Ovaries absolute weight (g) (% control)	-	-	-	-	-	0.09	0.098	0.105	0.12[a] (133.3)	0.114[a] (126.7)
Relative weight (%), organ to body (% control)	-	-	-	-	-	0.0267	0.0303	0.0313	0.043[a] (161.0)	0.042[a] (157.3)
Relative weight (%), organ to brain* (% control)	-	-	-	-	-	4.59	4.92 (107.2)	5.07 (110.5)	5.94 (129.4)	5.76 (125.5)
group incidences of histopathological changes in liver and adrenals										
Liver peracinar hepatocyte hypertrophy	0/10	0/10	2/10	6/10[a]	10/10[c]	0/10	0/10	0/9	6/10[a]	9/10[c]
centriacinar fatty vacuolation	0/10	3/10	1/10	1/10	2/10	3/10	1/10	4/9	7/10	10/10[c]
Adrenals degeneration of *zona reticularis*	0/10	1/10	0/10	0/10	0/10	0/10	0/10	0/9	9/10[c]	10/10[c]
Cortical droplet vacuolation *zona fascicularis*:										
● slight	2/10	2/10	3/10	3/10	2/10	-	-	1/9	3/10	8/10
● mild	-	-	-	3/10	1/10	-	-	-	-	-
● moderate	-	-	-	2/10	2/10	-	-	-	1/10	2/10
● marked	-	-	-	2/10	4/10	-	-	-	-	-
● severe	-	-	-	-	1/10	-	-	-	-	-

a ($p \leq 0.05$); b ($p \leq 0.01$); c ($p \leq 0.001$) significantly different from controls

*no statistical analysis conducted by RMS for ovary to brain weight ratio

Conclusion

Continuous dietary administration of triticonazole to CD rats during 90 days produced clear evidence of toxicity at 12500 and 25000 ppm, with the liver and the adrenals being identified as major target organs.

On the basis of the histopathological changes noted in the adrenals of males, the dose level of 25 ppm was firstly considered to be the NOAEL in the report. However, in the light of the peer-review that concluded that there were no treatment-related effects on the adrenal cortex of males receiving 25 and 250 ppm, the NOAEL was set at 250 ppm (equivalent to 19.8 and 22.3 mg/kg bw per day in males and females, respectively). The RMS 2016 confirms the NOAEL of 250 ppm, based on decreased body weight gain and reduced food consumption and food utilisation ratio in males and females, decreased number of erythrocytes in males, decreased haemoglobin, MCV and MCH in females, increased cholesterol in males and females, increased liver weight with histopathological findings in males and females, increased ovaries weight in females and histopathological findings in adrenals, all effects observed at 12500 ppm and above.

B.6.3.2.2. Mouse

Previous evaluation:	DAR (2003)
DRAR (2016)	Some additional information added in the results; conclusion of the study from DAR 2003 not changed

Reference:	RPA 400727: Preliminary toxicity study by dietary administration to CD-1 mice for 13 weeks
Author(s), year:	[redacted] 1991
Report/Doc. number::	R013027 / -
Guideline(s):	OECD 408 (1981); US EPA guideline 82-1
GLP:	Yes
Deviations from OECD 408 (1998):	- no functional observations conducted, - no clinic-chemical and haematological parameters investigated - histopathological examination conducted only on liver
Acceptability:	Yes (supplementary information only)

Material and methods

Groups of 12 male and 12 female CD-1 mice (source: [redacted].) received triticonazole (batch no. BD1074; purity 99.3 %) in their diet at concentrations of 0, 2500, 5000 or 8000 ppm (equivalent to 0, 382.8, 807.6 and 1426.2 mg/kg bw per day in males and 0, 503.8, 969.2 and 1657.6 mg/kg bw per day in females) for 90 days. Stability, homogeneity and concentrations of triticonazole in the diet were determined periodically during the study period.

Animals were observed at least twice daily for clinical signs and mortality. Body weights were recorded prior to dosing, on days 3 and 7 and then weekly. Food consumption was recorded weekly.

Neither haematology nor clinical chemistry parameters were investigated.

At necropsy, all animals were subjected to a detailed macroscopic pathology examination and the weights of selected organs (brain, heart, kidneys, liver, lungs, spleen, testes, and uterus) were recorded. Histopathological examinations were performed on the liver only.

Results

General observations: Neither treatment-related clinical signs nor mortalities which could be related to treatment with triticonazole were noted throughout the study period.

Concerning body weight, there was a significant depression of weight gain in all treated groups. This effect was particularly marked during the first week with initially body weight loss during the first 3 days of dosing (table 6.3.2.2-1). Nevertheless, food consumption was unaffected by treatment at any dose group when compared with controls.

Table 6.3.2.2-1: 13 weeks dietary repeat dose study in mice; Group mean body weight gain (g) and terminal body weight (g)

Treatment period	Dose group level (ppm)							
	Males				Females			
	0	2500	5000	8000	0	2500	5000	8000
Group mean body weight gain: week 0 – 1	1.3	-0.1**	-2.7**	-4.3**	0.8	0.3	-1.3**	-2.6**
week 1 – 13	15.1	12.7	12.3	10.9**	8.2	5.2	6.8	6.7
week 0 – 13 (% of control)	16.3 -	12.6** (77)	9.7** (60)	6.7** (41)	9.0 -	5.5 (61)	5.5 (61)	4.1* (46)
Terminal body weight (g) (% control)	41.0	37.0** (90.2)	34.3** (83.7)	31.5** (76.8)	29.6	25.9* (87.5)	26.2* (88.5)	23.5** (79.4)

* ($p \leq 0.05$); ** ($p \leq 0.01$); significantly different from controls (Student's t-test)

Organ weight analysis revealed dose-related increases in absolute and relative liver weights compared with controls. In addition, dose–related lower organ weights of uterus plus cervix were evident among all treated females, but statistical significance was achieved only at the highest dose group.

Table 6.3.2.2-2: 13 weeks dietary repeat dose study in mice; Organ weight changes of liver and uterus (mean group values)

	Dose group level (ppm)							
	Males				Females			
	0	2500	5000	8000	0	2500	5000	8000
Liver								
absolute weight (g) (% control)	2.04	3.53** (173.0)	4.07** (199.5)	4.71** (230.9)	1.55	2.23** (143.9)	2.80** (180.6)	3.11** (200.6)
Relative weight (%) (% control)	4.976	9.514** (191.2)	11.88** (238.7)	14.96** (300.6)	5.264	8.625** (163.8)	10.66** (202.5)	13.23** (251.3)
Uterus plus cervix								
absolute weight (g) (% control)	-	-	-	-	0.228	0.191 (83.8)	0.16 (70.2)	0.104** (45.6)
Relative weight (%) (% control)	-	-	-	-	0.802	0.749 (93.4)	0.631 (78.7)	0.443** (55.2)

* ($p \leq 0.05$); ** ($p \leq 0.01$); significantly different from controls (Bartlett's test for homogeneity of variance and Dunnett's test thereafter)

Macroscopic examination revealed high incidences of enlarged livers in all treated groups. Additionally, the livers of several animals were described as "swollen". There were no other findings which were attributed to treatment.

Histologically, periacinaror and/or panacinar hepatocytic fatty vacuolation was evident in all treated mice. These vacuoles were confirmed as fat on examination with Oil-Red-O. Further findings which occurred in all treated animals comprised hepatocyte hypertrophy, coagulative necrosis of hepatocytes hepatocyte necrosis and bile plug formation. In addition, increased mitotic activity of hepatocytes was seen in males and females which received 8000 ppm and in females which received 5000 ppm. Incidences of these findings are given in table 6.3.2.2-3.

Table 6.3.2.2-3: 13 weeks dietary repeat dose study in mice; group incidences of histopathological changes in the liver

	Dose group level (ppm)							
	Males				Females			
	0	**2500**	**5000**	**8000**	**0**	**2500**	**5000**	**8000**
Periacinar vacuolation	0/12	7/12	4/12	0/12	0/12	10/12	10/12	1/12
Panacinar vacuolation	0/12	12/12	12/12	12/12	0/12	12/12	10/12	12/12
Hepatocyte hypertrophy	0/12	8/12	12/12	12/12	0/12	12/12	11/12	12/12
Coagulative necrosis	0/12	4/12	8/12	5/12	0/12	1/12	3/12	4/12
Hepatocyte necrosis	0/12	7/12	4/12	8/12	0/12	1/12	4/12	3/12
Mitotic activity	0/12	0/12	0/12	2/12	0/12	0/12	3/12	7/12
Bile plug	0/12	2/12	7/12	10/12	0/12	0/12	1/12	10/12

Conclusion:
Based on treatment-related findings, particularly of significant hepatic toxicity, which were seen at all dose groups, a NOAEL could not be established for this study. However, it was concluded that a dose level of 1500 ppm is suitable as the highest dose for the subsequent mice carcinogenicity study.

B.6.3.2.3. Dog

No 90-days dog has been conducted. Since 1-year dog study is included in the dossier, 90-days dog study is not considered necessary.

B.6.3.3. Oral 1-year dog study

Previous evaluation:	DAR (2003)
DRAR (2016)	Some additional information and re-assessment of histological slides (Millar, 2015) added in the results; conclusion of the study from DAR 2003 slightly expended but not changed

Reference:	RPA 400727: Toxicity study by oral (capsule) administration to Beagle dogs for 52 weeks.
Author(s), year:	[redacted] 1993
Report/Doc. number::	R000173 / -

Guideline(s):	US EPA/FIFRA guideline 83-1 (1984)
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and methods

Groups of 4 male and 4 female beagle dogs (source: [redacted] received triticonazole (batch no. DA 646; purity 96.6 – 97.1 %) in gelatine capsules at daily dose levels of 0 (empty capsule), 2.5, 25 and 150 mg/kg bw per day for 52 weeks. Dosing was conducted after feeding.

Animals were observed daily for physical conditions, clinical signs and mortalities. Body weights were recorded prior to dosing and weekly thereafter whereas food consumption per animal was calculated weekly.

Ophthalmoscopic examinations were made in all animals prior to the first administration and after 12, 18, 24, 31, 38, 45 and 52 weeks of treatment with photographs taken at the later stages. After 18 weeks of treatment, the rate of tear secretion of dogs receiving 0 and 150 mg/kg bw was examined using the "Schirmer tear test".

Clinical chemistry (total protein, electrophoretic protein fractions, calcium, inorganic phosphate, sodium, chloride, potassium, urea, creatinine, glucose, cholesterol, bilirubin, chloride, AST, ALT, AP), haematology (haematocrit, haemoglobin, RBC, MCHC, MCV, MCH, WBC total, WBC differential, platelet count, reticulocyte count, Prothrombin time, activated thromboplastin time) and urinalysis measurements (colour, volume, pH, specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, nitrite, blood, sediment) were conducted in all animals prior to dosing and after 12, 24 and 50 weeks of treatment.

At termination of the study, all animals were subjected to gross pathological examinations and the following organs were weighed: adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostate, spleen, testes, thymus, thyroid, uterus. Following fixation, histopathological examinations of a wide range of organs/tissues (adrenals, aorta, brain, bronchi, caecum, colon, duodenum, epididymides, eyes and optic nerve, gall bladder, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, mammary gland, oesophagus, ovaries, pancreas, pituitary, prostata, rectum, salivary glands, sciatic nerve, skeletal muscle, skin, spinal cord, spleen, sternum and marrow, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus, vagina) were performed on all animals.

Results

General observations: There were no mortalities which could be related to treatment at any dose level.

Remark: One female dog receiving 25 mg/kg was killed for animal welfare reasons during week 5 following a six days period of deterioration in condition including severe pain of the joints. Examination of blood samples indicated high neutrophil and monocyte count, markedly high plasma AP and high plasma cholesterol and albumin concentrations. Significant findings at histopathology revealed myofibre degeneration, inflammation of skeletal muscles, multifocal perivascular changes in the brain and slight erythrocytoses in lymph nodes. Since the symptoms and severe findings were not repeated in any other dog, this death was considered incidental and not related to treatment.

Clinical signs as a reaction to treatment were confined to animals receiving 150 mg/kg and comprised neurological changes, higher incidences of red and thickened pinnae and skin, red gums, externally-visible lenticular opacities, ocular discharge and partially "closed eyes". Neurological changes were seen between weeks 6 and 11 of treatment, generally taking the form of tremor of the head and neck (noted in 2 males and all 4 females between 30 minutes and 4 hours after dosing) , short-duration convulsions (noted in one male and female during week 11, both before and after dosing), ataxia and reluctance to walk (observed in one female during week 9). There was complete recovery for all these signs.

Overall body weight gains of males and females receiving 150 mg/kg were significantly lower when compared with those of the controls. In addition, although the difference did not reach statistical significance, the overall body weight gains of females receiving 25 mg/kg were also marked lower (- 25 %) in comparison with controls. Weight gains of males receiving 2.5 mg/kg were lower than the controls, but in the absence of effects in males at 25 mg/kg, this was not considered related to treatment (table 6.3.3-1). In males, no effects > 10% on terminal body weight were observed, while in females at 25 mg/kg bw per day the terminal body weight was 13% lower than in controls and 30% in the 150 mg/kg bw per day group.

Table 6.3.3-1: 52 weeks repeat dose study in dogs; group mean body weight gain and terminal body weight (kg)

Treatment period	**Dose group level (mg/kg bw per day)**							
	Males				**Females**			
	0	**2.5**	**25**	**150**	**0**	**2.5**	**25**	**150**
Group mean body weight gain (kg) (week 0 – 13)	4.1	3.5*	3.7	2.9***	3.7	3.5	3.1	2.0**
week 13 - 26	1.5	1.1	1.4	1.2	1.3	1.0	0.7	0.4*
week 26 - 39	0.7	0.5	0.5	0.5	0.6	0.5	0.6	0.1*
week 39 - 52	0.2	0.0	0.3	0.4	- 0.1	- 0.1	- 0.2	- 0.1
week 0 – 52 (% of control)	6.6 -	5.1* (77.3)	5.9 (89.4)	4.8* (72.7)	5.6 -	5.0 (89.3)	4.2 (75)	2.3** (41.1)
Terminal body weight (kg) (% control)	14.495	13.440 (92.7)	14.260 (98.4)	13.437 (92.7)	13.707	12.730 (92.8)	11.910 (86.9)	9.630** (70.3)

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's t-test)

Food consumption was considered to have been unaffected to treatment.

Ophthalmoscopic examinations: Early cataract formation was noted in 4 males and 2 females receiving 150 mg/kg at week 13 with a further female of the top dose affected at week 19. These changes originally presented as anterior and posterior capsular opacities of varying severity progressed to total cataract formation with opacification of the lens nucleus, equatorial vacuolation and a clear cortical zone between the two. During cataractogenesis concomitant uveitis including conjunctivitis, blepharospasms, miosis and photophobia were observed. From week 32 onwards, no examination of the retina was possible in animals displaying a cataract. The rate of tear secretion was unaffected

at any dose level. There were no ophthalmoscopic changes that were considered treatment-related at 2.5 and 25 mg/kg bw per day.

Remark: It was stated by the study author that the cause of this lenticular degeneration is not clearly known but such effects were observed with some hypocholesterolaemic agents, and the administration of triticonazole was clearly associated with lowered plasma cholesterol concentrations.

Haematological treatment-related changes were limited to higher platelet counts at week 24 and 50 in female dogs receiving 150 mg/kg and also higher haematocrit values and haemoglobin concentrations evident in one male and 2 females of the top dose at week 12 of the study, when compared with control values. There were no other changes at 2.5 and 25 mg/kg considered to be related to treatment.

Clinical chemistry analysis revealed a number of treatment-related changes in animals receiving 150 mg/kg. In comparison with controls, alkaline phosphatase (ALP) activity was markedly high after 12 weeks, and became higher as the study progressed. In addition, lower cholesterol concentrations, total plasma protein and albumin concentrations were noted at this dose level when compared with controls. Plasma alanine amino-transferase (ALT) activities were consistently higher whereas creatinine concentration was consistently lower than in controls at this dose level. The significance of a reduction of plasma creatinine concentration is unknown and this change is not considered to be of toxicological significance.

Among animals receiving 25 mg/kg, similar treatment-related findings on AP activities, cholesterol and protein concentrations were observed although these changes tended to be less marked, and in some cases were only transient. However, statistical significant inter-group difference from control values (after exclusion of high dose values) were reached for AP activities in females at weeks 12, 24 and 50, and for cholesterol and protein concentrations in females at week 24, when compared with controls. One female receiving 25 mg/kg also had a consistently higher plasma ALT activity than the controls. There were no clinical chemistry changes in animals receiving 2.5 mg/kg bw per day.

Table 6.3.3-2: 52 weeks repeat dose study in dogs: Relevant haematological and clinical chemistry findings (group mean values)

Parameter	Dose group level (mg/kg bw per day)							
	Males				Females			
	0	2.5	25	150	0	2.5	25	150
Platelet count (10^{-9}/l)								
week 24	280	298	302	336	327	302	272	418*
week 50	345	338	376	431	380	383	406	517*
Alkaline phosphatase (iU/L)								
week 12	68	74	88	437***	83	71	111*	326***
week 24	51	73	86	705***	63	64	107***	385***
week 50	68	66	85	1029***	72	72	113**	600***

Parameter	Dose group level (mg/kg bw per day)							
	Males				Females			
	0	**2.5**	**25**	**150**	**0**	**2.5**	**25**	**150**
Alanine amino-transferase (iU/l)								
week 12	24	32	33	44**	28	25	34	43
week 24	27	29	32	52*	27	24	32	47*
week 50	37	36	37	60**	27	32	36	58**
Cholesterol (mg%)								
week 12	163	140	144	95**	145	143	137	121
week 24	161	144	128	78**	146	138	103*	105*
week 50	163	143	124	73**	159	163	157	112
Protein (g/l)								
week 12	56	52	53	51	51	54	52	51
week 24	59	56	54	50**	55	55	51**	51**
week 50	60	58	57	54**	60	58	58	53**

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's t-test)

Urinalysis: Treatment-related changes were limited to animals of the top dose level consisting of significantly increased specific gravity in males at weeks 24 and 50 and in females at all sampling times and a lower urinary volume in females at weeks 24 and 50.

Organ weight analysis revealed increased absolute and relative adrenal weights (organ to body weight ratio), resp., in males and females receiving 150 mg/kg when compared with control values. Adrenals to brain ratio has also been included by RMS in the DRAR 2016 (no statistical analysis conducted) and showed no effects in males while in females of the high dose group this comparison was in line with adrenals to body weight ratio. While no statistical differences were observed for absolute liver weight, the relative liver weight in females of high dose group showed statistical significance. At the highest dose group, relative liver weight in males was 16.8% higher than in controls while in females of the high dose groups relative liver weight was 42.5% higher than in control animals.
In addition, an increase in relative kidney weights in females (33% compared to control) and marked decreases in absolute and relative prostate weights in males were noted at highest dose level (table 6.3.3-3). The effects on the prostate weight in the high dose group were not accompanied by any effects in the lower dosed animals; based on marked manifestation the treatment related effect is questioned (however no information in the study report on unexpected circumstances in the analysis given).

Table 6.3.3-3: 52 weeks repeat dose study in dogs; Organ weight changes (mean group values)

	Dose group level (ppm)							
	Males				Females			
	0	**2.5**	**25**	**150**	**0**	**2.5**	**25**	**150**
Adrenals								
absolute weight (g)	1.41	1.45	1.30	1.88	1.78	1.60	1.58	1.69
HCD of 8 studies from 1987 to 1992								
Males: 1.15-1.86 (mean±SD: 1.43±0.17)								
Females: 1.12-2.06 (mean±SD: 1.52±0.2)								
Relative weight (%) (organ to body weight ratio)	0.0098	0.0108	0.0092	0.0139	0.0130	0.0125	0.0132	0.0174[a]
(% control)		(110.2)	(93.9)	(141.8)		(96.2)	(101.5)	(133.8)
HCD of 8 studies from 1987 to 1992								
Males: 0.007-0.014 (mean±SD: 0.0098±0.0014)								
Females: 0.008-0.0141 (mean±SD: 0.0113±0.0016)								
Relative weight (%) (organ to brain weight ratio*)	1.78	1.83	1.66	2.6	2.37	1.97	2.05	2.31
(% control)		(102.8)	(93.3)	(146.1)		(83.1)	(86.5)	(97.5)
Kidneys								
absolute weight (g)	61	62	66	64	58	55	53	54
Relative weight (%)	0.42	0.46	0.46	0.47	0.42	0.44	0.45	0.56[b]
(% control)								(133.3)
Liver								
absolute weight (g)	496	417	504	539	472	424	415	475
(% control)		(84.1)	(101.6)	(108.7)		(89.8)	(87.9)	(100.6)
Relative weight (%)	3.45	3.12	3.53	4.03	3.46	3.38	3.48	4.93[a]
(% control)		(90.4)	(102.3)	(116.8)		(97.7)	(100.6)	(142.5)
Prostata								
absolute weight (g)	8.18	8.93	8.92	2.86[a]	-	-	-	-
(% control)				(34.9)				
Relative weight (%)	0.0570	0.0651	0.0626	0.0211[a]	-	-	-	-
				(37.01)				

a = ($p \leq 0.05$); b = ($p \leq 0.01$); significantly different from controls (Bartlett's test for homogeneity of variance and Dunnett's test thereafter)

*no statistical analysis performed by RMS

Macroscopic pathology: An apparent enlargement of the liver was observed in two males treated at 25 mg/kg bw and in two males and two females treated at 150 mg/kg bw, although a similar finding was also recorded for one control male. Another observation at the macroscopic examination was thickening of the skin (predominantly pinnae and hocks) at a higher incidence in dogs treated with 150 mg/kg bw, compared to controls.

Microscopic examinations of animals at the terminal sacrifice revealed histopathological changes in the eyes and adrenals of dogs treated at 150 mg/kg. In the eyes, marked degeneration of the lens was observed in all males and 3 females at this dose level. In addition, one of these females showed a slight retinal oedema. Cholesterol content and synthesis are important for the structural and functional integrity of the fiber cell plasma membrane in the lens and

disturbance in cholesterol synthesis and/or the accumulation of cholesterol-oxides or -precursors can lead to cataract. The measured lower cholesterol levels in blood (please see under blood chemistry) may also lend weight to the assumption of an impairment of cholesterol synthesis in analogy to triticonazole mode of action in fungi, where it impairs ergosterol synthesis. Therefore the observed cataracts in almost all animals after prolonged treatment with triticonazole at high dose levels might have been mediated by an impairment of cholesterol synthesis in the eye.

According to study author, a higher incidence of slight to minimal vacuolation of the *zona fasciculata* in adrenals was evident in animals receiving 150 mg/kg bw than that in the controls. In 2015 the notifier provided an external pathologist (*Millar, P.M.,* 2015) with the old histological slides for reassessment/confirmation of study results (1993) regarding adrenals. The external pathologist stated that differences in incidence and severity between the original data and re-examination can largely be attributed to variation in the level of reporting between pathologists. Essentially the results were very similar. The results of the slide review confirmed that there was a greater incidence and/or severity of multifocal vacuolation in the zona fasciculata of both sexes given 150 mg/kg/day than in controls. At 25 mg/kg/day there was a slight but clear increase in severity and incidence of the finding in males but, in females, the difference was marginal. In animals given 2.5 mg/kg/day the severity and incidence was considered comparable to that of controls. The lesion was characterized by the presence of focus(i) of corticocytes enlarged by clear vacuolation. The foci tended to lay within the zona fasciculata near the junction between the zona fasciculata and zona reticularis but were also occasionally present in the outer zona fasciculata. Occasional affected corticocytes showed ballooning degeneration with rupture of the cell membrane and centralized pyknotic nuclei. In some instances a small infiltrate of mononuclear inflammatory cells was present within or surrounding the lesion.

The external pathologist (*Millar, 2015*) added in his report that the interpretation of the significance of adrenal findings is difficult: "*In support of the former is the absence of any changes in the 4 week study at twice the dosage. On the other hand there was an increase in mean absolute and body-weight relative adrenal weights recorded for animals given 150 mg/kg/day although it is considered unlikely, given the focal nature and extent of the lesions seen at the highest dosage, that they would have been responsible for the weight increases. Access to contemporaneous control data from the test facility might provide some help in interpretation although this may be of limited use since 52 week dog studies are comparatively rare and individual variations in the level of reporting and grading of the finding are likely to be large. In any event it is doubtful if this minor exacerbation in a spontaneous finding would have significance for humans.*

During the DRAR evaluation, notifier provided historical control data (eight studies conducted from 1987 – 1992 in the same laboratory) on adrenal histopathology:

Table 6.3.3-4: Historical control data in dogs (8 studies, 1987 – 1992) for adrenal histopathology

Adrenal gland finding	Incidence	
	Males	Females
Vacuolation (zona fasciculata)	2	2
Vacuolation (zona glomerulosa)	2	1
Vacuolation (cortical region unspecified)	1	0
Number of animals examined	33	33

Historical control data from eight 1-year studies in dogs from the same laboratory revealed changes in adrenals rather as a rare finding. No discrimination of findings as minimal, slight or moderate was provided.

Group incidences of relevant treatment-related histological findings are presented in table 6.3.3-5.

Table 6.3.3-5: 52 weeks repeat dose study in dogs; Group incidences of relevant histopathological findings

	Dose group level (ppm)							
	Males				Females			
	0	**2.5**	**25**	**150**	**0**	**2.5**	**25**	**150**
Adrenals ([redacted]1993)								
Vacuolation of *zona fasciculata*								
● minimal	1/4	0/4	1/4	1/4	0/4	0/4	0/3	0/4
● slight	0/4	1/4	1/4	3/4	0/4	0/4	1/3	3/4
Adrenals (Millar, 2015, re-examination)								
Vacuolation of *zona fasciculata*								
● minimal	2/4	1/4	1/4	1/4	2/4	2/4	2/3	0/4
● slight	0/4	1/4	3/4	1/4	1/4	0/4	0/3	2/4
● moderate	0/4	0/4	0/4	2/4	0/4	0/4	1/3	2/4
Eyes								
Lenticular degeneration	0/4	0/4	0/4	4/4	0/4	0/4	0/4	3/4

It has to be noted that in the light of the apparent neurological clinical signs, microscopic examination of brain, spinal cord and sciatic nerves did not reveal any abnormal treatment-related finding.

Conclusion:

The continuous administration of triticonazole to Beagle dogs for 52 weeks produced clear evidence of toxicity at 150 mg/kg bw per day. The liver, the eyes and the adrenals were identified as target organs. The increased adrenal weight at 150 mg/kg bw was associated with histopathological changes in the adrenal cortex (presence of focus(i) of corticocytes enlarged by clear vacuolation), although the correlation of adrenal weight increase and observed histopatholgical findings is doubted by the external pathologist (Millar, 2015). In the eyes, cataractogenic effects were observed after high dose administration. At this dose level also lower plasma cholesterol level was measured. Therefore the observed cataracts in almost all animals after prolonged treatment with triticonazole at high dose levels might have been mediated by an impairment of cholesterol synthesis in the eye. No eye effects were noted at the next lower dose level of 25 mg/kg. The clinical chemistry findings along with higher liver weight and macroscopic liver enlargement suggest effects on this organ, but there were no histopathological findings seen at any dose level in the liver.

In alignment with the conclusion in the DAR 2003 the NOAEL of the study is set at 2.5 mg/kg bw per day, based on decreased terminal body weight in females (87% of control), statistically significantly, almost two-fold increased ALP in females and histopathological findings in adrenals (males and females), all effects observed at 25 mg/kg bw per day.

B.6.3.4. Other routes

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	3-Weeks dermal toxicity study with triticonazole in rats
Author(s), year:	[redacted] 1997
Report/Doc. number::	R012966 / -
Guideline(s):	OECD 410 (1981); US EPA guideline 82-2
GLP:	Yes
Deviations from OECD 419 (1981):	-adrenals not weighed -only a limited number of organs/tissues was examined microscopically
Acceptability:	Yes

Material and methods

4 Groups of 5 male and 5 female rats (strain: Crl:CD(SD)BR VAF/Plus; source: [redacted].) were exposed dermally to triticonazole (batch no.: OP9550347; purity 97.2 %; dissolved in water) at dose levels of 0 (vehicle control), 100, 300 and 1000 mg/kg bw per day. The test article was applied 6 hours per day, on seven consecutive days per week for 3 weeks (23 days). The fur on the dorsal area of the animals was clipped to reveal an area of skin, equivalent to approximately 10 % of the body surface (approx. 25 cm^2). Triticonazole was applied to a gauze patch which was moistened with the appropriate amount of reverse osmosis water (approx. 2 µl/mg test material). The gauze patch was placed on the skin and secured with non-irritating tape. The application site was covered with an elastic latex bandage. At the end of each exposure period, the treated skin area was cleaned with water to remove any residual test substance.

Observations included twice daily examinations for clinical signs and mortality, recording of body weights and food consumption (weekly) and scoring of dermal irritation (daily, immediately before each dose application). Blood samples were collected from each animal at termination of the study for haematology (haematocrit, haemoglobin concentration, RBC, MCHC, MCV, MCH, WBC total, WBC differential, platelet count) and clinical chemistry measurements (ALT, AST, gamma-GT, urea, creatinine, glucose, bilirubin, cholesterol, total protein, albumin, globulin, calcium, phosphate, sodium, potassium, chloride). Each animal was subjected to a gross pathology examination and selected organs (brain, kidneys, liver, ovaries, and testes) were weighed. Histopathological examination was performed on a range of tissues (adrenals, brain, heart, kidneys, liver, ovaries, skin [treated and untreated], spleen, testes) from each animal in the control and high dose groups.

Results

General observations: No treatment-related mortalities occurred at any dose level. One female given 1000 mg/kg was found dead on day 17. There was no obvious cause of death apparent at necropsy; therefore the death of this animal was not considered to be related to treatment.

There were no differences in mean body weights, body weight gains and food consumption.

The cutaneous application of triticonazole produced neither apparent clinical signs of systemic toxicity nor local effects on the treated skin sites.

Haematology and clinical chemistry data showed no treatment-related effects at any dose level. Small but statistically significant differences for several parameters were considered incidental and unrelated to administration because they were inconsistent between the sexes and did not exhibit a relationship to dose.

Pathology: There were no organ weight findings, and no gross necropsy or microscopic findings indicative of an adverse effect of treatment after repeated dermal application of up to 1000 mg/kg triticonazole.

Conclusion

Neither signs of systemic toxicity nor local treatment effects of the skin were observed in treated animals under the study conditions. The NOAEL was found to be 1000 mg/kg bw per day (the highest dose tested).

B.6.4. GENOTOXICITY

B.6.4.1. In vitro studies

B.6.4.1.1. Bacterial assay for gene mutation

B.6.4.1.1.1. First study

Previous evaluation: DRAR (2016)	DAR (2003) No crucial remarks on the original assessment; in the renewal assessment the study was considered of limited information since no E.coli WP2 strains or S. typhimurium TA102 were tested

Reference:	RPA 400727: Assessment of mutagenic potential in histidine auxotrophs of Salmonella typhimurium (Ames Test)
Author(s), year:	May, K., 1991
Report/Doc. number::	R013016 / -
Guideline(s):	OECD 471 (1983) and US EPA guideline § 798.5265 (1985)
GLP:	Yes
Deviations from OECD 471 (1997):	-The stability of the test compound in the solvent was not determined -No statistical analysis of the results was performed -No E.coli WP2 strains or S. typhimurium TA102 were tested
Acceptability:	Yes; limited information since no E.coli WP2 strains or S. typhimurium TA102 were tested which have an AT base pair at the primary reversion site

Material and methods

Triticonazole (batch no. DA 646; purity 97.1%) was tested in the Ames test using histidine dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA 1535, TA 1537, TA 98 and TA 100). Triticonazole (dissolved in dimethylsulfoxide; DMSO) was added at concentrations of 0 (solvent control), 25, 79, 250, 790 and 2500 µg/plate (0.1 ml of each concentration/plate) in the presence and absence of S-9 mix (liver preparations from Aroclor 1254-induced male Sprague-Dawley rats). Dose levels were established on the basis of a preliminary range finding test with the strain TA 98. (In this pre-test, triticonazole at a concentration of 2500 µg/plate induced slight thinning of the background lawn and a reduction of revertant cells.) In two separate occasions three replicates per concentration were incubated at 37°C for 2 days.

As positive controls sodium azide (2 µg/plate administered to TA 1535 and TA 100, resp.), 2-aminoanthracene (2 µg/plate administered to TA 1535), 9-aminoacridine (80 µg/plate administered to TA 1537), 2-nitrofluorene (1 µg/plate administered to TA 98), and benzo[a]pyrene (5 µg/plate to TA 1537, TA 100 and TA 98) were used.

Evaluation criteria are not given in the report.

Results

In both trials, cytotoxicity (thinning of the background lawn of non-revertant cells and reduction of revertant colony numbers) was observed in all strains at 2500 µg/plate with and without S-9 mix. However, no increases in revertant colony numbers over control counts were obtained with any of the strains tested following treatment with triticonazole at any concentration, either in the presence and absence of metabolic activation. The positive control materials elicited the expected positive responses.

Conclusion

It can be concluded that triticonazole was not mutagenic when tested at dose levels up to 2500 µg/plate in this *in vitro* test system.

B.6.4.1.1.2. Second study

A new AMES test has been submitted for the purpose of renewal. Since in the AMES from the DAR (2003) no E.coli WP2 strains or S. typhimurium TA102 were included this new study is considered necessary to complete the information on gene mutation in bacterial cells.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	BAS 595 F (Triticonazole) - Salmonella typhimurium / Escherichia coli reverse mutation assay
Author(s), year:	Woitkowiak C., 2014
Report/Doc. number::	2014/1092377 / -
Guideline(s):	OECD 471 (1997), EPA 870.5100
GLP:	Yes
Deviations from OECD 471 (1997):	-No special statistical tests were performed
Acceptability:	Yes

Materials and methods

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and a strain of *E. coli* WP2 uvrA were exposed with Triticonazole (Batch: COD-001440, Purity: 91.3%) in the presence and absence of metabolic activation for 48 hours. Vehicle (DMSO) and positive controls were included in each experiment. In the Ames standard plate test (SPT) and the pre-incubation test (PIT), the test item was tested in six concentrations in a range of 33 to 5500 µg/plate with and without S9 mix (phenobarbital/ß-naphthoflavone-induced rat liver S9 fraction).

Test Material	BAS 595 F (Triticonazole)
Description:	solid / white
Lot/Batch #:	COD-001440
Purity:	91.3% (tolerance +/-1.0%)
Stability of test compound:	The stability of the test substance is guaranteed until 01-Jan-2019
Solvent used: DMSO	

Control Materials

Negative control: sterility controls were performed in accordance with the experimental design, but without addition of bacterial suspension.

Vehicle control: The vehicle control with and without S9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.

Solvent/final concentration: 100 µL/plate

Positive control compounds tested without addition of metabolic activation system:

Strain	**Mutagen**	**Solvent**	**Conc. [µg/plate]**
TA 100	N-methyl-N'-nitrosoguanidine (MNNG)	DMSO	5
TA 1535	N-methyl-N'-nitrosoguanidine (MNNG)	DMSO	5
TA 1537	9-Aminoacridine (AAC)	DMSO	100
TA 98	4-nitro-o-phenylenediamine (NOPD)	DMSO	10
WP2 uvrA	4-Nitroguinoline-N-oxide (4-NQO)	DMSO	5

Positive control compounds tested with addition of rat metabolic activation system:

Strain	**Mutagen**	**Solvent**	**Conc. [µg/plate]**
TA 100	2-Aminoanthracene	DMSO	2.5
TA 1535	2-Aminoanthracene	DMSO	2.5
TA 1537	2-Aminoanthracene	DMSO	2.5
TA 98	2-Aminoanthracene	DMSO	2.5
WP2 uvrA	2-Aminoanthracene	DMSO	60

To demonstrate the efficiency of the S9 mix in the assay, the S9 batch was characterized with benzo(a)pyrene.

Activation

S9 was produced from the livers of approximately 5 male Wistar rats (Crl:WI(Han); source: Charles River Laboratories Germany GmbH, Germany), weighting 200 – 300 g, that received 80 mg/kg bw phenobarbital i. p. and 80 mg/kg bw ß-naphthoflavone orally on 3 consecutive days.

The rat liver S9-mix was prepared freshly prior each experiment and had the following composition:

Component	**Concentration**
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10%

Test organisms

S. typhimurium strains: TA 98, TA 100, TA 1535, TA 1537, E. coli strains: WP2 uvrA

The bacterial strains are checked for the following characteristics at regular intervals: deep rough character (rfa), ampicillin resistance (R factor plasmid), and UV-light sensitivity (absence of uvrB and uvrA genes in Salmonella and E. coli strains, respectively).

Histidine and tryptophan auxotrophy was automatically proven in each experiment via the spontaneous rate.

Test concentrations

SPT/PIT: TA 98, TA 100, TA 1535, TA 1537 and WP2 uvrA were exposed to dose levels of 0, 33, 100, 333, 1000, 2750 and 5500 µg/plate with and without metabolic activation.

Standard plate test (SPT)

A mixture of 2-mL portions of warm (42 - 45°C) soft agar (0.8% agar and 0.6% NaCl, supplemented with 10% of 0.5 mM L-histidine + 0.5 mM (+)-biotin or 0.5 mM L-tryptophan), 0.1 mL test substance preparation or vehicle, 0.1 mL fresh bacterial culture, 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL of 100 mM sodium phosphate buffer (in tests without metabolic activation) was poured onto minimal agar in Petri dishes. Each Petri dish contained about 20 mL minimal agar (1.5% agar supplemented with 2.0% salts of Vogel-Bonner Medium E and 2.0% glucose). Each concentration and the controls were tested in triplicates. After incubation at 37°C for 48 - 72 hours in the dark, the bacterial colonies (his^+ or try^+ revertants) were counted using the Sorcerer Image Analysis System with the software program Ames Study Manager (Perseptive Instruments Ltd., Haverhill, UK). Colonies were counted manually, if precipitation of the test substance hindered the counting using the Image Analysis System.

Pre-incubation test (PIT)

0.1 mL test substance preparation or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9 mix (in tests with metabolic activation) or 0.5 mL phosphate buffer (in tests without metabolic activation) were incubated at 37°C for 20 minutes using a shaker. Subsequently, 2 mL soft agar was added and the mixtures were poured onto the agar plates within approximately 30 seconds. Each concentration and the controls were tested in triplicates. After incubation at 37°C for 48 - 72 hours in the dark, the bacterial colonies (his^+ or try^+ revertants) were counted using the Sorcerer Image Analysis System with the software program Ames Study Manager (Perseptive Instruments Ltd., Haverhill, UK). Colonies were counted manually, if precipitation of the test substance hindered the counting using the Image Analysis System.

Statistics

No special statistical tests were performed.

Evaluation criteria

Generally, the experiment was considered valid if the following criteria were met:

- The number of revertant colonies in the negative controls was within the range of the historical negative control data for each tester strain.
- The sterility controls revealed no indication of bacterial contamination.
- The positive control substances both with and without S9 mix induced a distinct increase in the number of revertant colonies within the range of the historical positive control data or above.
- Fresh bacterial culture containing approximately 109 cells per mL were used.

The test substance was considered positive in this assay if the following criteria were met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. at least doubling (bacteria strains with high spontaneous mutation rate, like TA 98, TA 100 and E.coli WP2 uvrA) or tripling (bacteria strains with low spontaneous mutation rate, like TA 1535 and TA 1537) of the spontaneous mutation rate in at least one tester strain either without S9 mix or after adding a metabolizing system.

A test substance was generally considered non-mutagenic in this test if:

- The number of revertants for all test strains were within the historical negative control data range under all experimental conditions in at least two experiments carried out independently of each other.

Results

Homogeneity of the test substance preparations at room temperature was verified analytically for a period of 4 hours and was ensured by mixing before generation of the test substance preparations and application.

In SPT, a weak bacteriotoxic effect (slight decrease in the number of his^+ revertants) was occasionally observed at 2750 and 5500 µg/plate with and without S9, respectively.

In PIT, bacteriotoxicity (reduced his^- or trp^- background growth, slight decrease in the number of his^+ and trp^+ revertants) was observed depending on the strain and test conditions from about 333 µg/plate onward.

Test substance precipitation was observed from about 1000 µg/plate onward with and without S9 mix.

In the SPT and PIT experiments with and without metabolic activation no relevant increase in number of revertants was observed in any strain tested (see Table 6.4.1.1.2-1). The positive controls yielded revertant numbers in a range expected for the respective strains and thus demonstrated the sensitivity of the test system. The vehicle control induced number of revertants that was within the historical data range of each strain.

Table 6.4.1.1.2-1: Standard plate and pre-incubation tests with BAS 595 F (Triticonazole) - Mean number of revertants

Experiment 1: Standard plate test										
Strain	**TA 100**		**TA 1535**		**TA 1537**		**TA 98**		**WP2 uvrA**	
Metabol. Activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Vehicle control										
DMSO	58.0	60.7	12.0	11.7	5.0	8.0	20.0	28.7	51.7	64.7
SD	7.9	8.1	1.0	2.5	3.6	3.5	4.4	4.0	5.5	4.2
Test item [µg/plate]										
33	61.3	54.3	9.3	7.7	3.7	5.3	17.3	25.0	54.0	70.7
SD	8.0	9.3	1.2	1.5	1.2	3.1	4.2	6.2	7.2	1.5
100	70.7	62.0	11.3	7.7	6.0	5.0	14.7	29.3	52.3	66.0
SD	4.2	5.6	1.2	2.3	2.6	1.0	2.1	3.5	13.1	3.0
333	62.3	55.7	12.7	7.0	6.7	5.0	15.7	23.7	54.0	74.0
SD	6.0	14.8	3.8	1.7	0.6	3.6	1.5	5.7	6.2	8.5
1000	70.3	55.3	13.0	12.3	5.7	6.7	17.3	23.7	43.0	72.3
SD	14.8	1.5	3.5	3.1	1.5	2.9	2.3	5.5	2.0	8.0
2750	59.0	49.7	10.0	7.3	4.7	6.7	15.7	21.0	49.7	47.0
SD	1.0	13.0	3.0	1.5	0.6	1.2	2.5	3.6	1.5	36.9
5500	52.3	62.3	10.3	10.0	5.0	4.7	14.3	17.3	53.0	56.7
SD	8.1	2.5	1.5	2.0	1.0	0.6	2.5	1.5	4.6	1.5
Pos. control										
§	5178.0	1568.3	5894.0	203.7	2413.7	185.3	393.7	1622.0	925.3	157.0
SD	257.6	92.4	64.2	5.0	296.4	37.8	44.2	41.3	11.5	9.0
Experiment 2: Pre-incubation test										
Strain	**TA 100**		**TA 1535**		**TA 1537**		**TA 98**		**WP2 uvrA**	
Metabol. activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Vehicle control										
DMSO	63.0	65.3	9.3	8.3	7.3	8.3	18.3	29.7	44.0	47.7
SD	12.5	4.5	1.2	1.5	3.5	0.6	3.1	2.5	6.0	5.5
Test item										

33	57.0	59.7	9.3	7.3	8.3	7.3	19.0	22.0	33.0	50.7
SD	1.7	2.3	3.2	1.2	3.5	2.5	5.3	5.0	5.3	9.5
100	62.0	54.3	10.0	7.0	6.7	9.0	24.3	25.3	34.7	42.3
SD	4.4	7.6	4.4	1.0	3.2	2.6	4.5	7.8	5.0	9.5
333	68.7	55.3	11.3	10.7	3.3	6.7	24.0	24.0	30.0	47.3
SD	6.4	8.1	4.0	0.6	1.2	1.5	4.4	2.6	9.8	4.5
1000	49.0	65.0	7.3	8.7	4.7	5.0	17.0	23.7	28.7	35.3
SD	7.0	3.0	3.1	2.1	1.2	1.0	3.6	6.0	2.5	3.8
2750	35.0	51.3	8.0	7.7	3.7	2.7	13.0	22.7	23.7	41.0
SD	4.4	7.0	2.0	0.6	1.5	1.2	4.4	4.2	4.7	1.7
5500	38.3	35.0	7.3	3.7	3.0	2.3	14.0	18.7	27.7	32.3
SD	7.1	9.0	1.5	1.5	1.0	1.5	3.0	1.5	7.1	5.5
Pos. control										
[§]	2930.0	2091.7	962.3	138.0	1701.0	151.0	399.3	1387.3	380.3	128.7
SD	88.1	108.5	226.6	25.9	113.1	10.4	9.1	70.8	73.6	21.2

[§] = Compound and concentrations please see Material and Methods

Conclusion

Based on the results of the present study, the test substance BAS 595 F (Triticonazole) is not mutagenic in the Ames standard plate test and pre-incubation test under the experimental conditions chosen.

B.6.4.1.1.3. Third study

An additional Bacterial assay for gene mutation (2015), with technical material and additionally spiked impurities has been included in the dossier. Since the content of the impurities is given in the title of the study, the study has been evaluated in Volume 4. The results were all throughout negative.

B.6.4.1.2. Test for clastogenicity/aneugenicity in mammalian cells

B.6.4.1.2.1. First study

Previous evaluation: DRAR (2016)	DAR (2003) Only some additional information on deviation from OECD 473 added. Conclusion of the original assessment not changed

Reference:	In vitro assessment of the clastogenic activity of RPA 400727 in cultured human lymphocytes
Author(s), year:	Dance C. A., 1992
Report/Doc. number::	R013062 / -
Guideline(s):	OECD 473 (1983)
GLP:	Yes
Deviations from OECD 473 (1997):	- no reduction of 50% or more in the mitotic index achieved in the presence of S9 mix-Only one sampling time was included; -the number of metaphases scored is in excess of that required by the mentioned guideline
Acceptability:	Yes

Material and methods

Cultures of human lymphocytes from a single male donor were exposed to triticonazole (batch no DA646; purity 97.1 %, dissolved in DMSO) without and with metabolic activation (liver preparations from Aroclor 1254-induced male CD rats) and the lymphocytes examined for chromosomal damage. The concentrations used in the main cytogenetic study (0, 10, 20, 40, 50 and 60 µg/ml without S-9, and 0, 125, 250 and 500 µg/ml with S-9) were based on a preliminary toxicity test with concentrations of 62.5, 125, 250, 500 and 1000 µg/ml tested.

In the cytogenetic study, triplicate cultures per concentrate were incubated either for 24 hours (without S-9) or 3 hours (with S-9) followed by a recovery period of 21 hours. In each case, colcemid was added (0.5 µg/ml) 3 hours before the end of the incubation. As positive control substances, chlorambucil (2 µg/ml) and cyclophosphamide (6 µg/ml) were used for the non-activation test and for metabolic activation test, resp.

Evaluation criteria: The mitotic indices were calculated for each culture based on the number of metaphases observed per 1000 cells scored. Cytogenetic analysis was based on scoring 100 metaphases per culture (300 metaphases per concentration) and the frequency of structural aberrant cells (excluding and including gaps) statistically analysed (Fisher's Exact test).

Results

In the preliminary cytotoxicity test, precipitation of the test material was apparent in cultures treated at 500 and 1000 µg/ml. In the presence of S-9 mix, no significant toxicity was seen in all cultures treated with triticonazole at concentrations $\leq$ 250 µg/ml; but reductions in mitotic activity of approx. 25 % and 37 % of solvent control were evident at 500 and 1000 µg/ml, resp. In the absence of S-9 mix, marked cytotoxicity was apparent at all test concentrations with reductions in mitotic activity of approx. 81 %, 96 %, 72 %, 82 % and 80 % at 62.5, 125, 250, 500 and 1000 µg/ml, resp.

In the cytogenetic study, precipitation of test material was apparent in cultures treated with triticonazole at 500 µg/ml. In the absence of S-9 mix, a dose-related cytotoxicity was apparent at the higher concentrations tested (40, 50 and 60 µg/ml) with reductions in mitotic index (compared to solvent control) of 14 %, 27 % and 50 % resp. No toxicity was evident at the lower concentrations; therefore slides from these cultures were not analysed for chromosomal damage. In the presence of S-9 mix, there was no evidence of toxicity at any concentration tested.

Metaphase analysis revealed no biologically or statistically significant increases ($p>0.05$) in the frequency of cells with structural aberrations including or excluding gaps in cultures treated with triticonazole with and without metabolic activation (table 6.4.1.2.1-1). Clear increases of chromosome aberrations were apparent in both positive controls demonstrating the sensitivity of the test system.

Incidence of polyploid cells: No increase in the incidence of polyploid cells was observed in cultures treated with triticonazole in the absence of S-9 mix. In the presence of S-9 mix, some increase in the number of polyploid cells (outside the historical control range of 0 – 3 per culture) was noted in two cultures treated with 250 µg/ml and, to a lesser extent, in one culture at 500 µg/ml. However, also in one solvent control culture (without S-9 mix) a higher incidence of polyploid cells (4) than in the HCD was noted.

It was suggested by the study author that the increased incidence of polyploidy might be indicative of an effect on the spindle formation or function or may be due to a non-specific effect associated with precipitation of the test compound.

However, it has to be noted that there was no clear dose-response in the absence of any effect on the mitotic index seen in this study. Also, no such effect on numerical aberrations was evident in the more recent chromosomal

aberration study in human lymphocytes (*Marshall R.; 1997*) with a comparable concentration range tested (the study described below). Therefore, the treatment-related significance of this finding in in this study can be questioned.

Table 6.4.1.2.1-1: Mitotic indices and mean % cells with chromosomal aberrations in cultured lymphocytes treated with triticonazole

Treatment (µg a.i./ml)	without metabolic activation				with metabolic activation			
	Mitotic index # (mean)	mean % cells with aberrations incl. gaps	excl. gaps	number of polyploid cells Δ	Mitotic index # (mean)	mean % cells with aberrations incl. gaps	excl. gaps	number of polyploid cells Δ
0 (solvent)	13.9	1.7	0.3	4,0,0	12.1	1.7	0.0	0,0,0
Triticonazole 40	11.9	2.7	0.0	0,1,0	-	-	-	-
50	10.2	2.3	2.0	2,0,1	-	-	-	-
60	6.9	3.7	0.7	1,1,0	-	-	-	-
125	-	-	-	-	17.5	0.7	0.7	0,0,0
250	-	-	-	-	13.0	3.0	1.0	9,1,7
500	-	-	-	-	11.6	4.0	1.0	0,3,5
Positive control Chlorambucil	12.3	35.0***	27.3***	1,1,0	-	-	-	-
Positive control Cyclophosph.	-	-	-	-	13.1	22.3***	17.0***	2,0,0

mitotic index = number of metaphases x 100
number of lymphocytes
*** = ($p \leq 0.001$) significantly different from controls
Δ number of polyploid cells per culture (3 cultures examined) when scoring 100 metaphases

Conclusion

It was concluded that triticonazole, under the conditions of the test, caused no increase in the incidence of structural chromosomal aberrations. The apparent increase in polyploid cells at 250 and 500 µg/ml, without any dose-response but above historical control solvent data cannot be easily interpreted.

B.6.4.1.2.2. Second study

Previous evaluation: DRAR (2016)	DAR (2003) Results of the original assessment so far slightly changed that the outcome of the first experiment without S9 is now considered to be equivocal (considered negative in DAR 2003). Some details (historical control data, separation of replicates for better understanding, etc..) added in the results. Remark: a batch of much lower purity (90.9%) than the technical specification ($\geq$ 95%) was used for the study

Reference:	Triticonazole: Induction of chromosome aberration in cultured human peripheral blood lymphocytes
Author(s), year:	Marshall R., 1997
Report/Doc. number::	R01210 / -
Guideline(s):	OECD 473 (1983)
GLP:	Yes

Deviations from OECD 473 (1997):	No
Acceptability:	Yes

Material and methods

Cultures of human lymphocytes (stimulated to divide by treatment with phytohaem-agglutinin) from a male [first experiment] and a female donor [second experiment] were exposed to triticonazole (batch no. OP9550347; purity: 90.9 %; dissolved in DMSO) with and without metabolic activation (liver preparations from Aroclor 1254-induced male Sprague-Dawley rats) in two independent experiments at concentrations of 0 (solvent control), 7.751, 11.07, 15.82, 22.6, 32.28, 46.12, 65.88, 94.12, 134.5, 192.1, 274.4, 392.0, 560 and 800 µg/ml (limit of solubility in the vehicle) [first experiment] and 0 (solvent control), 33.79, 45.05, 60.07, 80.09, 106.8, 142.4, 189.8, 253.1, 337.5, 450, 600 and 800 µg/ml [second experiment]. All samples were run in duplicate (A, B) for each concentration tested. It is stated in the study report that lymphocytes from two healthy, none smoking volunteers (male for experiment 1 and female for Experiment 2), not being suspected of any virus infection were used.

In the first experiment, cells were exposed to triticonazole either for 20 hours (without S-9) or 3 hours followed by a recovery period of 17 hours (with S-9). In the second experiment, an additional delayed sampling time, 24 hours after the first sample, following treatment in the absence and presence of S-9 was included for the highest concentration evaluated. In each case, colchicine (1 µg/ml) was added 1.5 hours before the end of the incubation. After hypotonic treatment, fixation of the cells and staining of the slides, metaphase analysis was carried at the dose level (and the next two lower concentrations) causing a decrease in mitotic index of 50 – 80 % of the solvent control value (based on at least 1000 cells counted). As positive controls, 4-nitroquinoline 1-oxide (NQO; 1.25 - 2.5 µg/ml) and cyclophosphamide (CPA; 25 µg/ml) were used for non-activation series and for metabolic activation series, resp.

Evaluation criteria: 100 metaphase figures from each duplicate culture and 25 cells from the positive control cultures, resp., were examined. The cells were evaluated for the presence of structural chromosomal aberrations (including and excluding gaps) and numerical aberrations (polyploid, endoreduplicated or hyperdiploid cells) and statistical analysis (Fisher's exact test) was performed. The test article was considered positive if (i) a statistically significant increase in the proportion of cells with structural aberrations (excl. gaps) occurred at one or more concentrations and (ii) the proportion of cells with structural aberrations exceeded the normal range and (iii) the results were confirmed in the second experiment.

Results

Based on the evaluation of the mitotic index, concentrations of 274.4, 392 and 560 µg/ml (mitotic inhibition 51 % [- S-9] and 56 % [+ S-9] at 560 µg/ml) were used in the first experiment for analysis of chromosomal aberrations. In the second experiment, the selected concentrations for analysis were 253.1, 337.5 and 450 µg/ml (mitotic inhibition 50% [- S-9] at 450 µg/ml) and 337.5, 450 and 600 µg/ml (mitotic inhibition 49 % [+ S-9] at 600 µg/ml), resp.

In first experiment, treatment of cells with triticonazole in the absence of S-9 resulted in statistically significant increased frequencies of cells with structural aberrations at 392µg/ml and 560 µg/ml (table 6.4.1.2.2-1). The statistical analysis has been conducted on the sum of results from replicate A and B.

The number of cells with structural aberrations (without S9) at 392µg/ml and 560 µg/ml were above the results from the positive control 4-nitroquinoline 1-oxide, however lesser number of cells has been scored for the positive control.

Slightly differently than in the evaluation in DAR 2003, RMS would currently draw the conclusion that in the first experiment there were equivocal results at 392µg/ml and 560 µg/ml triticonazole (positive (above HCD) always only in one of two replicates), with the remark that at 560 µg/ml a cytotoxicity of 50% was achieved.

No effects were observed with metabolic activation.

Table 6.4.1.2.2-1: Mitotic indices and number of chromosomal aberrations in cultured lymphocytes treated with triticonazole; 1st experiment

Treatment (µg a.i./ml)	Cells scored in the two replicates	without metabolic activation			with metabolic activation		
		Mitotic index (%)	cells with aberrations incl. gaps	excl. gaps	Mitotic index (%) (mean)	cells with aberrations incl. gaps	excl. gaps
20 hour sampling time (without S-9); 3 + 17 hour sampling time (with S-9)							
0 (solvent) A	100	4.2	2	1	5.4	3	2
0 (solvent) B	100	5.1	1	1	5.4	1	1
Total solvent or mean (mitotic index)	**200**	**(4.7)**	**3**	**2**	**(5.4)**	**4**	**3**
Triticonazole							
274.4 µg/ml A	100	4.0	2	2	5.6	1	1
274.4 µg/ml B	100	5.0	2	1	5.3	1	1
Total 274.4 µg/ml or mean (mitotic index)	**200**	**(4.5)**	**4**	**3**	**(5.5)**	**2**	**2**
392 µg/ml A	100	3.9	8[C]	8[D]	4.7	3	2
392 µg/ml B	100	3.6	4	3	3.9	2	2
Total 392 µg/ml or mean (mitotic index)	**200**	**(3.8)**	**12****	**11****	**(4.3)**	**4**	**4**
560 µg/ml A	100	2.1	6	5	2.5	3	2
560 µg/ml B	100	2.5	11[C]	10[D]	2.3	2	2
Total 560 µg/ml or mean (mitotic index)	**200**	**(2.3)**	**17*****	**15*****	**(2.4)**	**5**	**4**
positive control NQO A	25	-	5	4	-	-	-
positive control NQO A	25	-	4	4	-	-	-
Total NQA	**50**	**-**	**9*****	**8*****	**-**	**-**	**-**
positive control CPA A	25	-	-	-	-	10	10
positive control CPA B	25	-	-	-	-	9	8
Total CPA	**50**	**-**	**-**	**-**	**-**	**19*****	**18*****

** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls, Fisher's exact test

C = above HCD for solvent controls of 0 to7 cells/100 cells

D = above HCD for solvent controls of 0 to5 cells/100 cells

In the second experiment (table 6.4.1.2.2-2), a total of only 137 cells (sum of both replicates) could be analysed from cultures treated with 337.5 µg/ml in the presence of S-9 and sampled at 20 hours. Since the frequency of aberrations in the cells examined was very low, and no evidence of an increase in aberrant cells was apparent at higher concentrations, this deficiency was not considered to have affected the validity at this dose level.

The only finding in this experiment was a small but statistically significant increase in cells with structural aberrations (without S-9) at the 44 hour sampling, but according to the HCD included in the report the frequency of aberrant cells fell within the historical negative control ranges (including gaps: 0-9 aberrant cells/100 cells; excluding gaps: 0-6 aberrant cells/100 cells).

Table 6.4.1.2.2-2: Number of chromosomal aberrations in cultured lymphocytes treated with triticonazole; 2nd experiment

Treatment (µg a.i./ml)	Cells scored in the two replicates	without metabolic activation			with metabolic activation		
		Mitotic index (%) (mean)	cells with aberrations incl. gaps	excl. gaps	Mitotic index (%) (mean)	cells with aberrations incl. gaps	excl. gaps
20 hour sampling time (without S-9); 3 + 17 hour sampling time (with S-9)							
0 (solvent) A	100	2.8	1	1	2.9	1	0
0 (solvent) B	100	1.8	5	1	2.0	2	1
Total solvent or mean (mitotic index)	**200**	**(2.3)**	**5**	**2**	**(2.5)**	**3**	**1**
Triticonazole							
253.1 µg/ml (A)	100	1.6	3	2			
253.1 µg/ml (B)	100	2.1	2	0			
Total 253.1 µg/ml or mean (mitotic index)	**200**	**(2.3)**	**5**	**2**			
337.5 µg/ml (-S9, total)	200	1.2	6	4	-	-	-
337.5 µg/ml (+S9, total)	137				1.9	3	2
450 µg/ml (-S9, total)	200	1.2	6	4			
450 µg/ml (+S9, total)	197				1.9	1	1
600 µg/ml (A)	100				1.2	3	3
600 µg/ml (B)	70				1.3	0	0
Total 600 µg/ml or mean (mitotic index)	**170**				**(1.3)**	**3**	**3**
positive control NQO (A)	25	-	6	5			
positive control NQO (B)	25	-	6	5			
Total NQO	**50**	**-**	**12*****	**10*****			
positive control CPA (A)	25				-	6	5
positive control CPA (B)	4				-	1	1
Total CPA	29				-	7***	6***
44 hour sampling time (without and with S-9)							
0 (solvent) (A+B)	200	3.0	0	0	4.0	2	2
Triticonazole							

Treatment (µg a.i./ml)	Cells scored in the two replicates	without metabolic activation			with metabolic activation		
		Mitotic index (%) (mean)	cells with aberrations incl. gaps	excl. gaps	Mitotic index (%) (mean)	cells with aberrations incl. gaps	excl. gaps
450 µg/ml (A+B)	200	2.6	8*[E]	4*[F]			
600 µg/ml (A+B)	200				2.1	8	2

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls, Fisher's exact test

E = below HCD for solvent controls of 0 to 9 cells/100 cells

F = below HCD for solvent controls of 0 to 6 cells/100 cells

Concerning numerical aberrations (including endoreduplication, hyperploidy and polyploidy) the frequencies of cells with aberrations seen in all test-article-treated cultures fell within the historical negative control ranges (proven and confirmed by RMS).

In all negative controls, chromosome aberration frequencies were within historical normal ranges while they were significantly increased in both positive control cultures.

Conclusion

Following two independent experiments that were performed in the absence and presence of a rat liver metabolic activation system, triticonazole did not induce numerical chromosomal aberrations in cultured human lymphocytes when tested at dose levels up to toxic concentrations. Regarding structural chromosomal aberrations RMS (2016) is of the opinion that the results, based on *weight of evidence*, were negative. This conclusion is based on the fact that equivocal results although observed in the first experiment (positive only in one of two replicates at two concentrations) were not confirmed throughout the second experiment.

B.6.4.1.3. Test for gene mutation in mammalian cells

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Investigation of mutagenic activity at the HGPRT locus in an Chinese Hamster V79 cell mutation system
Author(s), year:	Lloyd, J. M., 1991
Report/Doc. number::	R013019 / -
Guideline(s):	OECD 476 (1984)
GLP:	Yes
Deviations from OECD 476 (2015):	No
Acceptability:	Yes

Material and methods

Triticonazole (batch no. DA 646; purity 97.1 %; dissolved in DMSO) was tested for its ability to induce point mutations at the *HGPRT*-locus in Chinese Hamster V79 cells (V79-4/I clone 9 3/12) obtained from Shell Research Ltd. England. The V79 cells (7.5 x 10^5 cells per flask) were exposed to triticonazole for a period of 3 hours at concentrations of 0, 62.5, 125, 250, 500 and 1000 µg/ml without and with metabolic activation (1.5 ml liver S-9 homogenate fraction from Aroclor 1254 induced male CD rats) in two replicate cultures. These dose levels were established on the basis of preliminary toxicity test with dose concentrations of 0, 5.6, 28, 140, 700 and 3500 µg/ml

(with and without S-9). DMSO was used as negative control and ethylmethanesulfonate (EMS, 1000 µg/ml) and 7,12-dimethylbenzanthracene (10 µg/ml) were used as positive controls without and with S-9 activation resp. Immediately following treatment with triticonazole, toxicity of the test substance and the cell survival was determined by plating a cell suspension containing 100 cells/plate to assess plating efficiency (determined by colony counts).

After an expression time of 7 days at 37°C, 6-thioguanine was added to the cultures as selective agent and 3 plates/concentration/replicate were seeded with 10^5 cells/plate. In addition, cell samples for the toxicity assessment (3 plates/concentration/replicate; 200 cells/plate) were also seeded without addition of the selective agent to determine plating efficiency. After further 6 incubation days, the colonies were fixed, stained and assessed for survival and 6-thioguanine resistance (6-TG^r). Two independent tests in the absence as well as in the presence of metabolic activation were carried out.

Evaluation criteria: The plates were scored and the mutation frequency per 10^5 survivors determined as: number of cells plated for plating efficiency (relative to solvent control value) x mean number of 6-TG revertant colonies per 10^5 survivors, divided by the mean number of plating efficiency colonies. No statistical analysis was applied to the results of the study.

Results

In the preliminary cytotoxicity test, there was no evidence of dose-related cytotoxicity observed at any concentration of triticonazole tested in either activated or non-activated cultures. However, precipitation of the test material was observed at 700 µg/ml, and there was extensive precipitation at 3500 µg/ml, forming aggregates of the test material and so dose levels of 62.5 – 1000 µg/ml were selected for the mutagenicity experiment.

Also in the main mutation assay, precipitation of the test material was noted at concentrations of 500 and 1000 µg triticonazole/ml, both with and without S-9 mix. There was no indication of dose-related cytotoxicity (assessed by plating efficiency) immediately following treatment with triticonazole, and no obvious effect following the expression period at any dose level, neither in the absence nor in the presence of S-9 mix in either of the two experiments.

Exposure of V79 cells to triticonazole up to 1000 µg/ml produced no increase in 6-TG^r colony numbers or in mutant frequencies in either of the two mutation assays, compared to the solvent control. Isolated small increases in mutation frequencies in some cultures were noted: In the first assay without S-9 mix, an increased mutation frequency was observed in one replicate at 250 µg/ml compared to solvent control but this effect was neither reproduced in the second replicate of this experiment nor in the second experiment. Additionally, no dose-response was observed since the same number of colonies as in the control was observed at higher doses of 500 and 1000 µg/ml. In the mutation assays with S-9 mix, very small increases in mutation frequencies were observed in cultures exposed to concentrations of 62.5 and 125 µg/ml (first test) and in cultures treated with 250 and 500 µg/ml (second test), but no increases were seen in the respective replicate cultures. Additionally, no dose-response was observed since almost the same number of colonies as in the control was observed at the highest dose of 1000 µg/ml. No historical control data were included in the study report.

A marked positive response was seen with the positive controls EMS and DMBA

Table 6.4.1.3-1: Plating efficiency and mutant frequency of two replicates (A, B) in CH V79 cells treated with triticonazole (mean values of 3 plates/replicate)

Treatment and concentration		without metabolic activation				with metabolic activation			
		plating efficiency		mutation frequency#		Plating efficiency		mutation frequency#	
		1st test	2nd test	1st test	2nd test	1st test	2nd test	1st test	2nd test
Solvent control	A	87.9	105.5	0.3	0.3	97.0	120.9	0.0	0.2
	B	84.9	71.5	1.5	0.0	91.2	125.2	0.0	0.0
Triticonazole (62.5 µg/ml)	A	81.7	77.2	1.6	0.0	95.9	119.9	3.9	0.3
	B	93.7	74.4	0.3	1.3	85.9	109.5	0.3	0.0
Triticonazole (125 µg/ml)	A	100.7	119.7	1.0	0.0	112.2	77.9	4.7	0.4
	B	124.4	99.2	0.0	0.7	99.9	96.5	2.0	1.0
Triticonazole (250 µg/ml)	A	110.4	54.5	6.1	0.0	91.7	93.2	0.0	3.5
	B	86.4	78.5	0.3	0.0	106.4	72.9	0.7	0.0
Triticonazole (500 µg/ml)	A	85.7	126.0	0.0	0.0	107.2	89.0	0.0	4.5
	B	82.5	105.5	0.0	0.0	99.9	93.7	1.0	0.0
Triticonazole (1000 µg/ml)	A	98.9	77.9	0.0	0.0	88.5	126.5	0.0	0.0
	B	67.9	61.5	1.5	0.0	58.2	102.0	0.5	0.0
EMS (1000 µg/ml)	A	112.0	72.9	123.2	128.1	-	-	-	-
	B	76.2	91.5	120.3	84.2	-	-	-	-
DMBA (10 µg/ml)	A	130.4	105.4	0.0	0.9	71.2	85.9	89.0	94.7
	B	132.0	121.2	0.0	0.0	123.0	81.0	44.7	75.3

= mutation frequency per 10^5 survivors

Conclusion

It was concluded that, under the conditions of this test, triticonazole showed no evidence of mutagenic activity at the HGPRT gene locus, when V79 cells were treated in the absence or presence of S-9 mix.

B.6.4.1.4. Test for DNA damage and repair

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	RPA 400727: Induction of unscheduled DNA synthesis (UDS) in rat hepatocytes in vitro
Author(s), year:	Foster, B., 1992
Report/Doc. number::	R0103061 / -
Guideline(s):	OECD 482 (1986)
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and methods

Hepatocytes were isolated from male Sprague Dawley CD rats (source: Charles River UK Ltd.) and cultured for a preliminary toxicity test using concentrations of 0 (solvent control), 62.5, 125, 250, 500 and 1000µg/ml triticonazole (batch no. DA646; purity 97.1 %; dissolved in DMSO). Cultures were incubated at 37°C for 18 hours and the toxicity of the test material was then evaluated using the Neutral Red Test.

In the main UDS assay, triplicate cultures per concentration were exposed to the test substance at concentrations of 0 (solvent control), 7.81, 15.6, 31.3, 62.5 and 125 µg/ml, and [^{3}H]-labelled thymidine (10 µCi/ml) for 18 hours, washed and then incubated for a further 24 hours before fixing. A second set of cultures were treated identically but without [^{3}H] thymidine to determine viability of the cells. Two slides per concentration from cultures with [^{3}H] thymidine treatment were investigated for UDS by autoradiography, with 50 non-S phase cells per slide being scored for nuclear and cytoplasmic grain count. As positive control, 2-Acetylaminofluorene (2.2 µg/ml) was used.

Evaluation criteria: The cytoplasmic grain count (CG) from each cell was subtracted from the nuclear grain count (NG) to derive the net nuclear grain count (NNG) and the percentage of cells in repair (NNG) was calculated for each slide. The test article was considered positive if the mean net nuclear grain count was ≥ 5, or more than 20 % of cells were in repair, and values for treated groups should be significantly higher than for control group (Students t-test).

Results

In the preliminary toxicity test, precipitation was noted at concentrations of ≥ 250 µg/ml. In addition, assessment using the Neutral Red Test revealed that hepatocytes treated with 125 and 250 µg/ml showed a marked reduced viability (36.1 % and 37 %, resp.) compared with the solvent control (100 %). Cultures treated with 62.5 µg/ml showed a slight reduced viability of 90.7 %.

In the main assay, hepatocyte viability was slightly reduced at concentrations ≤ 62.5 µg/ml compared with solvent control as determined by the Neutral Red Test. However, the cells appeared healthy when viewed microscopically. At 125 µg/ml a marked decrease in viability was seen (59.3 %).

The nuclear and cytoplasmic grain counts of triticonazole-treated cultures did not differ significantly from the solvent controls (table 6.4.1.4-1). The net nuclear grain counts from cultures treated at ≤ 62.5 µg/ml were also similar to those in negative controls. The only evidence for an increase in mean net nuclear grain count was at the toxic concentration of 125 µg/ml, but this was only slight compared with vehicle control, and there was no increase in the number of cells in repair.

A clear significant increase in NNG count was seen with the positive control substance 2 AAF.

Table 6.4.1.4-1: Rat hepatocyte UDS following treatment with triticonazole (mean values of two slides/concentration)

Treatment	% Viability	Nuclear grain count	cytoplasmic grain count	Net nuclear grain count	% cells in repair
0 (solvent)	100	4.41	7.64	-3.23	0
Triticonazole 7.81 µg/ml	91.7	6.52	10.17	-3.65	2
15.6 µg/ml	86.9	5.67	9.45	-3.79	0
31.3 µg/ml	86.2	5.75	9.38	-3.63	1
62.5 µg/ml	87.6	4.50	7.90	-3.40	3
125 µg/ml	59.3	2.87	5.00	-2.13*	0

Treatment	% Viability	Nuclear grain count	cytoplasmic grain count	Net nuclear grain count	% cells in repair
positive control 2 AAF(2.2 µg/ml)	93.8	31.65**	8.18	23.47***	95

* ($p \leq 0.05$); ** ($p \leq 0.01$); *** ($p \leq 0.001$) significantly different from controls (Student's t-test)

Conclusion:

It was concluded that under the conditions of this test, there was no evidence of DNA damage leading to UDS in rat hepatocytes treated *in vitro* with triticonazole.

B.6.4.2. In vivo studies in somatic cells

Previous evaluation: DRAR (2016)	DAR (2003) No remarks on the original assessment, only information on systemic availability is added in results

Reference:	RPA 400727: Assessment of clastogenic action on bone marrow erythrocytes in the Micronucleus Test
Author(s), year:	[redacted] 1992
Report/Doc. number::	R012061 / -
Guideline(s):	OECD 474 (1983)
GLP:	Yes
Deviations from OECD 476 (1997):	No
Acceptability:	Yes

Material and methods

Triticonazole (batch no. DA 646, purity 97.1 %, suspended in 0.5 % methyl cellulose in distilled water) was administered to groups of male and female CD-1 mice (source: [redacted] by gastric intubation: Single treatments have been performed at doses of 0 (vehicle control), 25, 125 and 625 mg/kg bw. These dose levels were based on a preliminary study using dose levels of 625, 1250, 2500 and 5000 mg/kg bw in order to estimate the maximum tolerated dose (including histological evaluation of bone marrow smear).

In the main study, 5 male and 5 female mice from all dose levels were killed 24 hours after administration of the test substance, and further groups of 10 mice each having received 0 and 625 mg/kg bw, resp., were killed after 48 and 72 hours, resp. From each mouse, bone marrow smears from the femurs were obtained, and the frequencies of micronucleated cells per 2000 erythrocytes were scored. Calculated values of micronuclei per 1000 polychromatic erythrocytes were analysed statistically (Mann-Withney U-test). The ratio of polychromatic : mature cells was also calculated for each animal.

The positive control group (5 males and 5 females) received a single oral dose of chlorambucil (30 mg/kg bw) and bone marrow smears were taken 24 hours after dosing.

Evaluation criteria: A positive response is indicated by a significant increase in the incidence of micronucleated polychromatic erythrocytes compared to the incidence in vehicle control for at least one of the sampling times.

Results

In the preliminary test, all animals treated at ≥ 1250 mg/kg bw were sacrificed in extremis between 1 and 2 hours after dosing. Mice dosed at 625 mg/kg showed transient signs like hunched posture, hyperactivity and piloerection. In addition, mice treated at this dose level lost weight during the 24 hours post dosing but had regained the weight loss within 48 or 72 hours. Histological evaluation of the bone marrow smear did not show evidence of changes in the ratio of polychromatic : mature erythrocytes.

In the main study, all mice treated at 125 and 625 mg/kg bw showed transient overactivity after dosing. In addition, some mice treated at 625 mg/kg showed transient piloerection and hunched posture. There was also a slight body weight loss in six of 10 mice treated at 625 mg/kg and sacrificed after 24 hours and in nine of the ten mice given chlorambucil. No signs were seen in the vehicle control groups.

Histological investigations did not show any significant differences ($p > 0.05$) in the frequencies of micronucleated polychromatic cell in any dose group treated with triticonazole and at any of the three time points investigated (table 6.4.2-1). Group mean values were closely similar to mean control group values at all termination times. There was also no effect on the ratio polychromatic: mature erythrocytes. In mice given the positive control, a clear increase in the incidence of micronucleated polychromatic erythrocytes was seen. It is assumed that triticonazole was systematically available since in ADME studies after single oral dose of 500 mg/kg bw the exposure of bone marrow was demonstrated.

Table 6.4.2-1: Incidence of micronucleated erythrocytes and the ratio of polychromatic to normochromatic erythrocytes (group mean values)

Sampling time	Treatment	Dose (mg/kg)	Ratio p/n		Incidence mnp		Incidence mnn	
			♂	♀	♂	♀	♂	♀
24 hours	Vehicle control	-	1.0	0.8	1.0	0.2	0.0	0.2
	Triticonazole	25	0.8	1.0	0.4	0.0	0.5	1.1
		125	0.9	1.0	1.0	0.6	0.3	0.2
		625	0.9	1.0	0.8	0.4	0.9	0.4
	Chlorambucil	30	0.8	0.6	56.4**	39.1**	1.6	1.5
48 hours	Vehicle control	-	0.9	1.0	0.4	0.6	0.8	0.4
	Triticonazole	625	0.8	0.8	0.8	0.2	0.1	0.3
72 hours	Vehicle control	-	0.9	0.8	0.4	0.9	0.8	0.4
	Triticonazole	625	1.0	1.0	0.6	0.6	0.6	0.6

** significantly different from vehicle control at $p \leq 0.01$
p/n ratio of polychromatic to normochromatic erythrocytes
mnp number of micronucleated cells per 1000 polychromatic erythrocytes examined
mnn number of micronucleated cells per 1000 normochromatic erythrocytes examined

Conclusion

It can be concluded that under the conditions of this test, triticonazole did not induce chromosomal damage leading to micronucleus formation in polychromatic erythrocytes of treated mice at doses up to 625 mg/kg bw.

B.6.4.3. In vivo studies in germ cells

No study provided, not considered necessary.

B.6.4.4. Other (non-standard) genotoxicity studies

Notifier provided as a result of a literature search a publication on high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals. In this evaluation also triticonazole was included.

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals
Author(s), year:	Knight A.W. et al., 2009
Report/Doc. number::	2009/1130462/-
Guideline(s):	No
GLP:	No
Deviations from Guideline	No Guideline
Acceptability:	Yes; supplementary information

Executive Summary

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

For triticonazole negative results were observed in all three screening assays, indicating that triticonazole has no genotoxic properties supporting available *in vitro* and *in vivo* data.

Material and methods

Test Material:	Triticonazole
Description:	not specified
Batch/purity #:	not specified
Stability of test compound:	not specified

Control Materials:	
Vehicle control:	GreenScreen HC GADD45a-GFP: DMSO
	CellCiphr p53: DMSO
	CellSensor p53 RE-bla: DMSO
Positive control:	GreenScreen HC GADD45a-GFP: not specified
	CellCiphr p53: mentioned, but not specified
	CellSensor p53 RE-bla: Nultilin-3 (12 µM)
Test Organisms:	GreenScreen HC GADD45a-GFP: human lymphoblastoid TK6 cell line (GADD45a-GFP reporter strain and out-of-frame EGFP gen control strain)
	CellCiphr p53: human HepG2 cell line
	CellSensor p53 RE-bla: HCT-116 cell line
Test Concentrations:	GreenScreen HC GADD45a-GFP: 50, 100 and 200 µM
	CellCiphr p53: 0.39 – 200 µM
	CellSensor p53 RE-bla: 1.2 nM – 92 µM

Cytotoxicity assay

GreenScreen HC GADD45a-GFP:

Inhibition of cell proliferation was detected by reduction of optical absorbance.

CellCiphr p53:

Cell loss was recorded by measuring the cell counts after Hoechst 3342 staining with Arrayscan HCS Reader, and IC_{50} values were calculated.

CellSensor p53 RE-bla:

not specified

Genotoxicity assay

GreenScreen HC GADD45a-GFP:

Human *GADD45a* mediated growth arrest and DNA damage was recorded by p53 regulated induction of GFP protein via fluorescence. A control strain containing an out-of-frame EGFP gene with non-functional GFT protein was used for corrections of auto-fluorescence or non-specific induced cellular fluorescence.

CellCiphr p53:

DNA damage was recorded by measurement of p53 activation via a fluorescent anti-p53 antibody (Alexa Fluor 488). Half-maximal activity (AC_{50}) values were determined by fitting the data to the Hill equation using the Condoseo module of Genedata Screener (Genedata AG, Basel, Switzerland).

CellSensor p53 RE-bla:

Activation of the p53 controlled beta-lactamase were recorded via proprietary "GeneBLAzer" technology based on fluorescence resonance energy transfer (FRET). Data were expressed as the ratio of emissions at 460 nm/530 nm (excitation at 405 nm). For primary data analysis, readings for each titration point were first normalized relative to the Nultilin-3 control (12 µM, 100%) and wells containing the vehicle only (basal, 0%), and then corrected by applying a pattern correction algorithm using control plates containing the DMSO diluent alone. Concentration–response titration points for each compound were fitted to the Hill equation and concentrations of half-maximal activity (AC_{50}) and maximal response (efficacy) values were calculated.

Evaluation criteria

GreenScreen HC GADD45a-GFP:

If the cell density relative to a vehicle-treated control fell below 80% at 1 test concentration the compound was deemed cytotoxic and if extended over 2 or 3 concentrations, strongly cytotoxic. Otherwise the compound was considered negative for cytotoxicity.

If induction of GFP fluorescence relative to a vehicle-treated control exceeded 50% at 1 test concentration the compound was deemed genotoxic and if extended over 2 or 3 concentrations, strongly genotoxic. Otherwise the compound was considered negative for genotoxicity.

CellCiphr p53:

A positive result was concluded if the p53 AC_{50} was calculated to be below 200 µM, provided the AC_{50} was lower than the IC_{50} for cell loss/cytotoxicity at for that time point.

CellSensor p53 RE-bla:

A positive result was concluded if the p53 AC_{50} was calculated to be below 92 µM.

Results

Negative result were obtained with triticonazole in the GreenScreen HC GADD45a-GFP, CellCiphr p53 and CellSensor p53RE-bla assays tested up to 200 µM.

Conclusion

According to the results of the present study, the test substance triticonazole is not mutagenic in the GreenScreen HC GADD45a-GFP, CellCiphr p53 and CellSensor p53RE-bla assays under the experimental conditions chosen here.

B.6.5. LONG-TERM TOXICITY AND CARCINOGENESIS

B.6.5.1. Rat

Previous evaluation:	DAR (2003)

DRAR (2016)	Some additional information/tables and re-assessment of histological slides (Millar, 2015) added in the results; conclusion of the study from DAR 2003 not changed

Reference:	RPA 400727: Combined oncogenicity and long-term toxicity study by dietary administration to CD rats
Author(s), year:	[redacted] 1994
Report/Doc. number::	R013100 / -
Guideline(s):	OECD 451 (1981) and US EPA Pesticide Assessment Guidelines, Subdivision F, No. 83-5
GLP:	Yes
Deviations from OECD 451 (2009):	No
Acceptability:	Yes; The intended duration of the study was 104 weeks, but given the survival rates, the terminal sacrifice was initiated after 99 weeks for males and 100 weeks for females to ensure sufficient survival of at least 25 % for terminal investigations. It is noted that the survival rate did not meet the requirements for a 50 % survival rate at 24 months specified in the relevant guidelines in order to establish a negative result for carcinogenicity. It was stated in the report that the early termination reflects the decline in CD rats longevity noted in this laboratory and others over recent years. Although the value of the study is limited by the poor survival, detailed examination of pathological and histopathological data exhibited no convincing indication of any treatment-related hyperplastic or oncogenic response. Therefore, it can be concluded that the rats survived long enough to allow a valid interpretation of the potential oncogenicity of the test compound

Material and method:

Groups of 50 male and 50 female CD rats (source: [redacted].) received triticonazole (batch no. DA 646; purity 97 %) with the diet at dose levels of 0, 5, 25, 750 and 5000 ppm (equivalent to mean achieved doses of 0, 0.2, 1.0, 29.4 and 203.6 mg/kg bw per day [males] and 0, 0.3, 1.3, 38.3 and 286.6 mg/kg bw per day [females] for two years (99 weeks [♂], 100 weeks [♀]). Additional 15 animals/sex and dose group were sacrificed after 26 and 53 weeks of treatment.

Test diets were prepared twice weekly; concentrations, stability and homogenicity of the test diets were confirmed periodically by analysis.

All animals were inspected at least daily for morbidity, mortality and clinical signs, and weekly for a more detailed examination including palpation. Body weights were recorded at weekly intervals for the first 14 weeks of treatment and once every two weeks thereafter. Food consumption was recorded weekly.

Ophthalmoscopy was performed before commencement of treatment (all animals) and after 24, 50 and 98 weeks (control groups and high dose group animals only).

Biochemistry (total protein, albumin, globulin, calcium, phosphate, sodium, potassium, urea, creatinine, glucose, cholesterol, bilirubin, chloride, AST, ALT, AP, CPK), haematology (haematocrit, haemoglobin concentration, RBC, MCHC, MCV, MCH, WBC total, WBC differential, platelets count, reticulocyte count, prothrombin time) and urinalysis (colour, volume, pH, specific gravity, protein, glucose, ketones, bilirubin, urobilinogen, nitrite, blood cells, sediments) were conducted in 10 males and females from each dose group after 24, 52 and 76 weeks and at termination.

At the end of dosing, each animal was subjected to a gross pathology examination and selected organs were weighed (adrenals, brain, heart, kidneys, liver, lungs, ovaries, pituitary, prostata, spleen, testes, thymus, thyroid, uterus).

Histopathology was performed in a wide range of tissues (adrenals, brain, caecum, colon, duodenum, epididymides, eyes and optic nerve, femur and marrow, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes [mandibular,

mesenteric], mammary gland, oesophagus, ovaries, pancreas, pituitary, prostata, rectum, salivary glands, sciatic nerve, smooth muscle, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus and vagina) on all interim (26 or 52 weeks) and terminal phase control and high-dosed animals. In addition, the kidneys, liver and lungs were examined for all animals, and the adrenals for all animals assigned to the 26 week interim phase and for all females assigned to the 52 week interim phase. Microscopy was also performed in all animals killed or dying during the study from all groups and from all rats in respect of tissues considered to exhibit a reaction to treatment.

Results

General observations: There were no treatment-related clinical signs of toxicity at any dose level. There were also no treatment-related significant adverse intergroup differences in mortalities and survival, resp. However, a trend towards higher survival rates was evident in treated animals (table 6.5.1-1).

Table 6.5.1-1: Combined oncogenicity/toxicity study in CD rats. Number of surviving animals among the 50 rats assigned to each group and time taken to reach < 50 % survival

	0 ppm		5 ppm		25 ppm		750 ppm		5000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Survival rate (week 99 for males and week 100 for females)										
Surviving animals (%)	14/50 (28)	19/50 (38)	23/50 (46)	14/50 (28)	14/50 (28)	16/50 (32)	17/50 (34)	18/50 (36)	21/50 (42)	28/50 (56)
Time taken to reach < 50 % survival										
Weeks	85	94	96	94	93	94	87	90	94	>100

At 5000 ppm, there was a significant reduction in body weight gain of approximately -20 % and -29 % in males and females resp., during the first week of dosing when compared with the respective control values. Body weight gains of females in this group remained lower than controls throughout the study, being 79 % of controls at week 88 and 90% at week 100 while males receiving 5000 ppm were similarly but less markedly affected (92 % of controls at week 76). At the end of the study no effects on body weight gain was observed in other treated groups.

Food consumption was not affected by treatment. However, lower food conversion efficiency was evident in females receiving 5000 ppm during the first 14 weeks of treatment and in males during the first week (table 6.5.1-2).

Table 6.5.1-2: Body weight and body weight gain

	0 ppm		5 ppm		25 ppm		750 ppm		5000 ppm	
	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Body weight gain										
Week 0-1										
No of animals	80	80	80	80	80	80	80	80	80	80
bw gain (g)	64	31	65	29	65	29	64	31	51**	22**
(% of control)			102	94	102	94	100	100	80	71

	0 ppm ♂	0 ppm ♀	5 ppm ♂	5 ppm ♀	25 ppm ♂	25 ppm ♀	750 ppm ♂	750 ppm ♀	5000 ppm ♂	5000 ppm ♀
Week 0-76 (♂) and 0-88 (♀)										
No of animals	32	29	39	31	39	29	40	29	42	38
bw gain (g)	798	465	781	456	833	396	797	412	738	367**
(% of control)			98	98	104	85	100	89	92	79
Week 0-98 (♂) and 0-100 (♀)										
No of animals	14	19	23	14	16	16	17	18	22	28
bw gain (g)	698	404	732	423	748	417	771	472	690	363
(% of control)			105	105	107	103	110	117	99	90
Final body weight										
Week 98 (♂) and 100 (♀)										
bw (g)	877	558	913	571	930	570	946	623	866	515
(% of control)			104	102	106	102	108	112	99	92
Food efficiency, group mean values (%)										
Week 1	33.3	20.3	33.1	18.4	33.7	18.9	33.4	20.6	27.6	14.8
Week 1-14	14.4	8.5	14.5	8.5	14.6	8.3	14.7	8.5	14.0	7.3

** ($p \leq 0.01$) (Behrens-Fisher and Dunnett's test)

Ophthalmoscopic examination of control and high dosed animals did not reveal any treatment-related findings after 24 or 50 weeks of treatment.

There was an apparent increase in the incidence of lenticular sclerosis in males receiving 5000 ppm after 98 weeks of treatment when compared with control animals. It was, however, mentioned in the study report that the morphological characteristics were representative of the normal ageing nuclear sclerosis change in rats.

There was also a stronger increase in the incidence of posterior capsular opacity plaque of the lens in males receiving 5000 ppm compared with controls. In females no effects which could be considered treatment related were observed. Incidences of all lens findings at the ophthalmoscopic examination after 98 weeks of treatment are given in table 6.5.1-3.

Table 6.5.1-3: Combined oncogenicity/toxicity study in CD rats. Incidence of lens findings at ophthalmoscopic examination after 98 weeks of treatment

Findings in the lens	Males		Females	
	Control	5000 ppm	Control	5000 ppm
Number of animals examined	14 (100 %)	22 (100 %)	22 (100 %)	30 (100 %)
Anterior suture line opacity	7 (50 %)	6 (27.3 %)	7 (31.8 %)	13 (43.3 %)
Anterior subcapsular opacity	0	0	2 (9.1 %)	1 (3.3 %)
Posterior capsular opacity (central)	4 (28.6 %)	5 (22.7 %)	2 (9.1 %)	5 (16.6 %)
Posterior capsular opacity plaque (HCD males: 3.7-19%, females: 0-21.4%)	1 (7.1 %)	**5 (22.7 %)**	1 (4.5 %)	1 (3.3 %)
Anterior polar opacity	1 (7.1 %)	2 (9.1 %)	1 (4.5 %)	3 (10 %)
Anterior polar cataract	0	1 (4.5 %)	0	0
Arcuate opacity	0	0	3 (13.6 %)	1 (3.3 %)

Findings in the lens	Males		Females	
	Control	5000 ppm	Control	5000 ppm
Sclerosis (HCD males: 0-11%, females: 0-20%)	1 (7.1 %)	**9 (40.9 %)***	15 (68.1 %)	21 (70 %)
Nuclear sclerosis	1 (7.1 %)	0	1 (4.5 %)	1 (3.3 %)
Ventral forward protrusion	0	0	1 (4.5 %)	0
Obscured (due to keratitis or poorly) dilated pupil	0	3 (13.6 %)	0	0

* (p=0-03) (Fisher's exact test)

In three position papers (*Bouvier G., 1998; Pallen, 2002; Renault D., 2002*), historical control data concerning the findings sclerosis and posterior capsular opacity plaque in CD rats have been provided: Only few studies performed with CD rats are available for the appropriate time period (1989 – 1993) at the same laboratory. It was stated that historical control data showed that the control incidence of lenticular sclerosis in CD rats was ranging from 0 % – 11 % in males and 0 % – 20 % in females. While in males the incidence of sclerosis in the control group was within HCD, the incidence in females of the concurrent control group (68.1%) exceeded the HCD remarkably (3-fold). It is stated in the position paper that also the longer survival rate of males treated at 5000 ppm most probably contributed to the late development of this age-related lesion. This view is supported by the fact that 21 animals were still alive at the end of the study in the 5000 ppm-treated group compared to 14 animals in the control group. In the position paper *Bouvier, 1998* it is stated that the observed sclerosis of the lens is present as a hardening of the nucleus of the lens but is visible only as a spot in the centre of the lens of equivalent size to the foetal nucleus of the lens. It is added that this is a different change comparing to the characteristic lens sclerosis noted in dogs and humans.
Concerning the posterior capsular opacity (PCO), the incidence in the lens of male rats (22.7 %) was slightly above the upper limit of historical control data in CD rats (3.7 % – 19 % in males, 0 % – 21.4 % in females). No statistical significance was demonstrated for this finding and it was considered therefore an incidental finding.

Overall, it can be concluded that both posterior capsular opacity plaque and sclerosis of the lens might represent normal ageing changes in CD rats and need not be regarded as compound-related. This assumption is supported by the fact that these observations appeared only after 98 weeks but were not evident during the intermediate examinations at 24 and 50 weeks. It is also noteworthy that no associated histopathological changes were reported in the eye of any examined animal, including those which exhibited PCO or nuclear sclerosis at opthamological examination.

Haematological analysis of blood samples obtained after 76 and 97 weeks of treatment indicated, in comparison with controls, significantly lower platelet counts (-16 % at week 76 and –19 % at week 97) and a longer prothrombin time in females receiving 5000 ppm at week 76 and 97. Platelet counts in males receiving 5000 ppm were also slightly but significantly lower after 24 (-10 %) and 76 weeks (-17 %) of treatment. Variations of these parameters in other treated groups were seen on isolated occasions, but no clear pattern was observed. Also other inter-group differences occasionally attained statistical significance but were not considered to be of toxicological relevance. There were no haematological findings that were considered treatment-related at 5, 25 and 750 ppm.

Clinical chemistry analyses revealed slightly but consistently and significantly lower plasma ALT activities in animals receiving 5000 ppm. A similar change was apparent among animals receiving 750 ppm after 50 and 76 weeks of treatment, although statistical significance was not attained. The plasma AST activity of animals receiving 5000 ppm was slightly but consistently lower than controls, although statistical significance was not reached on any occasion (table 6.5.1-4). The reduction in transaminase ALT and AST was considered as very minor at 5000 ppm and lacked dose-response in lower doses. In general, no clear dose-response was observed and the adversity of slightly decreased liver enzymes, for which no comparable disease in human is known, is toxicologically doubtful.

There were significantly lower cholesterol concentrations at week 76 in males receiving 5000 ppm and in females at week 76 and 97 in almost all dosed groups. However, throughout the whole study no evidence of a clear dose- and time-related pattern was observable and the observed decreases and increases were considered of minor extent. Since the fungicidal MoA of triticonazole is inhibiting ergosterol production and fungal cell wall synthesis it cannot be excluded that some of the observed effects on decrease in cholesterol might be treatment-related. However, based on very minor change the adversity is questionable. Also the effects observed in eyes of rats (one of the organs with the highest need for cholesterol) did not correlate with cholesterol level – while statistically significantly decreased cholesterol was observed in females of almost all treated groups, no effects in eyes of females treated with 5000 ppm could be considered treatment-related.

There were no other haematological or clinical chemistry finding considered to be associated with treatment in any dose group.

Table 6.5.1-4: Clinical chemistry findings (group mean values)

	Dose group level (ppm)									
	Male					**Female**				
	0	**5**	**25**	**750**	**5000**	**0**	**5**	**25**	**750**	**5000**
Alanine amino transferase, ALT (iU/l)										
week 24	34	35	31	34	**25**[b]	30	32	30	28	**22**[a]
(% control)		(103)	(91)	(100)	(74)		(107)	(100)	(93)	(73)
week 50	43	42	50	28	24	44)	49	46	34	**29**[a]
(% control)		(98)	(116)	(65)	(56)		(111)	(105)	(77)	(66)
week 76	49	99	38	28	27	42	43	37	29	**28**[a]
(% control)		(202)	(78)	(57)	(55)		(102)	(88)	(69)	(67)
week 97	43	**25**[a]	28	31	**24**[a]	41	29	32	39	**25**[a]
(% control)		(58)	(65)	(72)	(56)		(71)	(78)	(95)	(61)
Aspartate amino transferase, AST (iU/l)										
week 24	97	100	80	97	83	76	86	76	78	67
(% control)		(103)	(82)	(100)	(86)		(113)	(100)	(103)	(88)
week 50	88	91	95	78	76	97	117	111	95	84
(% control)		(103)	(108)	(89)	(86)		(120)	(114)	(98)	(87)
week 76	97	225	83	84	79	89	92	79	89	76
(% control)		(232)	(86)	(87)	(81)		(103)	(89)	(100)	(85)

	Dose group level (ppm)									
	Male					Female				
	0	**5**	**25**	**750**	**5000**	**0**	**5**	**25**	**750**	**5000**
week 97 (% control)	83	74 (89)	83 (100)	73 (88)	72 (87)	83	75 (90)	74 (89)	99 (119)	70 (84)
Total cholesterol (mg/dL)										
week 24 (% control)	61	63 (103)	66 (108)	75 [a] (123)	68 (111)	94	78 (83)	106 (113)	79 (84)	118 (126)
week 50 (% control)	103	120 (117)	129 (125)	91 (88)	102 (99)	117	114 (97)	107 (91)	135 (115)	110 (94)
week 76 (% control)	134	110 (82)	120 (90)	137 (102)	78[b] (58)	157	111[a] (71)	103[b] (66)	101[b] (64)	116[a] (74)
week 97 (% control)	194	155 (80)	171 (88)	166 (86)	158 (81)	192	185 (96)	112[a] (58)	130[a] (68)	130[a] (68)

a ($p \leq 0.05$); b ($p \leq 0.01$); significantly different from controls (Student's t-test)

There were no significant results recorded at urinalysis throughout the study. However, generally lower urinary volumes and higher specific gravities among animals receiving 5000 ppm were noted at most occasions, although the differences did not always attain statistical significance.

Pathology: There were no clearly treatment-related macroscopic abnormalities detected at any of the scheduled necropsies or at the necropsies of premature decedents.

Organ weight analysis revealed slightly but significantly increased relative liver (+23 %; $p<0.01$) and spleen weights (+21 %; $p<0.01$) in females receiving 5000 ppm sacrificed after 26 weeks of treatment. The absolute weights were also slightly higher than controls in these animals but statistical significance was not attained. Males at this interim sacrifice were not affected. There were no treatment-related effects on organ weights of either of these organs after 52 weeks or at termination.

Histopathological examination at the interim sacrifices after 26 and 53 weeks of treatment revealed no neoplastic findings considered related to treatment.

Non-neoplastic findings were limited to the adrenals of animals treated with 5000 ppm. After 53 weeks of treatment, chronic inflammation was observed in the *zona fasciculata* of 4/14 females receiving 5000 ppm (compared to 0/15 in the control group). A higher incidence of multinucleated cells in the *zona fasciculata* were noted in 9/15 females after 26 weeks of treatment (compared to 0/15 in controls) and in 3/14 females after 53 weeks of treatment (compared to 0/15 in controls). Additionally, there was a higher incidence of cortical fatty vacuolation in males after 26 and 53 weeks of treatment, although statistical significance was not achieved.

At the terminal phase, no significant increases in the incidence of findings in the adrenals were observed in any group. It was suggested by the author of the study, that the degenerative changes in the adrenal cortex (showing the highest incidence after 26 weeks of treatment) were associated with an early insult, although the mechanism of

action on the adrenals is unclear. (Degenerative changes in the adrenal cortex were also noted in the 13-week study in CD rats; B.6.3.1.1). In 2015 the notifier provided again an external pathologist (*Millar, P.M.,* 2015) with the old histological slides (all female controls and females given 5000 ppm from the 26 week and 53 week sacrifices and twenty-five control females and twenty-five females given 5000 ppm from animals assigned to the terminal sacrifice) for reassessment/confirmation of evaluation from 1994. Most of the findings from 1994 were confirmed but also new findings/incidences identified (included in table 6.5.1-5). The external pathologist provided following assessment:

- In females assigned to the 26 week sacrifice the presence of multinucleated cells, again interpreted as macrophage giant cells, was confirmed. However, within the area of the junction between *reticularis* and *fasciculata* 9/15 females given 5000 ppm were considered to have minimal degenerative changes similar to those observed in the multigeneration and 13 week studies. The lower incidence and severity of the findings, when compared to those observed in the multigeneration or 13 week studies was most likely a result of the lower dosage employed. Minimal mononuclear inflammatory cell infiltrates, increased pigment deposition and minimal or mild hypertrophy of the reticularis were also present in these animals. Thus the same lesion was observed at this sacrifice as in previous studies but at a much reduced severity.
- At the 53 week sacrifice re-examination confirmed the presence of multinucleated cells in 3/14 animals. In addition, after careful examination at high power, minimal degenerative changes at the junction between reticularis and fasciculata were considered to be present in only 3/14 females given 5000 ppm. Ten of the fourteen females, from the same dosage group, had minimal or mild hypertrophy of the zona reticularis. A finding that had not previously been reported but might be an early indicator of the pathological process
- In females assigned to the terminal sacrifice it was noticeable that the incidence of the common spontaneous background changes of cortical hypertrophy and cortical haemorrhagic degeneration were more prevalent in controls than females given 5000 ppm (N.B. the latter finding appeared to be used by the original pathologist to encompass the diagnoses cystic degeneration and telangectasis that are now more usually recorded as separate diagnoses). No clear evidence of treatment-related degenerative changes were observed

Non-neoplastic findings at the terminal sacrifice considered to be treatment-related were confined to the liver and the lungs: In the liver, a significant increased incidence of centriacinar fatty vacuolation was reported for females receiving 5000 ppm and some evidence (no statistical significance was achieved) of increased fatty vacuolation of hepatocytes in males at 5000 ppm and in females at 750 ppm. (However, no special staining techniques, e.g. to confirm the presence of fat were used.) The lung findings were a significant increase in the incidence of accumulation of alveolar macrophages in females receiving 5000 ppm, but as it was only observed in a few animals of one sex, it was considered of doubtful toxicological significance.
Group incidences of relevant non-neoplastic findings are given in table 6.5.1-5.

Table 6.5.1-5: Group incidences of relevant non-neoplastic findings at the interim and terminal sacrifice (scheduled and unscheduled sacrifice); original evaluation from the study report ([redacted], 1994) and re-evaluation Millar 2015

Findings	Dose level (ppm)									
	Males					Females				
	0	5	25	750	5000	0	5	25	750	5000
Adrenals (26 weeks), [redacted], **1994**										
multinucleated cells	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	**9/15[c]**
cortical fatty vacuolation	4/15	2/15	2/15	3/15	7/15	0/15	0/15	1/15	0/15	1/15
(26 weeks), **Millar, 2015**										
degeneration of the junction between *reticularis* and *fasciculate*						0/15				9/15
Minimal mononuclear inflammatory cell infiltrates, increased pigment deposition and minimal or mild hypertrophy of the *reticularis*						0/15				9/15
(53 weeks) [redacted], **1994**										
multinucleated cells	0/14	-	-	-	0/14	0/15	0/15	0/14	0/14	3/14
chronic inflammation	0/14	-	-	-	0/14	0/15	0/15	0/14	0/14	**4/14[a]**
cortical fatty vacuolation	1/14	-	-	-	3/14	1/15	0/15	0/14	0/14	0/14
(53 weeks), **Millar, 2015**										
minimal or mild hypertrophy of the zona *reticularis*						0/15				10/14
minimal degenerative changes at the junction between *reticularis* and *fasciculata*						0/15				3/14
(terminal), [redacted], **1994**										
multinucleated cells	0/50	0/35	0/42	0/40	0/50	0/50	0/46	0/48	0/46	3/50
cortical fatty vacuolation	11/50	10/35	7/42	10/40	13/50	8/50	4/46	4/48	6/46	11/50
Liver (terminal)										
centriacinar fatty vacuolation	6/50	3/50	5/50	5/50	9/50	16/50	15/50	11/50	23/50	**33/50[b]**
Lungs (terminal)										
accumulation of alveolar macrophages	2/50	1/50	0/50	1/50	2/50	0/50	3/50	1/50	1/50	**7/50[a]**

a = ($p \leq 0.05$); b = ($p \leq 0.01$); c = ($p \leq 0.001$) significantly different from controls (Fisher's exact test)

Concerning neoplastic findings, statistically significant increases in the incidences of benign lesions were limited to the pituitary gland and the skin:

In the pituitary gland, benign adenomas were observed in males (29/50; $p < 0.05$) treated at 5000 ppm compared to 19/50 in control males. It was stated in the report that the statistical significance for this tumour is coincidental due to an unusually low incidence in control animals. Historical control data (studies from the same laboratory, studies conducted from 1991 to 1993) concerning this type of tumour have been submitted (*Renault D., 2002).* The incidence in the males receiving 5000 ppm (58 %) is within the historical range of this tumour for this strain of male rats at this laboratory when full length studies (104 weeks) were considered (30.7 – 62 %), but slightly outside the historical range of 36.7 – 54.5 % for abbreviated studies (90 – 93 weeks). However, the incidences of this finding in the intermediate dose groups showed no indication of any relationship to dose (see table 6.5.1-6). Therefore, the

slightly higher incidence of this benign tumour, which is well known as a spontaneous age-related lesion in the rat was not considered to be of biological relevance. Among animals which died or were killed during the treatment period there was also a statistically significantly higher incidence of benign pituitary adenomas among females treated at 750 ppm, but it was suggested in the study report that the lack of dosage-relationship and the lower incidence of histogenically related carcinomas among these animals indicate this finding also does not have biological significance.

In the skin, incidences of keratoacanthomas in males that received 5 and 5000 ppm achieved statistical significance. If the incidence of keratoacanthoma were considered together with the incidence of the histologically similar benign papilloma of the skin, no significant differences were attained. It was concluded in the study that this fact, together with the lack of a dose-relationship confirms that the tumour is very unlikely to be related to treatment with triticonazole.

Besides the findings observed in the pituitary and skin, the only remarkable observation was an increased incidence (not statistically significantly different to control, no positive trend in the trend-test) of benign follicular cell adenomas noted in the thyroids of males treated at 5000 ppm. However, this finding was considered very unlikely to be treatment-related because:

- Tumour type and background incidence: The incidence of thyroid follicular adenomas in male rats was only slightly above the historical control range and no statistical significance (not statistically significantly different to control, no positive trend in the trend-test) or dose-response was observed
- Multi-site responses: In no organ the effects were considered treatment-related, so no multi-site response was observed.
- Progression of lesions to malignancy: Only benign tumours were observed.
- Whether responses are in a single or in both sexes: Only males in the high dose were affected slightly above HCD
- Whether responses are in single species or several species: Increased incidence of thyroid follicular cell adenoma was observed only in male rats but not in female rats or mice
- Pattern from HCD (higher incidence in males than in females) was reflected in the study results
- Thyroid was not the target organ of triticonazole in any of the submitted studies
- No increase in precursor leasions, such as follicular cell hypertrophy was observed
- No effects on thyroid hormone receptors and no inhibition on TPO or deiodinase type 1 enzyme activity was observed in the US EPA ToxCast screening programme

All other tumours were of the types commonly seen in CD rats and occurred with the expected frequency. It is noteworthy to mention that neither the evidence for treatment-related adrenal cortical adenocarcinoma (males: 0/50, 0/35, 0/42, 0/40, 0/50; females: 1/50, 1/46, 0/48, 1/46, 0/50), adrenal cortical adenoma (males: 0/50, 1/35, 0/42, 0/40, 1/50; females: 3/50, 1/46, 0/48, 0/46, 3/50) nor for adrenal medullary phaeochromocytoma (males: 5/50, 6/35, 5/42, 3/40, 4/50; females: 1/50, 2/46, 0/48, 0/46, 1/50) was observed.

Table 6.5.1-6: Group incidences of relevant neoplastic findings at the terminal phase

Findings	Dose level (ppm)									
	Males					Females				
	0	5	25	750	5000	0	5	25	750	5000
Pituitary (animals killed or dying during the treatment period)										
number examined	38	30	38	36	30	32	38	32	33	23
-Adenomas	13	16	19	18	17	21	25	23	**29[a]**	16
-Carcinomas	0	1	0	2	0	3	5	1	1	2
Pituitary (all animals)										
number examined	50	38	43	43	50	49	45	44	44	50
- Adenomas	19	24	24	25	**29[a]**	32	31	34	40	32
(%)	(38)	(63)	(56)	(58)	(58)	(65)	(69)	(77)	(90)	(64)
HCD 104 weeks studies from 1987 – 1993: males: 30.7-60.2%										
- Carcinomas	0	1	2	2	0	3	5	1	1	2
Skin (all animals)										
number examined	20	22	21	24	27	9	11	10	10	8
-Papilloma	4	2	1	1	3	0	1	1	0	0
-Keratoacanthoma	0	**5[a]**	2	2	**6[a]**	0	1	0	0	1
(%)		(23)			(22)					
Thyroid (all animals)										
Number examined	47	30	34	39	49	49	38	34	35	50
Follicular cell adenoma	3	1	0	0	7[(i)]	1	0	1	1	1
(%)	(6.4)	(3.3)	(0)	(0)	(14.3)	(2.0)	(0)	(2.9)	(2.9)	(2.0)
HCD 104 weeks studies from 1987 – 1993: males: 2 – 10%, females: 0 – 4.2%										

a = ($p \leq 0.05$) significantly different from controls (Fisher's exact test)

(i)= neither statistically significantly different to control (Fisher's exact test) nor any positive trend in (one-sided) Cochran-Armitage Trend-test using STATXACT

Conclusion

The continuous dietary administration of triticonazole to CD rats during lifetime period produced clear evidence of toxicity at 5000 ppm with reduction in body weight gains and food efficiency, more specifically in females. Statistically significant treatment-related findings in clinical chemistry parameters were reported in both sexes but they were minor and frequently not time- and dose-related. For changes in the eye lens (evident in males at 5000 ppm after 98 weeks of treatment) it cannot be completely excluded that they are also an effect of treatment, although most of the eye effects were considered to be normal age-related changes. Significant treatment-related non-neoplastic lesions were noted in the adrenal cortex (interim sacrifice, females only) and in the liver of high dose females (5000 ppm) at terminal sacrifice.

Statistically significantly increased incidences of benign pituitary adenoma (within historical control data) were noted in males at 5000 ppm, and of keratoacanthoma of the skin in males treated at 5 or 5000 ppm. However, these findings were considered to be coincidental and not indicative of an oncogenic potential. Slightly increased incidence (above HCD) of thyroid follicular cell adenoma in males of high dose group was considered to reflect the spontaneous occurrence of this finding.

The NOAEL for this study was confirmed by RMS 2016 to be 750 ppm (29.4 – 38.3 mg/kg bw per day) based on decreased bodyweight gain and significant histopathological findings in the liver and adrenals evident at 5000 ppm.

B.6.5.2. Mouse

Previous evaluation:	DAR (2003)
DRAR (2016)	Some additional information/tables added in the results; conclusion of the study from DAR 2003 not changed

Reference:	RPA 400727: Oncogenicity study by dietary administration to CD-1 mice for 78 weeks
Author(s), year:	[redacted] 1994
Report/Doc. number::	R013143 / -
Guideline(s):	OECD 451 (1981) and US EPA Pesticide Assessment Guidelines, Subdivision F, No. 83-5
GLP:	Yes
Deviations from OECD 451 (2009):	No
Acceptability:	Yes;

Material and methods

Groups of 52 male and 52 female CD-1 mice (source: [redacted]) were treated with triticonazole (batch no. DA646; purity 96.4 – 97.1 %) via diet at dose levels of 0, 15, 150 and 1500 ppm (equivalent to 0, 1.8, 17.4 and 202.2 mg/kg bw per day in males and 0, 2.1, 20.1 and 209.5 mg/kg bw per day in females) for 78 weeks. Additional 16 animals/sex and dose group were sacrificed after 26 weeks of treatment.

Test diets were prepared each week; concentrations, stability and homogenicity of the test diets were confirmed periodically by analysis.

Animals were observed daily for mortalities, clinical signs and behavioural pattern. All animals were also given a more detailed clinical examination weekly. Body weights were recorded before treatment, at weekly intervals until week 15 and once every two weeks thereafter and at necropsy. Food consumption by each cage was determined at weekly intervals throughout the study period. After 24 weeks of treatment, ophthalmoscopic examination was performed on both eyes of all mice from the control groups and test groups receiving 1500 ppm scheduled for interim sacrifice.

Blood smears for haematological investigations were obtained after 50 and 76 weeks of treatment from all animals of the control and high dose treated groups and differential leucocyte counts were performed. In addition, at necrospy blood samples from ten male and ten female mice from each dose group were taken and detailed haematological investigations conducted (haematocrit, haemoglobin concentration, RBC, MCHC, MCV, MCH, WBC total, WBC differential, platelets count).

At terminal necropsy, the weights of selected organs (adrenals, brain, heart, kidneys, liver, lungs, spleen, testes, uterus) were recorded. Histopathology was performed in a full range of tissues from all animals in the control and

high dose groups (adrenals, aorta, brain, caecum, colon, duodenum, epididymides, eyes and optic nerve, femur including marrow, gall bladder, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, mammary gland, oesophagus, ovaries, pancreas, pituitary, prostata, rectum, salivary glands, sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord, spleen, sternum, stomach, testes, thymus, thyroid with parathyroid, trachea, urinary bladder, uterus and vagina), from animals that died or were killed in extremis, and on lungs, liver and kidneys from animals in the low and intermediate dose groups.

Results

General observations: There were no treatment-related clinical signs or changes in the incidence of palpable swelling at any dose level.

At the end of the treatment period at week 78, the survival rates of males were 63 %, 62 %, 50 % and 58 %, and of females 73 %, 73 %, 73 % and 88 % in the control, low, intermediate and high dose groups, resp. No treatment-related effects on the distribution and timing of premature deaths were reported.

Body weight: In animals receiving 1500 ppm, lower body weight gains were reported during the majority of the dosing period with a depression of 25 % (males) and 31 % (females) at week 52 compared with controls. The overall body weight gains for the entire dosing period of males and females at 1500 ppm were 85 % and 77 % of control values, resp. There were no clear effects of treatment on body weight gains of animals treated at 5 or 150 ppm.

Food consumption was similar between treated and control groups; however, efficiency of food conversion was slightly lower during the first 14 days of treatment for males at 1500 ppm.

Ophthalmoscopy: There were no findings at the ophthalmoscopic examinations of high dose and control animals after 24 week which could be attributed to treatment. Superficial corneal opacities were evident in 3 males and 3 females receiving 1500 ppm compared to one male and one female in the controls, but this was stated to be a common finding in mice of this strain and the distribution was considered to be incidental.

Haematology: Examination of blood smears after 50 and 76 weeks, and a detailed haematological examination after 77 weeks of treatment did not indicate any effects of treatment.

Organ weight analysis revealed significantly higher liver weights (absolute and relative) of males and females receiving 1500 ppm at both the interim and terminal sacrifices. Absolute and relative adrenal weights in this group were also slightly higher than in controls at the interim sacrifice, although statistical significance was achieved for the relative weights in males only (table 6.5.2-1).

Table 6.5.2-1: Organ weight changes (mean group values)

Parameter	**Dose group level (ppm)**							
	Males				**Females**			
	0	**15**	**150**	**1500**	**0**	**15**	**150**	**1500**
Interim phase (26 weeks)								
Adrenals absolute weight (g) (% control)	0.005	0.005 (100)	0.005 (100)	0.007 (140)	0.007	0.008 (114)	0.007 (100)	0.009 (129)

Parameter	Dose group level (ppm)							
	Males				Females			
	0	**15**	**150**	**1500**	**0**	**15**	**150**	**1500**
relative weight (%) (% control)	0.0098	0.0095 (96)	0.0116 (118)	0.0171[a] (174)	0.0224	0.0261 (117)	0.0212 (95)	0.0292 (130)
Liver absolute weight (g) (% control)	2.52	2.55 (101)	2.44 (97)	3.19[b] (127)	1.82	1.69 (93)	1.75 (96)	2.08[b] (114)
relative weight (%) (% control)	5.23	5.19 (99)	5.51 (105)	7.89[b] (151)	5.54	5.28 (95)	4.98 (90)	6.58 (119)
Terminal phase (78 weeks)								
Adrenals absolute weight (g) (% control)	0.006	0.004 (67)	0.005 (83)	0.004 (67)	0.006	0.007 (117)	0.006 (100)	0.006 (100)
relative weight (%) (% control)	0.0129	0.0094 (73)	0.0101 (78)	0.0098 (76)	0.0158	0.0179 (113)	0.0173 (109)	0.0188 (119)
Liver absolute weight (g) (% control)	2.88	2.46[a] (85)	2.58 (90)	3.36[a] (117)	1.90	1.95 (103)	1.95 (103)	2.19[b] (115)
relative weight (%) (% control)	6.05	5.40 (89)	5.31 (88)	7.65[b] (126)	4.84	5.12 (106)	4.97 (103)	6.23[b] (129)

a ($p \leq 0.05$); b ($p \leq 0.01$); significantly different from controls (Student's t-test)

Pathology: There were no macroscopic findings considered treatment-related at the interim phase. At the terminal phase, there was an increased incidence of enlarged livers among males receiving 1500 ppm. In addition, there was a slightly higher incidence of large and dark mesenteric lymph nodes among males of the top dose compared with their controls.

Histopathological examinations of the animals at the interim as well as terminal phase exhibited no neoplastic findings considered related to treatment with triticonazole.

Non-neoplastic findings revealed the liver as the main target organ:

At the interim phase, periacinar hepatocytic hypertrophy and centriacinar hepatocytic fatty vacuolation was noted among animals receiving 1500 ppm. At the terminal phase, there was also a statistically significant increase in the incidence of centriacinar hepatocytic fatty vacuolation in males and females of the top dose groups. In addition, there was an increased incidence of parafollicular hyperplasia of the mesenteric lymph nodes in males that had received 1500 ppm and been killed at termination. For this finding, significance was not reached when animals dying during the study period were included. An increased incidence of amyloidosis was evident in several organs (adrenals, gastro-intestinal tract, kidneys, lymph nodes, spleen thyroid) in males receiving either 150 or 1500 ppm. However, amyloidosis is known to occur spontaneously in aged mice of this strain and its occurrance is not regarded as a finding of toxicological importance. Notable non-neoplastic findings at the interim and terminal phase are summarized in table 6.5.2-2.

Table 6.5.2-2: Group incidences of non-neoplastic findings

Parameter	Dose group level (ppm)							
	Males				Females			
	0	15	150	1500	0	15	150	1500
Interim phase (26 weeks)								
Liver								
centriacinar fatty vacuolation	0/16	0/16	0/16	**5/14**[a]	0/16	0/16	2/16	**9/16**[c]
periacinar hepatocyte hypertrophy	0/16	0/16	0/16	3/14	0/16	0/16	0/16	0/16
Terminal phase (78 weeks)								
Liver								
centriacinar fatty vacuolation	1/52	0/52	0/52	**12/52**[b]	0/52	1/520	0/52	**8/52**[b]
periacinar hepatocyte hypertrophy	0/52	0/52	0/52	0/52	0/52	0/52	0/52	1/52
Mesenteric lymph nodes								
Parafollicular hyperplasia								
• terminal sacrifice only	0/31	1/4	1/5	**4/29**[a]	1/38	0/7	0/3	1/44
• all animals of terminal phase	1/49	1/24	1/28	4/46	1/52	0/21	0/16	1/49

a ($p \leq 0.05$); b ($p \leq 0.01$); c ($p \leq 0.001$) significantly different from controls (Fisher's exact test)

Conclusion

It can be concluded that dietary administration of triticonazole to mice up to and including 1500 ppm for 78 weeks showed no evidence on any treatment-related increase in the type or incidence of neoplastic findings in this study suggestive of a carcinogenic effect. Based on reduced body weight gains and clear effects on the liver (including increased organ weights and histopathological findings) the mid dose level of 150 ppm (equivalent to 17.4 [♂] and 20.1 [♀] mg/kg bw per day) is confirmed by RMS 2016 to be a NOAEL in this study.

B.6.6. REPRODUCTIVE TOXICITY

B.6.6.1. Generational studies

Previous evaluation:	DAR (2003)
DRAR (2016)	Additional information/tables added in the results; conclusion on the NOAEL of the study from DAR 2003 not changed. The discussion on C&L from the DAR (2003) is supported.

Reference:	Two-generation reproduction study with RPA400727 in rats
Author(s), year:	[redacted] 1993
Report/Doc. number::	R013085 / -
Guideline(s):	US EPA guideline 83-4 and OECD 416 (1983)
GLP:	Yes
Deviations from OECD 416 (2001):	-missing measurements for oestrus cycling, differential ovarian follicle count, sperm morphology and motility, implantation sites, some organ weights, anogenital distance, areola / nipple retention, sperm histopathology, detailed testicular histopathology, examination of intact epididymis, investigation on postlactational ovary - pairs without progeny not evaluated to determine the apparent cause of the infertility - no re-mating with proven sirs or dams conducted for F_1 generation where 8 animals per

	sex at 5000 ppm did not mate
Acceptability:	Yes; The lack of <u>some</u> parameters could be waived by following information: • Gonadal histopathology: No abnormalities in gonads in the 90-day rat study, 2-year chronic toxicity / carcinogenicity study. Also in the current study no abnormal gonadal histopathology was observed, however level of detail unknown

Material and methods

Groups of 28 rats/sex/dose group (strain: Sprague Dawley CRL:CD®BR VAF/plus; source: [redacted] of the F_0 generation received triticonazole (batch no. DA646; purity 97.1 %) via diet at dose levels of 0, 5, 25, 750 and 5000 ppm for 10 weeks before mating, and throughout mating, gestation and lactation period until terminal sacrifice. The rats were paired on a basis of 1 male : 1 female for a maximum of 21 days. Vaginal examinations were done daily during cohabitation, and the presence of sperm in the vaginal smear or a copulatory plug was considered evidence of positive mating. No attempts of re-mating are recorded in the study.

F_1-offspring were culled on day 4 post-partum to give four male and four female offspring per litter where possible. At weaning (day 21 postpartum) 28 male and 28 female F_1 pups were selected to form the F_1 parental generation. These animals were maintained for 10 weeks and were then paired. The F_1 parental animals received diets containing the same concentration of test material as their parents for at least 10 weeks prior to mating, then throughout mating, gestation and lactation of the F_2 litters. F_1 females were allowed to litter and rear their F_2 offspring to weaning. The F_1 animals and the F_2 pups were then examined at necropsy for abnormalities.

The observations included clinical signs (at least twice daily), recording of body weights (weekly during premating; on days 0, 7, 14 and 20 of gestation; on days 0, 4, 7, 14 and 21 of lactation) and food consumption (weekly during premating; days 0 - 4, 4 - 7, 7 - 14 and 14 - 20 of gestation; 0 - 4, 4 - 7, 7 - 10 and 10 – 14 of lactation) and reproductive performance (mating and fertility indices, conception rate, duration of gestation, gestation index, number, sex and weight of live and dead pups/litter and presence of external anomalies at birth; number and individual weight of pups were recorded on days 4, 7, 14 and 21 postpartum). At necropsy, in addition to macroscopic examination of adult animals (F_0 and F_1), male and female reproductive organs (epididymides, prostate, seminal vesicles, testes, uterus, vagina and ovaries), liver, pituitary and adrenals were weighed. Microscopic examination was performed on all these tissues from control and high dose group animals, with adrenals also being examined from all dose groups. F_1 pups culled on day 4 postpartum and post weaning offspring not selected for continuation of the study, and F_2 pups were examined macroscopically.

Results

<u>Test article intake</u>: No information on the test compound intake (mg/kg bw per day) is provided in the study report. Achieved daily intakes for both generations at the different segments of the study have been calculated by the notifier and are given in <u>table 6.6.1-1</u>.

Table 6.6.1-1: Group mean intakes of triticonazole (mg/kg bw per day) at different segments of the study

Dose level (ppm)	**F_0**	**F_1**	**F_0**	**F_1**	**F_0**	**F_1**
	♂/♀ (pre-mating)		**♀ (gestation)**		**♀ (lactation)**	
5	0.34/0.37	0.37/0.43	0.32	0.33	0.58	0.46

Dose level (ppm)	F_0	F_1	F_0	F_1	F_0	F_1
	♂/♀ (pre-mating)		♀ (gestation)		♀ (lactation)	
25	1.64/1.81	1.82/2.14	1.59	1.60	2.97	2.96
750	49.35/54.80	56.18/65.25	48.41	49.10	87.99	93.25
5000	350.8/389.3	445.3/493.8	337.64	339.08	592.99	528.05

Mortality and clinical signs: During gestation, one 5000 ppm F_0 female was sacrificed after prolonged parturition (# C79528) and 3 further F_0 females receiving 5000 ppm were sacrificed or found dead during late gestation (day 23; # C79549) or lactation (day 7; # C79538 and day 9; # C79536). No specific clinical signs were noted before death of these animals; animal # C79536 was thin, hunched and passive. At necropsy histopathological lesions of the adrenal cortex including degeneration of cells, focal acute inflammation and moderate to severe adrenal haemorrhage were observed in these females. It was considered that the four deaths could have been related to treatment.
In addition, a total of 5 premature deaths amongst F_0 (1 male at 750 ppm and 1 control female, with lesions of the urinary system seen at necropsy) and F_1 parental animals (1 male at 5000 ppm and 2 females at 5 and 750 ppm, resp., sacrificed due to hindlimb injury and poor health, resp.) occurred but were considered not to be treatment-related. There were no other deaths or clinical signs of toxicity considered related to treatment in any other group amongst F_0 and F_1 animals.

Body weights

In F_0 parental females at 5000 ppm, bodyweights and weight gains were significantly lower throughout premating, gestation and lactation, compared with control animals (Figure 6.6.1-1 and table 6.6.1-2). Also in males receiving 5000 ppm, bodyweight gains were significantly lower over the first two weeks of pre-mating (83% of control in first week and 92% of control in first two weeks) but were comparable to control afterwards. In F_1 parental animals at 5000 ppm, body weights and weight gains were significantly lower during pre-mating in males and females, and throughout gestation and lactation for females (Figure 6.6.1-3, 6.6.1-4 and Table 6.6.1-2, 6.6.1-3). There was also a significant ($p \leq 0.05$) reduction of body weight and body weight gain among males receiving 750 ppm for the first week of pre-mating. However, these reductions were very slight (93% of control) and considered to be coincidental.
At 5000 ppm there was an apparent difference in body weight and body weight development of F_0 males compared to F_1 males (table 6.6.1-1). While F_1 males treated with 5000 ppm had 20-35% less weight compared to control animals (over the treatment), F_0 males were almost unaffected by treatment with triticonazole. Also the body weight development of F_1 females was more affected by the treatment with triticonazole than F_0 females.

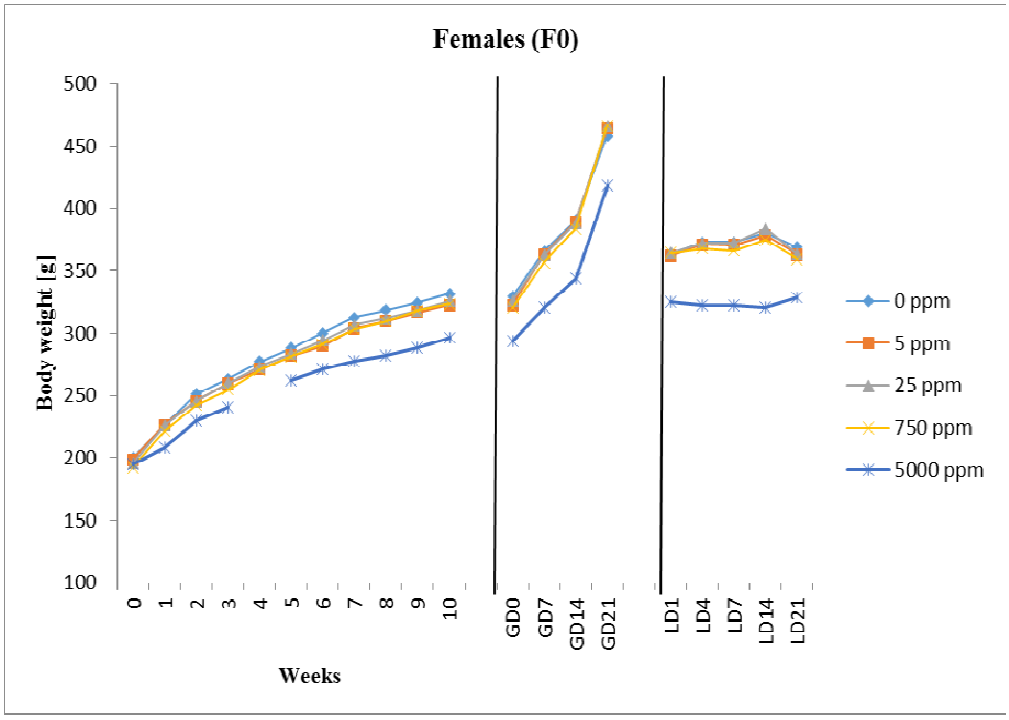


Figure 6.6.1-1. Body weight development in females (F0)

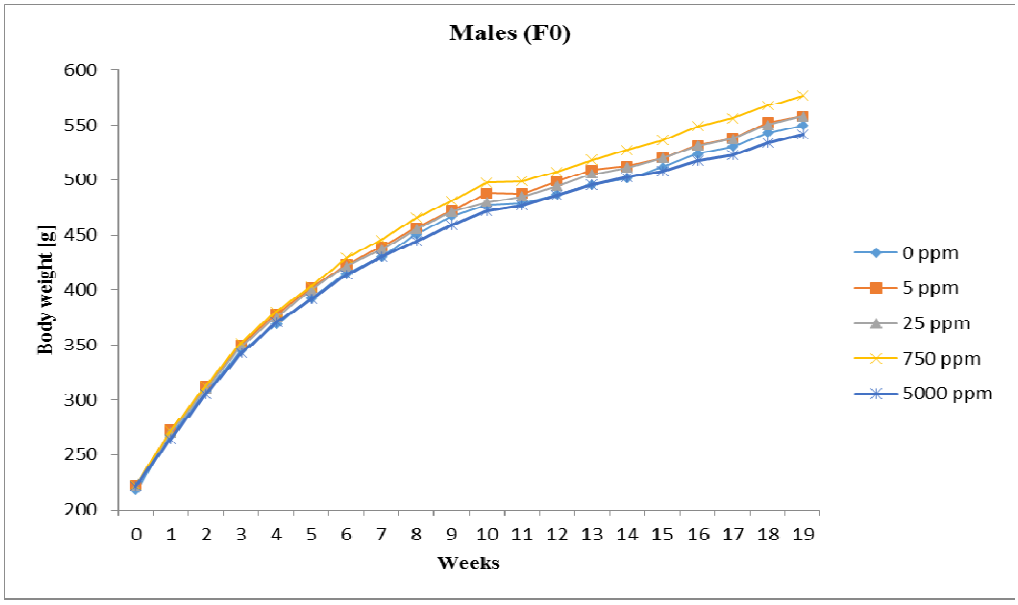


Figure 6.6.1-2. Body weight development in males (F_0)

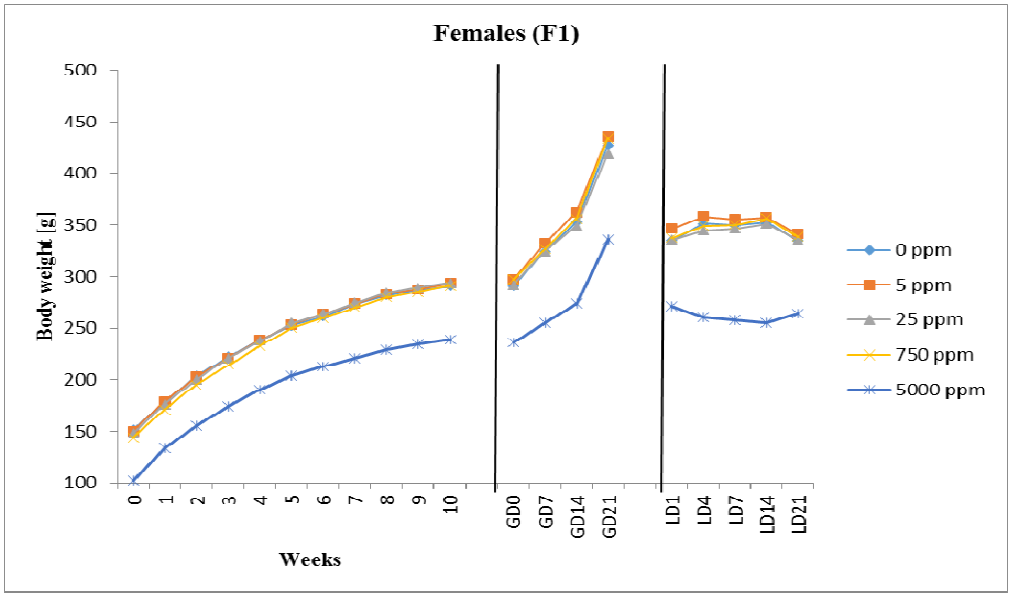


Figure 6.6.1-3. Body weight development in females (F_1)

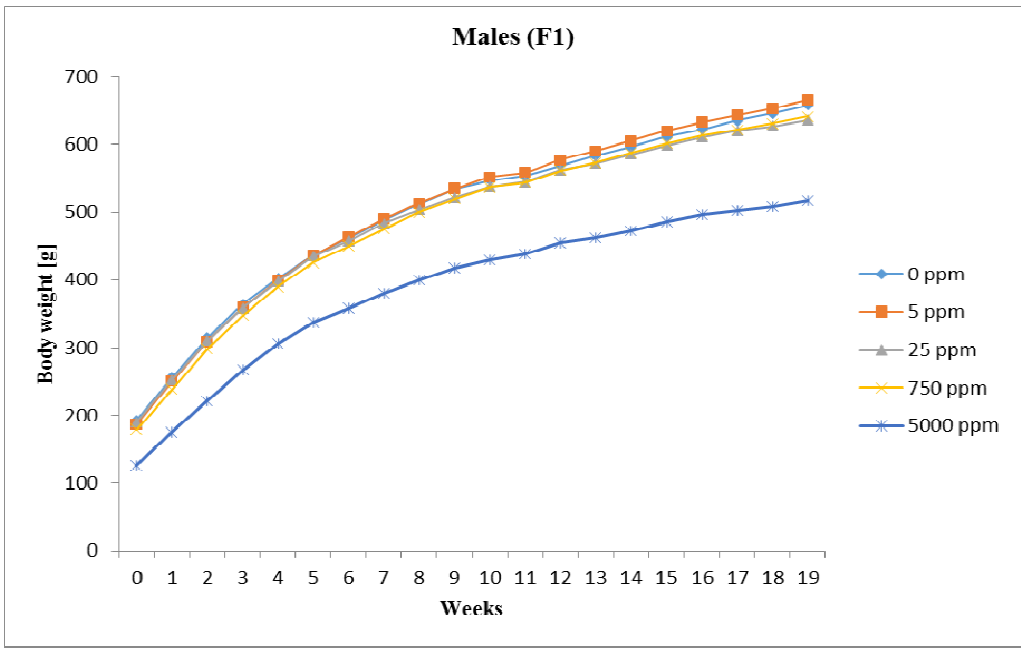


Figure 6.6.1-4. Body weight development in males (F_1)

Table 6.6.1-2: Mean body weights of F_0 and F_1 females (g and % control)

Week	Treatment group					
	ppm	**0**	**5**	**25**	**750**	**5000**
Week 0	F_0	200.11	198.57	195.74	192.64	194.89
	F_1	151.48	150.37	148.11	144.14	**102.41**** (67.6)
Week 2	F_0	251.25	245.37	245.50	242.06	**229.75**** (91.4)

	F_1	203.78	202.94	200.02	194.93	**155.51**** (76.3)
Week 4	F_0	277.40	270.85	273.20	270.67	**253.90**** (91.5)
	F_1	238.82	238.79	237.66	232.84	**190.66**** (79.8)
Week 6	F_0	300.16	289.83	294.36	291.13	**271.10**** (90.3)
	F_1	261.13	263.67	263.05	259.62	**213.17**** (81.6)
Week 10 (end of pre-mating)	F_0	331.62	322.48	325.81	323.44	**296.57**** (89.4)
	F_1	290.71	293.56	294.09	291.13	**239.07**** (82.2)
Overall premating weight gain (0-19)	F_0	*131.5*	*123.9*	*130.1*	*130.8*	***101.7**** *(77.3)*
	F_1	*139.2*	*143.2*	*146.0*	*147.0*	*136.7 (98.9)*
GD0	F_0	329.35	322.74	326.34	320.35	**293.75**** (89.2)
	F_1	290.80	297.14	292.14	296.90	**236.27**** (81.2)
GD7	F_0	365.9	362.87	362.34	356.71	**320.81**** (87.7)
	F_1	324.59	331.83	324.26	326.74	**255.45**** (78.7)
GD14	F_0	390.55	389.13	388.04	383.47	**343.96**** (88.1)
	F_1	352.72	362.30	348.76	355.96	**273.81**** (77.6)
GD21	F_0	458.16	463.80	465.68	466.35	**418.83**** (91.4)
	F_1	426.97	435.33	418.44	434.01	**335.59**** (78.6)
LD0	F_0	363.5	362.3	364.5	364.5	**324.7**** (89.3)
	F_1	334.7	346.2	335.6	337.3	**271.0**** (80.9)
LD4	F_0	372.9	371.1	372.0	367.8	**322.4**** (86.5)
	F_1	351.3	358.0	344.7	349.2	**260.1**** (74.0)
LD7	F_0	373.0	370.8	372.3	366.1	**322.1**** (86.4)
	F_1	349.4	355.3	346.6	349.8	**258.1**** (73.7)
LD14	F_0	380.4	378.4	383.7	374.5	**320.8****

						(84.3)
	F_1	352.3	357.2	350.7	356.2	**255.3**** (72.5)
LD21	F_0	368.7	363.2	363.4	359.2	**328.5**** (89.1)
	F_1	334.6	341.2	335.1	338.3	**264.2**** (78.9)

**** ($p \leq 0.01$); significantly different from control

Table 6.6.1-3: Mean body weights of F0 and F1 males (g and % control)

Week	Treatment group					
	ppm	**0**	**5**	**25**	**750**	**5000**
Week 0	F_0	217.45	222.26	220.96	222.27	222.08
	F_1	193.29	187.30	189.96	179.21* (92.7)	**125.53**** (64.9)
Week 2	F_0	307.56	312.47	310.01	312.91	304.96
	F_1	314.48	309.49	310.28	298.41	**220.48**** (70.1)
Week 4	F_0	368.43	378.02	374.96	380.02	370.15
	F_1	401.90	399.18	391.69	389.24	**305.54**** (76.0)
Week 6	F_0	414.95	423.04	421.96	428.90	414.09
	F_1	461.54	463.00	456.90	450.00	**357.64**** (77.5)
Week 10 (end of pre-mating)	F_0	476.82	487.96	479.81	497.41	471.94
	F_1	546.35	551.49	537.86	536.80	**429.60**** (78.6)
Week 19 (termination)	F_0	549.78	558.00	557.68	577.07	541.21
	F_1	658.36	665.25	635.88	641.13	**517.02**** (78.5)
Overall weight gain (0-19)	F_0	*332.3*	*335.7*	*336.7*	*354.1*	*319.1*
	F_1	*465.1*	*478.0*	*445.9*	*461.9*	*391.9** (84.2)*

* ($p \leq 0.05$), ** ($p \leq 0.01$); significantly different from control

Marked decreases in food consumption were reported in F_0 females receiving 5000 ppm throughout gestation, and in F_1 males and females receiving 5000 ppm during premating, and throughout gestation and lactation, resp. There were also sporadic reductions in food consumption amongst F_1 males receiving 25 and 750 ppm, but there was no consistent dose-related trend.

Effects on fertility: In the F_0 generation, there were no treatment-related effects on mating and fertility index, conception rate (no. pregnant animals/no. mated animals) and gestation index (no. live litters born/no. animals

pregnant) at any dose level. However, the mean duration of gestation was significantly increased at 5000 ppm (22.6 days compared to 22.1 days in the control group) but was within the HCD of the laboratory for F_0 generation. It was stated in the report that this was due to a longer gestation evident in two females (24 and 25 days) and is not related to treatment therefore, but there was a clear trend towards longer gestation times in the F_0 generation at this dose level (table 6.6.1-6). Female #C79528 (gestation day 25) had to be sacrificed moribund after prolonged parturition; however, the other female (gestation day 24) had surviving pups. In F_1 generation the effect on longer duration of gestation in the 5000 ppm group was less marked (table 6.6.1-6) but also lower number of animals presented the high dose group based on impairment of mating performance.

For the pregnant F_1 females duration of gestation was again 22.6 days, this time not statistically significant but outside the HCD of the laboratory. In the F_1 generation, there were significant decreases in mating (no. animals inseminated/no. animals paired) and fertility indices (no. animals pregnant/animals paired) at 5000 ppm. In F_1 generation 8 out of 28 animals did not mate (neither presence of sperm in the vaginal smear nor copulatory plug was observed) resulting in a mating index of 71% and being lower than the historical control data (81-100%). Also the fertility index with 64% was below the HCD, mainly caused by the fact that 8 females were not mated. The two apparently infertile F_1 high dose males (#84836 and #84845; mated but did not produce pregnancy) did not show histopathological findings that could explain infertility (testes and epididymis). No explanation for 8 males and females of the 5000 ppm group not mating was stated in the study report. RMS (2016) evaluated individual data of these 8 males (#C84847, #C84846, #C84825, #C84841, #C84852, #C84834, #C84851, #C84842) and females (#C84860, #C84868, #C84872, #C84878, #C84873, #C84867, #C84879, #C84877). In week 10 (just prior to the mating) 6 of the concerned males had 20% lower body weight compared to control animals, only males #C84851 and #C84842 had 17% and 15% lower body weight compared to control males, respectively. From 20 males who successfully mated, 12 males had more than 20% lower body weight than the mean of the control males, 6 males had 14 to 20% lower body weight and 2 animals had body weights less than 10% lower than the control. Regarding adrenal findings in males, all animals of the 5000 ppm group had vacuolation of adrenal cortex.

In females, 3 non-mated animals (#C84860, #C84868, #C84872) had 71-78% body weight compared to control animals while remaining 5 females had body weight 83% to 91% of the mean control value. The body weight change was partialy higher than in control animals but initial, markedly lower, body weight in F_1 generation could not be compensated. From these 8 females only 3 females (#C84873, #C84879 and #C84878) showed adrenal cortex degenerative effects or giant cells and only one female (#C84878) showed ovary vacuolation and the presence of giant cells. From 20 females who mated 8 females had more than 20% lower body weight than the mean of the control females, 10 females had 10 to 18% lower body weight and 2 animals had body weights less than 10% lower than the control. From these 20 females 10 females showed adrenal cortex degenerative effects and/or giant cells and only one female showed ovary with giant cells.

The reason for the decreased mating/fertility rates remain unclear, and it appears to be most probably the consequence of the excessive general toxicity, expressed as approximately 20% lower body weight than in control animals (already observed at the time of birth for F1 generation).

The summary of findings observed in not-mated animals is presented in table 6.6.1-4.

Table 6.6.1-4: Body weight, body weight change and adrenal findings observed in eight F1 males and females that did not mate

Group	**Findings in non-mated animals**				
5000 ppm	**Body weight (week 10 of pre-mating) - % control**	**Bw change (week 10 of pre-mating) - % control**	**Vacuoaltion of adrenal cortex**	**Degeneration of adrenal cortex**	**Giant cells**
Males					
#C84847	69	81	x	-	-
#C84846	70	78	x	-	-
#C84825	70	80	x	-	-
#C84841	76	89	x	-	-
#C84852	77	80	x	-	-
#C84834	78	87	x	-	-
#C84851	83	92	x	-	-
#C84842	85	93	x	-	-
Females					
#C84860	71	102	-	-	-
#C84868	76	77	-	-	-
#C84872	78	103	-	-	-
#C84878	83	88	-	-	x
#C84873	83	117	-	x	x
#C84867	86	103	-	-	-
#C84879	87	107	-	x	x
#C84877	91	94	-	-	-

X Finding observed
-No finding observed

There were no effects on fertility that were considered treatment-related at 5, 25 or 750 ppm for both generations.

Litter data: There were a number of effects on litter parameters at 5000 ppm seen in the F_1 and F_2 generations. The livebirth index (no. live pups at day 1/no. born pups) and viability index (no. pups alive on day 4/no. live born pups) were significantly lower for both F_1 and F_2 litters at this dose level compared with those of the respective controls whereas the total number of stillborn F_1 and F_2 pups was increased (table 6.6.1-5). In addition, total litter death was noted for four F_0 females given 5000 ppm during lactation between days 0 – 4, and the mean number of live pups/litter with live pups on days 0 and 4 of lactation were all significantly reduced among F_2 litters at 5000 ppm. Necropsy of dead pups showed that no milk in the stomach was the only notable finding (table 6.6.1-7).

Table 6.6.1-5: Summary of study findings on reproductive performance and delivery and litter data in the 2-generation study in rats

Parameter		Dose level (ppm)				
		0	**5**	**25**	**750**	**5000**
Pre-coital interval (days)	F_0	2.12	2.81	3.32	2.81	2.61
	F_1	3.07	3.18	2.54	3.38	3.30
Number of paired females	F_0	27	28	28	28	28
	F_1	28	28	28	27	28
Total number inseminated	F_0	26	28	28	27	28
	F_1	28	28	26	27	**20****
Total number pregnant (%)	F_0	23 (88)	27 (96)	22 (79)	25 (93)	28 (100)
	F_1	26 (93)	28 (100)	25 (96)	25 (93)	**18**** (90)
Number of dams delivering	F_0	23	27	22	25	27
	F_1	26	28	25	25	17
Gestation length (days) (HCD of laboratory, 1988 – 1993; F0 = 21.8 – 22.7 d; F1 = 22.0-22.5 d)	F_0	22.1	22.1	22.2	22.0	**22.6***
	F_1	22.1	22.2	22.1	22.1	22.6
Mating index (%) (no. animals inseminated/ no. animals paired x 100) (HCD of laboratory, 1988 – 1993; F0 = 90 – 100%, F1 = 81-100%	F_0	96	100	100	96	100
	F_1	100	100	93	100	**71****
Fertility index (%) (no. animals pregnant/ animals paired x 100) (HCD of laboratory, 1988 – 1993; F0 and F1 = 76 – 96%	F_0	85	96	79	89	100
	F_1	93	100	89	93	**64****
Gestation index (%) (% pregnancies yielding live litters)	F_0	100	100	100	100	93
	F_1	100	100	100	100	89
Livebirth index (%) (no. live pups at day 1/no. born pups x 100)	F_1-pups	93	98	99	98	**82****
	F_2-pups	99	98	99	98	**85****
Viability index (%) (no. pups alive on day 4/ no. live born pups)	F_1-pups	92	94	99	97	**82****
	F_2-pups	98	100	99	97	**89****
Weaning index (%) (days 4 - 21)	F_1-pups	95	100	100	100	94
	F_2-pups	100	100	100	100	100
No. stillborn pups/no. pups delivered (%)	F_1-pups	18/335 (5.3%)	7/419 (1.7%)	2/341 (0.6%)	7/411 (1.7)	46/382 (12.04%)
	F_2-pups	4/381 (1.1%)	4/408 (0.98%)	3/343 (0.87%)	7/388 (1.8%)	11/203 (5.4%)

Parameter		Dose level (ppm)				
		0	**5**	**25**	**750**	**5000**
Entire litter died, killed, missing, and/or cannibalised	F_1-pups	0	1	0	0	4
Day 0-4		0	0	0	0	0
Day 5-21	F_2-pups	0	0	0	0	1
		0	0	0	0	0
Sex ratio at day 0 (% males)	F_1-pups	48	47	51	49	49
	F_2-pups	51	49	54	54	53
Mean live pups/litter with live pups	F_1-pups	13.70	15.22	15.32	16.12*	12.35
Day 0		13.00	14.85	15.14	15.64*	12.64
Day 4	F_2-pups	14.46	14.32	13.52	15.20	**11.13****
		14.15	14.25	13.36	14.80	**11.07****

* ($p \leq 0.05$); ** ($p \leq 0.01$); significantly different from controls

Table 6.6.1-6: Summary of gestation times for the F_0 and F_1 generations in the 2 generation study in rats

Group	number of animals with gestation time (days)									
	F_0					F_1				
Gestation day	**21**	**22**	**23**	**24**	**25**	**21**	**22**	**23**	**24**	**25**
0 (control)	-	20	3	-	-	-	23	3	-	-
5 ppm	3	18	4	1	-	1	21	6	-	-
25 ppm	-	18	4	-	-	2	17	4	-	-
750 ppm	3	19	2	-	-	-	21	3	-	-
5000 ppm	1	11	13	1	1	-	10	5	1	1

Table 6.6.1-7: Incidence of gross necropsy observations in F_1 and F_2 pups

Dose [ppm]	0	5	25	750	5000
F_1 pups					
Litters evaluated	22	25	22	25	21
Pups evaluated	217	299	240	307	203
- Live	205	292	238	300	183
- Stillborn	12	7	2	7	20
No milk in stomach	16 (5)	7 (6)	1 (1)	9 (8)	26 (11)
Milk in stomach	1 (1)	3 (2)	2 (2)	3 (2)	1 (1)
Total pup necropsy observations	17	10	5	14	30
- % affected pups/litter	27	32	12	44	62
F_2 pups					
Litters evaluated	26	28	25	25	17
Pups evaluated	335	363	300	339	141
- Live	331	359	297	332	130
- Stillborn	4	4	3	7	11
No milk in stomach	3 (3)	2 (2)	5 (4)	6 (4)	13 (5)
Milk in stomach	2 (1)	1 (1)	1 (1)	2 (2)	2 (2)

Dose [ppm]	0	5	25	750	5000
			F_1 pups		
Litters evaluated	22	25	22	25	21
Pups evaluated	217	299	240	307	203
- Live	205	292	238	300	183
- Stillborn	12	7	2	7	20
Total pup necropsy observations	14	12	13	14	15
- % affected pups/litter	42	25	32	36	35

* $p \leq 0.05$, ** $p \leq 0.01$ (Wilcoxon-test, one-sided)
( ) values in brackets give litter incidence

Mean pup bodyweights were significantly lower for male and female F_1 pups at 5000 ppm on days 7, 14 and 21 and it seems that the effects on body weights in F_1 pups gain in severity when pups open their eyes and start self-feeding (PND 12-15). For F_2-pups at 5000 ppm mean bodyweights were significantly lower for males and females from day 0 to day 21 and achieved at day 21 only about 50% of the body weight of control pups (table 6.6.1-8). There were no effects on litter data that were considered treatment-related at 5, 25 or 750 ppm for both generations.

Table 6.6.1-8: Mean pup weights (g) in the 2-generation study in rats

Dose level	Males					Females				
	Day 0	**Day 4**	**Day 7**	**Day 14**	**Day 21**	**Day 0**	**Day 4**	**Day 7**	**Day 14**	**Day 21**
F_1 Litters (Body weight [g] (SD))										
0 ppm	6.46 (0.69)	9.94 (1.93)	17.33 (2.74)	36.31 (4.51)	59.25 (6.82)	6.03 (0.58)	9.44 (1.41)	16.10 (2.68)	34.28 (4.35)	54.98 (5.96)
5 ppm	6.55 (0.76)	10.70 (1.57)	17.98 (2.21)	37.07 (3.52)	59.32 (5.27)	6.16 (0.58)	10.16 (1.43)	17.17 (2.29)	35.63 (3.21)	56.77 (4.41)
25 ppm	6.59 (0.64)	10.95 (1.11)	18.43 (1.81)	37.96 (3.01)	61.11 (4.71)	6.17 (0.62)	10.42* (1.33)	17.39 (1.77)	36.21 (2.53)	57.99 (3.93)
750 ppm	6.40 (0.56)	10.47 (1.01)	17.65 (1.42)	36.50 (2.68)	58.35 (4.21)	6.10 (0.51)	10.06 (0.82)	16.81 (1.30)	34.89 (2.46)	55.41 (3.95)
5000 ppm	6.14 (0.81)	9.86 (1.60)	**15.21**** (2.16) *87.8% of control*	**26.83**** (4.10) *73.9% of control*	**41.82**** (7.22) *70.6% of control*	5.85 (0.68)	8.97 (1.51)	**14.13**** (2.16) *87.8% of control*	**25.54**** (4.04) *74.5% of control*	**39.27**** (6.66) *71.4% of control*
F_2 Litters (Body weight [g] (SD))										
0 ppm	6.35 (0.49)	10.56 (1.32)	17.32 (1.43)	34.62 (2.27)	56.21 (4.02)	5.94 (0.43)	10.01 (1.30)	16.52 (1.59)	33.60 (2.61)	53.96 (4.08)
5 ppm	6.58 (0.55)	11.12 (1.13)	18.25 (1.99)	36.32* (3.07)	58.57 (4.83)	6.18 (0.49)	10.63 (1.02)	17.55 (1.72)	35.04 (2.50)	56.10 (3.41)
25 ppm	6.38 (0.66)	10.49 (1.65)	17.69 (2.01)	35.95 (2.45)	58.43 (4.63)	6.11 (0.63)	10.21 (1.54)	16.84 (1.86)	34.45 (2.67)	55.38 (4.58)
750 ppm	6.42 (0.55)	10.68 (1.30)	17.55 (1.40)	35.36 (1.68)	57.31 (3.31)	6.07 (0.54)	10.24 (1.19)	16.65 (1.53)	34.17 (2.26)	54.76 (3.89)
5000 ppm	**5.71**** (0.47) *89.9% of control*	**7.83**** (1.35) *74.1% of control*	**12.07**** (1.55) *69.7% of control*	**19.81**** (1.83) *57.2% of control*	**28.49**** (4.01) *50.7% of control*	**5.37**** (0.55) *90.4% of control*	**7.16**** (0.90) *71.5% of control*	**11.28**** (1.16) *68.3% of control*	**18.67**** (1.73) *55.6% of control*	**26.66**** (3.62) *49.4% of control*

* ($p \leq 0.05$); ** ($p \leq 0.01$); significantly different from controls

Pathology: At necropsy of parental F_0 animals, absolute and relative left adrenal weights were significantly lower and absolute and relative liver weights were significantly higher for F_0 females at 5000 ppm compared with controls. Similar findings were observed in F_1 females. While in females a decrease in adrenal weight was observed, relative left and right adrenal weight in F_1 males was increased (table 6.6.1-9).

Table 6.6.1-9: Mean absolute and relative organ weights in the 2-generation study in rats

Organ weights		Group dose level (ppm)									
		♂					♀				
		0	5	25	750	5000	0	5	25	750	5000
Adrenals (left), absolute weight (g)	F_0	0.0248	0.0308**	0.0264	0.028	0.0261	0.0422	0.0424	0.0420	0.0423	0.0314**
	F_1	0.0296	0.0299	0.0292	0.0262*	0.0267	0.039	0.04	0.0366	0.0376	0.0268*
Adrenals (right), absolute weight (g)	F_0	0.0262	0.0272	0.0251	0.026	0.0244	0.0393	0.0414	0.0409	0.0387	0.0341
	F_1	0.0285	0.0293	0.0282	0.0251*	0.0255*	0.0361	0.0382	0.0342	0.0345	0.0266**
Adrenals (left), relative weight (%)	F_0	0.0045	0.0056**	0.0048	0.0049	0.0048	0.0117	0.012	0.0117	0.0118	0.0094** (80% control)
	F_1	0.0046	0.0046	0.0047	0.0041	0.0053* (115 %)	0.0120	0.0122	0.0111	0.0114	0.0097* (81%)
Adrenals (right), relative weight (%)	F_0	0.0047	0.0049	0.0045	0.0045	0.0045	0.0109	0.0117	0.0114	0.0108	0.0102
	F_1	0.0044	0.0045	0.0045	0.004	0.0051* (116 %)	0.0111	0.0116	0.0104	0.0105	0.0096* (86 %)
Liver, absolute weight (g)	F_0	20.67	20.16	21.04	21.57	21.09	14.59	14.3	14.4	15.0	16.59**
	F_1	23.21	23.17	22.63	22.63	18.89*	12.48	12.55	12.75	13.53	13.36
Liver, relative weight (%)	F_0	3.77	3.63	3.79	3.75	3.89 (103 %)	4.01	4.03	4.01	4.18	4.96** (124 %)
	F_1	3.56	3.49	3.58	3.55	3.68 (103%)	3.82	3.78	3.83	4.09	4.83* (126 %)

* ($p \leq 0.05$); ** ($p \leq 0.01$); significantly different from controls

At 5000 ppm microscopic findings in the adrenals comprised of increased incidence and severity of vacuolation in the adrenal cortex for males, and degenerative changes of the adrenal cortex in females (vacuolation, syncytial giant cell formation, deposition of collagen, large pigment laden cells, minimal inflammation). In F_1 parental animals in 5000 ppm group vacuoles and giant cells were present in the ovaries in four females which were considered treatment-related; no similar findings were observed in F_0 females.

In 2015 the notifier provided an external pathologist (*Millar, P.M.,* 2015) with the old adrenals histological slides (all female controls and females given 750 or 5000 ppm, F_0 and F_1). His conclusion was as follows:

- There was good correlation with the findings recorded by the original study pathologist.
- The lesion in the adrenal glands of females given 5000 ppm was essentially identical to that seen in the 13 week study except that the presence of degenerating corticocytes was less frequent but secondary mononuclear inflammatory cell infiltrates, in particular the appearance of macrophage 'giant cells' and to a lesser extent collagen deposition were more prevalent. The 'giant cells' were interpreted to be macrophage syncytial or foreign-body giant cells engaged in the ingestion of breakdown products from degenerate corticocytes. Several of them contained small intracytoplasmic inclusions of amorphous material. These differences most likely derived from the lower dosage level employed in comparison to the 13-week study and the time over which the animals were exposed to the test item.
- No treatment-related findings were observed in females given 750 ppm.

There were no treatment-related macroscopic findings present for F_1 and F_2 weanlings at 5000 ppm. In addition, no treatment-related effects were observed in parental animals given 5, 25 and 750 ppm.
The group incidences of relevant histopathological findings are given in table 6.6.1-10.

Table 6.6.1-10: Group incidences of histopathological changes in parental F_0 and F_1 animals in the 2 generation study in rats, original assessment

Findings		Group dose level (ppm)									
		♂					♀				
		0	5	25	750	5000	0	5	25	750	5000
Adrenals											
Vacuolation of adrenal cortex	F_0	7/28	6/28	4/28	6/27	**27/28**	0/27	0/28	1/28	1/28	0/24
	F_1	15/28	9/28	8/28	9/28	**27/27**	0/28	1/27	0/28	0/27	0/28
Degeneration of adrenal cortex	F_0	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/28	0/28	**22/24**
	F_1	0/28	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/27	**11/28**
Collagen deposition	F_0	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/28	0/28	6/24
	F_1	0/28	0/28	0/28	0/28	0/27	0/27	0/28	0/28	0/28	0/28
Giant cells	F_0	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/28	0/28	**16/24**
	F_1	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/28	0/28	**14/28**
Cortical pigment	F_0	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/28	0/28	**6/24**
	F_1	0/28	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/27	**7/28**
Chronic inflammation	F_0	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/28	0/28	2/24
	F_1	0/28	0/28	0/28	0/28	0/27	0/28	0/27	0/28	0/27	0/28
Ovaries											
Vacuolation	F_0	-	-	-	-	-	0/27	-	-	-	0/24
	F_1	-	-	-	-	-	0/28	-	-	-	4/28

Findings		Group dose level (ppm)									
		♂					♀				
		0	5	25	750	5000	0	5	25	750	5000
Giant cell formation	F_0	-	-	-	-	-	0/27	-	-	-	1/24
	F_1	-	-	-	-	-	0/28	-	-	-	2/28

Conclusion:

The NOAEL for parental toxicity in this study is 750 ppm (equivalent to 49.35 (♂) and 48.41 (♀) mg/kg bw per day), based on maternal mortality, significantly reduced body weight in both sexes and necropsy findings (histopathology and organ weights) in adrenals, liver and ovaries at 5000 ppm.

The NOAEL for reproductive effects is 750 ppm (equivalent to 49.35 (♂) and 48.41 (♀) mg/kg bw per day), based on significantly reduced mating and fertility index in F_1 generation at 5000 ppm.

The NOAEL for offspring in this study is 750 ppm (equivalent to 49.35 (♂) and 48.41 (♀) mg/kg bw per day), based on effects on survival and growth at 5000 ppm, consistently observed across both generations.

Assessment of criteria for classification and labelling for reproductive effects:

Already for the DAR (2003) and evaluation by ECB a position paper ([redacted], *1999*) on reproductive effects has been submitted by the notifier:

1) *Mortality observed in F_0 females only during the last phase of pregnancy and the first days of lactation*: It was stated that the period between the end of pregnancy and the first days of lactation corresponds to the period when the action of triticonazole has reached its most severe effects inducing the degeneration of the adrenal cortex. In addition, triticonazole intake for females was comparable between pre-mating and gestation periods, but during the 7 – 14 day lactation period when highest values were observed, the compound intake increased due to the increased food consumption representing 1.8 fold and 1.3 fold increases over the pre-mating compound intake values for the F_0 and F_1 dams, resp. This marked increase in compound intake during the lactation phase has contributed to the strong toxicity observed in dams indicated by a marked decrease in body weight gain and high treatment-related mortality in F_0 females (14 %).

2) *Significant increase of pregnancy duration in F_0 females at 5000 ppm*: It was stated that this effect was mainly due to two females with longer gestation periods (24 and 25 days. resp), and since the group mean values (22.6 days for F_0 and 22.5 days for F_1 generation) are in the range of historical control data (21.8 – 22.7 days for F_0 and 22.0 – 22.5 days for F_1 generation), the effect is not considered to be treatment-related.

3) *Reduction of male fertility and especially of female fertility in F_1 generation*: Concerning the significantly decreased mating and fertility indices evident at 5000 ppm, it was stated that these effects could be related to a decreased receptivity of females. Since the female sexual response in common laboratory animals is regulated by hormones produced by the ovaries and adrenals, a deficiency of hormonal secretion could be responsible for a weak or absent female sexual response. The degeneration of the adrenal cortex observed in female rats treated at 5000 ppm could be responsible for a reduced secretion of adrenal hormones. It was stated that in

contrast, human female sexual behaviour does not depend entirely upon the direct action of such hormones, but is facilitated by the central nervous system.

4) *Significant increase of stillbirths and neonatal mortality, significant decrease of the mean number of pups per litter in F_1 and F_2 and significant reduction of the weight gain in pups F_1 and F_2 up to the weaning*: As the reduction in the number of pups born, reduced pup viability and pup weights were observed only in litters from females dosed at 5000 ppm, these effects were considered to be the consequence of maternal toxicity and not considered as specific signs of pup toxicity. Furthermore, no developmental changes were observed in offspring, even, when parental animals were treated at the highest dose.

In the DAR 2003 it was concluded *that there is evidence that reproductive parameters like female fertility, number of live born pups and pup viability are adversely affected by triticonazole at a very high dose level, exceeding the maximum tolerated dose. No effects on reproductive parameters were seen in the absence of maternal toxicity. Therefore, the adverse effects on the reproductive function were considered in all probability as the consequence of distinct maternal toxicity* and no classification and labelling of triticonazole for reproductive toxicity was considered justified.

In the renewal evaluation (2016) RMS carefully re-assessed the results and originally provided argumentation, and also added additional information on some parameters not included in the first DAR (2003). Especially bearing in mind that triticonazole has already been discussed in ECB (summary record, ECBI/90/06 Rev. 8, Ispra, 22 August 2007) where no classification was proposed and no new studies on reproductive toxicity have been made available since then, the repetition of discussion on reproductive toxicity and classification should be considered carefully.

As per guidance on the application of the CLP criteria (July 2017), all findings on reproductive toxicity are considered for classification purposes irrespective of the level of parental toxicity. The RMS notes that many effects on offspring (such as survival and growth) can be a consequence of significant maternal toxicity (such as mortality, statistically significant decreases in body weight and body weight gain, especially if greater than 10% and severe pathological findings in organs such as the adrenal glands and liver). Such effects were observed in the present study for both generation parents and the effects on the offspring in this case were considered attributable to significant maternal toxicity. A very significant cumulative offspring body weight reduction was observed in F_1 and especially F_2 pups from the highest dose animals. A lactation effect was not supported by the data. Perinatal toxicity due to parental/maternal toxicity was much more likely since observations on the pups during necropsy ("no milk in stomach") indicated nursing deficits of the female rats. Classification for effects on or via lactation is not supported.

In relation to effects on fertility the Guidance on the Application of the CLP Criteria (July 2017) states that "*Adverse effects on fertility and reproductive performance seen only at dose levels causing marked systemic toxicity (e.g. lethality, dramatic reduction in absolute body weight, coma) are not relevant for classification purposes*". While mortality was observed at the top dose of 5000 ppm in the F_0 generation (doses: 338 (gestation) – 593 (lactation) mg/kg bw per dayay), where no reduced mating and fertility index was recorded, there was no mortality observed at the highest dose in the F_1 generation (doses: 339 (gestation) – 528 (lactation) mg/kg bw per dayay), where a markedly reduced fertility and mating index was observed. However, several noteworthy findings of toxicity were

observed in the F_1 generation adults such as excessively lower body weight (82% bw compared to controls in females and 79% in males at week 10, just prior to the mating), reductions in adrenal gland weight in females and increase in males, increases in liver weight in females and serious histopathological findings in the adrenals in both sexes (however with different histopathology). At doses below 5000 ppm there were no effects observed.

The data was not sufficient for the RMS to conclude that the reduced mating performance observed in the F_1 parental animals at the highest triticonazole dose was a consequence of a substance specific effect on sexual function and fertility. There was no dose response evident for any of the reported effects. Significant systemic toxicity at highest dose tested was detected in both males and females, observed as markedly lower body weight compared to control animals. Data was lacking with respect to the mechanism of action and cause of the reduced mating performance. The relationship and importance of adrenal insufficiency and hormonal disturbance to reduced mating performance remain speculative since comparison of individual data revealed no correlation. A slight increase in gestational length amongst the F_0 and F_1 females was not considered to be strong evidence in support for classification. It is not possible to identify the reduced mating performance as being a primary, substance-mediated effect. 5000 ppm (338 – 528 mg/kg bw per dayay) is considered well above the MTD and the observed findings do not justify classification regarding reproductive performance. In consideration of all the available data, the RMS does not support classification for fertility which is in line with the previous conclusion derived by ECB (2007).

B.6.6.2. Developmental toxicity studies

B.6.6.2.1. Rat

B.6.6.2.1.1. Range finding rat study

Previous evaluation:	No, only mentioned in DAR (2003) in the main study, but not evaluated
DRAR (2016)	Evaluation included in DRAR (2016)

Reference:	RPA 400727: Preliminary teratology study in the rat
Author(s), year:	[redacted] 1990
Report/Doc. number::	C018955 / -
Guideline(s):	US EPA guideline 83-3 and OECD 414 (1981)
GLP:	Yes
Deviations from OECD 414 (2001):	According to the most recent OECD TG 414, the recommended administration period is covering the whole gestation period and should continue until the day prior to scheduled caesarean section. While range-finding, lower number of dams (6) was included in the study.
Acceptability:	Yes; additional information

Materials and methods

Test Material:	BAS 595 F (RPA400727)
Description:	solid / white powder
Batch #:	Batch HUT423 (RPA400727)
Purity / content:	97.0% (RPA400727)
Vehicle:	0.5% Methylcellulose suspension in drinking water (0.5% MC)
Test animals:	
Species:	Rat
Strain:	CD (Sprague Dawley)

Sex:	female
Age (GD 6):	ca. 9-11 weeks
Weight:	195 to 237 g (at commencement of the study)
Source:	[redacted]
Acclimation period:	5 days
Diet:	Biosure Laboratory Animal Diet No. 1, Biosure, Lavender Mill, Manea, Cambridgeshire, England), ad libitum
Water:	Tap water, ad libitum
Housing:	Single housing in RB3 modified cages (North Kent Plastics Limited, Dartford, Kent, England) during gestation. The cages consisted of high density polypropylene bodies with lids and floors of stainless steel grid. During mating 1 male and 1 female animal were housed together. During acclimatisation 5 rats per cage were housed in RC1 cages (North Kent Plastics Limited, Dartford, Kent, England).

Environmental conditions:	
Temperature:	18 – 25°C
Humidity:	40 – 70%
Air changes:	approx. 15 air changes/hour
Photo period:	Alternating 12-hour light and dark cycles

In this study two substances were investigated side-by-side (RPA400727 and LS840606). RPA400727 is a synonym for triticonazole, thus only the observations/results obtained for RPA400727 are relevant and summarized below. The results of LS840606 (substance not further described) were not included in the evaluation.

Mating procedure

Females were paired on a one-to-one basis with stock males of the same strain. Each morning following pairing, the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepared from each female and examined for the presence of spermatozoa. The day on which a sperm positive vaginal smear or at least three copulation plugs were found was designated Day 0 of gestation.

Treatment

Six animals were allocated to each treatment group. The animals were treated either with the solvent alone (0.5% (w/v) aqueous methylcellulose suspension) or with triticonazole at 50, 250, or 1250 mg/kg bw per dayay. The animals were dosed daily by oral gavage from day 6 to day 15 of gestation at a volume-dosage of 10 mL/kg.

Test substance analysis

Samples of each concentration of triticonazole were taken during the first and last weeks of treatment.

Statistics

Where relevant, means and standard deviations of each test group were calculated. Because of the small sample size in this range-finding assay no statistical evaluation was performed. Biological significance was assessed by reference to historical control data.

Observations

The animals were examined once daily for any visible signs of reaction to treatment.

Body weight and food/water consumption

Maternal bodyweight was determined on days 0, 3, 6 to 16, 18, and 20 of gestation. Food and water consumption were recorded on days 0, 3, 6, 9, 12, 16, 18 and 20 of gestation.

Sacrifice

On GD 20, the dams were sacrificed by inhaled carbon dioxide and the foetuses 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:

- Number of corpora lutea
- Number of implantation sites
- Number of resorption sites (classified as early or late)
- Number and distribution of implantation sites in the uterus classified as
  - live fetuses or
  - dead implantations
- Individual placental weights

Based on the above the following parameters pre- and post-implantation loss was calculated:

Examination of foetuses:

The following examinations were performed with the foetuses:

- Weight and sex of individual foetuses
- External abnormalities of individual foetuses
- The neck and the thoracic and abdominal cavities of approximately two thirds of each litter were dissected and examined. Following examination and evisceration the foetuses were placed in industrial methylated spirit and stored.
- The remaining foetuses were placed in Bouin´s fixative and stored.

Results

No deaths occurred during the study.

Three females of the 1250 mg/kg bw per dayay group showed brown head, body or peri-genital staining which commenced early in the treatment period. In other respects, the general condition of treated females was similar to that of the control animals.

Food and water consumption of treated females was similar to that of control animals.

In the high dose group, reduced body weight gain (73% of control at day 14) and body weight (90% of control at day 14) were observed during treatment, but some recovery was observed following cessation of treatment. The mean body weight was not affected by the treatment in the 50 and 250 mg/kg bw per dayay groups.

Table 6.6.2.1.1-1: Body weight development in rats administered triticonazole

Dose level [mg/kg bw per dayay]	**0**	**50**	**250**	**1250**
Dams evaluated [n]	**6**	**6**	**6**	**6**
Body weight (mean±SD) [g]				
Day 0 (% control)	216 ± 6	210 ± 7 (97.2)	212 ± 10 (98.1)	211 ± 9 (97.7)
Day 7 (% control)	262 ± 12	249 ± 11 (95.0)	253 ± 13 (96.6)	247 ± 13 (94.3)
Day 14 (% control)	311 ± 16	293 ± 14 (94.2)	299 ± 14 (96.1)	280 ± 16 (90.0)
Day 20 (% control)	396 ± 19	371 ± 26 (93.7)	383 ± 21 (96.7)	353 ± 25 (89.1)
Relative body weight [%] control gain (mean)				
Day 0 to 14	100	87	92	73
Day 0 to 20	100	89	95	79

Gross necropsy in females at termination did not reveal any treatment-related findings. An overview of any findings is presented in table 6.6.2.1.1-2. In the low dose group two incidences of unilateral hydronephrosis were observed and one dam of the high dose also had a bilateral hydronephrosis, possibly indicating some genetic prevalence to develop this finding.

Table 6.6.2.1.1-2: Observations at necropsy of females on day 20 of gestation

Dose Level [mg/kg bw per day]	**0**	**50**	**250**	**1250**
Females examined	**6**	**6**	**6**	**6**
Red/brown staining on head	-	1	-	2
Misshapen muzzle	-	1	-	-
Unilateral hydronephrosis	-	2	-	-
Bilateral hydronephrosis	-	-	-	1
Yellow urogenital staining	-	-	-	1

The number of implantations, viable young, resorptions and the extent of pre- and post-implantation losses showed some inter-group variations, but were essentially unaffected by treatment. Almost all values were well within the historical control data from 177 range finding studies of the laboratory (except 17.6% pre-implantation loss at 250 mg/kg bw per day, but for this finding neither dose response was observed nor the final number of pups was effected). Although within historical control data, number of pups in high dose group was lower, basically as a consequence of the higher post-implantation loss (mean : 11.3%, two dams affected with 25.0 and 21.4%), which was however, also within HCD.

Mean foetal weight was slightly reduced in the 1250 mg/kg bw per dayay group only compared to the controls, but the mean value of 3.31 g was within the historical controls (3.00 – 3.55 with a mean of 3.32 g). The mean placental weight (0.55 g) was slightly increased in the high dose group compared to controls, but still within the historical controls (0.43 – 0.57 g with a mean at 0.50 g), whereas no effects were observed in the other dose groups.

Table 6.6.2.1.1-3: Group mean litter data – females killed on day 20 of gestation

Dose level	0	50	250	1250
Pregnancy status (mean ± SD) [n]				
- viable young (male) HC: 6.8 (5.2-8.2)	7.0 ± 1.4	6.3 ± 1.4	8.3 ± 1.6	5.7 ± 2.3
- viable young (female) HC: 6.9 (5.6-8.7)	6.3 ± 1.2	6.5 ± 1.0	6.3 ± 2.6	6.2 ± 1.9
- viable young (total) HC: 13.7 (11.1-15.3)	13.3 ± 1.5	12.8 ± 1.3	14.7 ± 2.3	11.8 ± 1.7
Cesarean section data				
- Corpora lutea [mean ± SD] HC: 16 (13.9-19)	15 ± 0.9	15 ± 2.8	18 ± 2.1	14.2 ± 2.5
- Implantations [mean ± SD] HC: 14.6 (12.0-16.7)	13.7 ± 1.0	13.2 ± 1.3	14.8 ± 2.4	13.3 ± 2.3
- Pre-implantation loss [mean %] HC: 8.7 (1.6-16.7)	9.9	13.2	17.6	7.0
- Post-implantation loss [mean %] HC: 5.9 (1.7-12.7)	2.4	2.5	1.1	11.3
- Resorptions [mean ± SD] HC: 0.85 (0.25-1.79)	0.3 ± 0.6	0.3 ± 0.6	0.2 ± 0.4	1.5 ± 1.2
- Early resorptions [mean ± SD] HC: 0.68 (0.05-1.68)	0.3 ± 0.6	0.3 ± 0.6	0.2 ± 0.4	1.5 ± 1.2
- Late resorptions [mean ± SD] HC: 0.18 (0.0-0.58)	0	0	0	0
Weight parameters (mean ± SD)				
Placental weight [g] HC: 0.5 (0.43-0.57)	0.50 ± 0.02	0.51 ± 0.02	0.48 ± 0.01	0.55 ± 0.02
Foetal weight [g] HC: 3.32 (3.00-3.55) (% control)	3.47 ± 0.06	3.55 ± 0.07 (102.3)	3.51 ± 0.07 (101.2)	3.31 ± 0.08 (95.4)

HC: Historical control data from 177 studies [mean (range)]

External examination of foetuses

No external findings were recorded that were considered treatment-related. Some findings were above the historical control data (which are exclusively given as fetal incidences); a comprehensive evaluation however is difficult due to the low number of animals investigated compared to a full study. There seems to be some evidence that the increased incidence of large placentae seen in the high dose group is treatment related, as that observation is seen in 3 litters at a fetal incidence of 4.2%, although the fetal incidence is well within historical controls (0.0 – 6.2%).

Table 6.6.2.1.1-4: Results from external examination of foetuses

Dose level [mg/kg bw per day]	0	50	250	1250
Litters Evaluated	6	6	6	6
Foetuses Evaluated	80	77	88	71
Number of males	42	38	50	34
Number of females	38	39	38	37
Observations [% incidence] (no. of litters)				
- Small foetus (less than 2.7 g) HC: 3.44 (0.0-16.9)	0.0 (0)	1.3 (1)	2.3 (2)	5.6 (3)
- Large foetus (more than 4 g) HC: 1.24 (0.0-8.7)	2.5 (2)	3.9 (2)	2.3 (1)	16.9 (1)
- Pale foetus HC: 0.03 (0.0-1.1)	0.0 (0)	0.0 (0)	1.1 (1)	0.0 (0)
- Shiny foetus HC: 0.24 (0.0-4.1)	0.0 (0)	1.3 (1)	0.0 (0)	1.4 (1)
- Domed head	0.0 (0)	0.0 (0)	0.0 (0)	1.4 (1)

Dose level [mg/kg bw per day]	0	50	250	1250
HC: 0.007 (0.0-0.4)				
- Kink in tail HC: 0.007 (0.0-0.4)	0.0 (0)	0.0 (0)	0.0 (0)	1.4 (1)
- Pale/white rimmed placenta HC: 0.09 (0.0-4.1)	0.0 (0)	1.3 (1)	0.0 (0)	5.6 (1)
- Large placenta (more than 0.7 g) HC: 1.33 (0.0-6.2)	0.0 (0)	1.3 (1)	0.0 (0)	4.2 (3)

HC: Historical control data from 175 studies including 44562 foetuses [mean (range)]

Internal examination

Macroscopic examination of foetuses of all dose groups showed low, but possibly dose-related incidence of hydronephrosis. In the 250 mg/kg bw increased unilateral hydronephrosis occurred in two litters (5.0% of the foetuses) and at the 1250 mg/kg bw three litters (6.4% of the foetuses) had this finding. Increased incidences of bilateral hydronephrosis was seen in 2 (3.9% foetal incidence), 1 (6.7% foetal incidence) and 2 (8.5% foetal incidence) litters in the 50, 250 and 1250 mg/kg bw group. All foetal incidence values except the 8.5% from the 1250 mg/kg bw dose group were within the historical control range. Hydronephrosis is characterized by dilatation of the renal pelvis, which is usually accompanied by compression of the parenchyma. Notifier stated that it is frequently observed in animal experiments, including control animals, and is usually classified as variation (Hoffmann et al, 2016). Variations of the urinary tract are however, also frequently observed with triazole compounds[3]. Due to foetal dose response (no dose response observed on litter basis) and occurrence above historical control data, bilateral hydronephosis is considered to be a treatment-related effect at 1250 mg/kg bw per day. No treatment-related increased incidences of hydroureter were seen in this range finding study.

In the main study no treatment-related effects on the incidence of hydronephrosis was observed, however in the main study the maximum dose was lower i.e.1000 mg/kg bw per day (limit dose). It cannot be excluded that hydronephrosis, already observed in dams, also might indicate some genetic prevalence in the animals used for the study.

Table 6.6.2.1.1-5: Results from external examination of fetuses

Dose level [mg/kg bw per day]	0	50	250	1250
Litters Evaluated	6	6	6	6
Foetuses Evaluated	54	51	60	47
Number of males	29	21	34	21
Number of females	25	30	26	26
Observations [% incidence] (no. of litters)				
- Prominent blood vessels on lungs	0.0 (0)	0.0 (0)	0.0 (0)	0.0 (0)
- Haemorrhagic edges to lungs	1.9 (1)	0.0 (0)	0.0 (0)	0.0 (0)
- Unilateral hydronephrosis HC: 0.99 (0.0-7.6)	0.0 (0)	0.0 (0)	5.0 (2)	6.4 (3)
- Bilateral hydronephrosis HC: 0.70 (0.0-7.6)	0.0 (0)	3.9 (2)	6.7 (1)	8.5 (2)
- Bilateral hydroureter HC: 2.76 (0.0-15.8)	1.9 (1)	3.9 (1)	0.0 (0)	0.0 (0)

HC: Historical control data from 141 studies including 21472 foetuses [mean (range)]

[3] EFSA Panel Panel on Plant Protection Products and their Residues (PPR Panel) Scientific Opinion on risk assessment for a selected group of pesticides from the triazole group to test possible methodologies to assess cumulative effects from exposure throughout food from these pesticides on human health on request of EFSA. 2009; 7 (9); 1167. [104pp.]. Available online: www.efsa.europa.eu

Conclusion

Based on the toxicological response of dams (reduced body weight and body weight gain) and foetuses in utero (bilateral hydronephrosis) to a dosage of 1250 mg/kg bw per dayay, a dosage of 1000 mg/kg bw per dayay was suggested as a suitable top dose to be used in the main prenatal developmental toxicity study in rats. The NOAEL of the range finding study is proposed at 250 mg/kg bw per day for both maternal and developmental toxicity.

B.6.6.2.1.2. Main rat study

Previous evaluation:	DAR (2003)
DRAR (2016)	Additional information/tables added in the results Conclusion on the foetal NOAEL of 200 mg/kg bw per day from DAR 2003 confirmed Maternal NOAEL proposed to be increased from 40 (DAR 2003) to 200 mg/kg bw per day

Reference:	RPA 400727: Teratology study in the rat
Author(s), year:	[redacted] 1991
Report/Doc. number::	C018955 / -
Guideline(s):	US EPA guideline 83-3 and OECD 414 (1981)
GLP:	Yes
Deviations from OECD 414 (2001):	According to the most recent OECD TG 414, the recommended administration period is covering the whole gestation period and should continue until the day prior to scheduled caesarean section. Otherwise the study conducted in 1991 broadly met the requirements of the 2001 version of the guideline. The study protocol is considered to be able to identify teratogenicity
Acceptability:	Yes; since one of the effects of triazoles is possible cleft palate formation, the duration of administration is considered acceptable (hard palate closure is finalised at end of organogenesis)

Material and methods

Groups of 23 presumed pregnant rats (strain: CD of Sprague Dawley origin; source: [redacted]) received triticonazole (batch no YG 2156/1; purity 99.5 %; suspended in 0.5 % w/v aqueous methylcellulose) from day 6 to 15 of pregnancy by oral gavage at a constant dosing volume of 10 ml/kg bw. Dose levels were 0 (vehicle control), 40, 200 and 1000 mg/kg bw per day. These dose levels were based on the results of a preliminary study with dose levels of 0, 50, 250 and 1250 mg/kg bw per day given to groups of 6 pregnant rats. Dosing suspensions were prepared freshly every day and concentrations were confirmed by analysis.

Females were paired on a one-to-one basis with stock males of the same strain. Each morning following pairing the trays beneath the cages were checked for ejected copulation plugs and a vaginal smear was prepeared from each female and examined for the presence of spermatozoa. Observations for mortality and clinical signs were made daily. Body weights were recorded on days 0, 3, 6, 6 – 16, 18 and 20 of gestation, food consumption was measured over two to four day periods on days 3, 6, 9, 12, 16, 18 and 20 of gestation. On gestation day 20, females were subjected to a post-mortem macroscopic examination. The reproductive tract was examined for the number of corpora lutea/ovary, implantation and resorption sites, number and distribution of live and dead foetuses, weight and sex of each foetus and external malformed foetuses. Half of the foetuses from each litter were processed for the examination of skeletal abnormalities and the remainder processed for visceral abnormalities.

Results

Maternal effects: There were no deaths among treated females and no treatment-related clinical signs of toxicity were observed in any dose-group. At the highest dose level, slight maternal toxicity was reflected in marginally, but consistently lower body weight gains during the treatment period compared to controls, particularly during the latter part of the study (days 12 – 16: 85.5% body weight gain compared to control), resulting in mean body weight of approx. 96 % of controls at termination (table 6.6.2.1.2-1). In addition, food intake was marginally reduced throughout the treatment period at 1000 mg/kg. There were no effects on these parameters at the two lower dose levels.

Table 6.6.2.1.2-1: Food consumption and body weight development in rats administered triticonazole

Dose level [mg/kg bw per dayay]	**0**	**40**	**200**	**1000**
Food consumption [g/animal/day] (% control)				
Day 0 to 2	25	26 (104)	26 (104)	25 (100)
Day 3 to 5	27	28 (103.7)	28 (103.7)	27 (100.0)
Day 6 to 8	29	29 (100.0)	29 (100.0)	27 (93.1)
Day 9 to 11	31	31 (100.0)	31 (100.0)	29 (93.5)
Day 12 to 15	33	33 (100.0)	33 (100.0)	31 (93.9)
Day 16 to 17	37	37 (100.0)	36 (97.3)	35 (94.6)
Day 18 to 19	36	35 (97.2)	35 (97.2)	33 (91.7)
Body weight [g] (% control)				
Day 0	226	227 (100.4)	228 (100.9)	225 (99.6)
Day 6	270	273 (101.1)	272 (100.7)	265 (98.1)
Day 15	347	351 (101.2)	348 (100.3)	336 (96.8)
Day 20	437	439 (100.5)	434 (99.3)	419 (95.9)
Body weight gain [g] (% control)				
Day 0 to 6	44	46 (104.5)	44 (100.0)	40 (90.9)
Day 6 to 12	47.6	48.2 (101.3)	46.5 (97.7)	44.7 (93.9)
Day 12 to 16	44.7	41.2 (92.2)	39.9 (89.3)	38.2 (85.5)
Day 0 to 20	211	212 (100.5)	206 (97.6)	194 (91.9)

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

At necropsy, pale areas on the median or lateral liver lobes were noted in 0, 0, 2 and 2 animals at 0, 40, 200 and 1000 mg/kg, respectively (table 6.6.2.1-2). There were no other findings considered treatment-related at terminal necropsy.

Table 6.6.2.1.2-2: Gross necropsy findings in rats administered triticonazole during gestation days 6 to 15

Dose group	Animal #	Observation
Control	10	raised white areas on surface of spleen
	22	Unilateral hydronephrosis
	23	Uterus distended with yellow fluid
40 mg/kg bw per dayay	46	Red/brown staining on head/nose/ears
	42	Scab on upper lip
	28	Unilateral hydronephrosis
	28	Unilateral hydroureter
200 mg/kg bw per dayay	69	Red/brown staining on head/nose/ears
	63, 64	(Punctate) pale areas on surface of median/lateral liver lobes
1000 mg/kg bw per dayay	81, 96, 99	Red/brown staining on head/nose/ears
	99	Hair-loss on upper fore-limb
	80, 97	(Punctate) pale areas on surface of median/lateral liver lobes

Litter data/foetal parameters: The number of implantations and of the viable young, the extent of pre- and post-implantation losses, and the mean foetal and placental weights gave no indication of any response to treatment in any dose group.

Table 6.6.2.1.2-3: Caesarean section data of rats

Dose level [mg/kg bw per dayay]	0	40	200	1000
Pregnancy status				
- mated [n]	25	25	25	25
- pregnant [n]	25 (24, one female died on day 8)	24	24	23
conception rate [%]	100	96	96	92
- aborted [n]	0	0	0	0
- dams with viable fetuses [n]	24	24	24	23
- dams with all resorptions [n]	0	0	0	0
- mortality	1	0	0	0
- pregnant at terminal sacrifice [n]	24	24	24	23
Cesarean section data[a]				
- Corpora lutea [n] [HCD: mean: 16.4 (14.3-18.5)]	16.8±2.2	17.8±2.5	16.7±2.7	16.1±1.9
- Implantation sites [n] [HCD: mean: 15.1 (13.5-17.1)]	15.4±1.7	16.1±2.8	15.7±3.3	15.3±1.6
- Pre-implantation loss [%] [HCD: mean: 8.0 (5.9-12.5)]	8.4	10.4	7.6	6.4
- Post-implantation loss [%] [HCD: mean: 5.3 (2.8-8.6)]	4.6	5.4	5.3	7.7
- Resorptions [n] [HCD: mean: 0.77 (0.40-1.38)]	0.7±0.8	0.9±0.9	0.8±0.9	1.2±1.1
- Early resorptions [HCD: mean: 0.75 (0.40-1.38)]	0.7±0.8	0.9±0.9	0.8±0.9	1.2±1.1
- Late resorptions [HCD: mean: 0.02 (0.00-0.05)]	0.0	0.0	0.0	0.0

Dose level [mg/kg bw per dayay]	**0**	**40**	**200**	**1000**
- Dead foetuses [n]	0	0	0	0
- Live foetuses [n] [HCD: mean: 14.3 (12.7-16.5)]	14.7±2.1	15.3±2.6	14.9±3.2	14.1±1.8
- Total live female foetuses [n] [HCD: mean: 7.2 5.9-8.8)]	7.0±2.5	6.8±2.2	7.3±2.5	6.6±2.1
- Total live male foetuses [n] [HCD: mean: 7.1 (5.8-8.6)]	7.6±2.0	8.4±2.5	7.6±2.2	7.5±2.2
- Sex ratio [% males]	51.7	54.9	51.0	53.2
Placental weights [g] [HCD: mean: 0.51 (0.46-0.54)]	0.56±0.02	0.53±0.02	0.56±0.02	0.56±0.02
Mean foetal weight [g]	3.74±0.05	3.74±0.06	3.73±0.07	3.77±0.07
- males [g]	3.83±0.21	3.84±0.23	3.84±0.22	3.87±0.19
- females [g]	3.64±0.22	3.61±0.22	3.61±0.18	3.67±0.22

[a] Mean ± SD on litter basis; Statistical evaluation: * $p \leq 0.05$; ** $p \leq 0.01$ (Dunnett-test, two-sided)

Examination of the foetuses at necropsy showed that the pattern of external gross abnormalities did not suggest any effect of treatment. In addition, no effects of treatment were identified at foetal examination for visceral abnormalities. The incidence of hydronephrosis (unilateral plus bilateral) was slightly increased in the top dose group compared with controls but was well within the historical control range of 18 studies from the laboratory, conducted in the period of 5 years. The increased incidence of hydronephrosis in the preliminary study at 50, 250 and 1250 mg/kg bw per day was thus not reproduced at comparable dose levels.

Examination of the foetuses at necropsy showed that the pattern of external gross abnormalities did not suggest severe effect of treatment. One remarkable finding was an increased incidence of large fetuses in the top dose, although the incidence at 1000 mg/kg bw per day was also within HCD. The fetal incidences and litters were 9.1% (12), 8.7% (12), 8.1% (12) and 10.5% (16) in the controls and treatment groups. Further the incidence of foetueses with large placenta was increased above historical control data at 250 and 1000 mg/kg bw. The fetal incidences and litters were 4.5% (6), 3.6% (8), 5.6% (9) and 6.2% (9) in the controls and treatment groups. In the absence of effect on the placenta weights this finding is considered of limited significance.

For the fetuses examined at necropsy (179, 189, 186 and 166 foetuses in the control, 40, 200, 1000 mg/kg bw), the incidence of hydronephrosis (unilateral plus bilaterial) was slightly increased in the top dose group (3 % of the fetuses affected) compared with controls (1.1 % of the fetuses affected), but was well within the historical control range (0 – 6.4 % for unilateral and 0-5.7% for bilateral hydronephrosis) for these findings in the laboratory. For the fetuses examined during the serial freehand sectioning (173, 177, 171, and 158 foetuses in the control, 40, 200, and 1000 mg/kg bw groups) no meaningful differences were observed for unilateral or bilateral hydronephrosis. Also the incidences for uni- or bilateral hydroureters were not different from controls in both groups of foetuses. Thus a treatment relationship for the occurrence of hydronephrosis and hydroureter can be excluded. The detailed data are shown in table 6.6.2.1.2-4 and 6.6.2.1.2-5.

Table 6.6.2.1.2-4: Incidence of necropsy findings in rat foetuses

Dose level [mg/kg]	**0**	**40**	**200**	**1000**
Litters Evaluated	24	24	24	23
Fetuses Evaluated	352	366	357	324
Individual findings				
- Large foetus (more than 4.10 g)				

Dose level [mg/kg]	0	40	200	1000
- Fetal incidence [%]	9.1	8.7	8.1	10.5
*HCD: mean: 6.60 (0.7 – 16.3)**				
- Litter incidence [No.]	12	12	12	16
- Large placenta (more than 0.70 g)				
- Fetal incidence [%]	4.5	3.6	5.6	6.2
HCD: mean: 1.34 (0.0-5.0)				
- Litter incidence [No.]	6	8	9	9
Litters Evaluated	24	24	24	23
Fetuses Evaluated	179	189	186	166
Individual findings				
- Unilateral hydronephrosis				
- Fetal incidence [%]	0.0	0.0	0.5	1.2
*HCD: mean: 0.72 (0.0 - 6.4)***				
- Litter incidence [No.]	0	0	1	2
- Bilateral hydronephrosis				
- Fetal incidence [%]	1.1	0.0	0.0	1.8
*HCD: mean: 0.68 (0.0-5.7)***				
- Litter incidence [No.]	2	0	0	3
- Unilateral hydroureter				
- Fetal incidence [%]	0.6	0.5	1.1	1.2
*HCD: mean: 0.49 (0.0-2.2)***				
- Litter incidence [No.]	1	1	2	2
- Bilateral hydroureter				
- Fetal incidence [%]	1.7	0.0	0.0	1.8
*HCD: mean 0.68 (0.0-2.6)***				
- Litter incidence [No.]	2	0	0	3

*Historical background data collected in 22 studies and 6926 foetuses and ** in 18 studies and 3076 foetuses conducted approximately up to the time of the present study.

Table 6.6.2.1.2-5: Incidence of findings in rat fetuses at free-hand serial sectioning

Dose level [mg/kg]	0	40	200	1000
Litters Evaluated	24	24	24	23
Fetuses Evaluated	173	177	171	158
Individual findings				
- Unilateral slight hydronephrosis				
- Fetal incidence [%]	0.6	0.0	0.6	1.3
*HCD: mean: 0.85 (0.0 - 3.6)**				
- Litter incidence [No.]	1	0	1	2
- Bilateral slight hydronephrosis				
- Fetal incidence [%]	0.6	0.0	1.8	0.0
*HCD: mean: 0.45 (0.0-1.4)**				
- Litter incidence [No.]	1	0	2	0
- Unilateral slight hydroureter				
- Fetal incidence [%]	1.2	0.0	2.3	2.5
*HCD: mean: 3.89 (0.0-9.2)**				
- Litter incidence [No.]	2	0	4	4
- Bilateral hydroureter				
- Fetal incidence [%]	5.2	0.0	3.5	1.9
*HCD: mean 2.84 (0.0-5.3)**				
- Litter incidence [No.]	5	0	3	3

*Historical background data collected in 16 studies and 2466 foetuses conducted approximately up to the time of the present study.

Examination of the foetuses for skeletal abnormalities revealed a dose-related increase in the incidence of foetuses with an additional (rudimentary) thoracolumbar rib (14th rib) or pair of ribs (13/14 and 14/14), slightly exceeding the historical control at 1000 mg/kg bw dose group, but being not statistically significant. The incidences of unilateral or bilateral 14th rib was slightly higher than the concurrent control values also at 40 and 200 mg/kg, did also not achieve statistical significance and were within the historical background range collected from studies

conducted before and after (1991 – 1994) the time, where the present study ran. The notifier cited the publication of Wickramaratne[4] where the spontaneous occurrence of supernumerary ribs as well as their prevalence post-partum was discussed. According to the author slight disturbances in development readily affect the thoraco-lumbar border which is a particularly labile region. After the birth, the frequency of (rudimentary) supernumerary ribs decline, accompanied by an increase in the prevalence of a fully ossified transverse process on the first lumbar vertebra as a consequence of the maturation. Based on additional literature[5] RMS concluded that the supernumerary ribs in rats, other than in mice (where they are very frequently response to stress), may represent a direct effect of the compound on the foetus, an indirect effect due to disturbance of maternal homeostasis or a combination of direct and indirect effects. However, according to Hood 2006, supernumerary ribs seem to be one of the few foetal effects which could be attributed to maternal toxicity with a higher level of certainty.

The incidences of the skeletal findings are summarized in the table 6.6.2.1.2 -6. The percentage of foetuses affected per litter (as litter is the experimental unit) has been calculated according to Hood RD, 2006 (Developmental and Reproductive Toxicology: A Practical Approach) as sum of percent of affected fetuses per litter divided by total number of litters. Since this parameter has been calculated by RMS, no historical control data are available. Historical control data are however available for percentage incidence of fetuses only.

Table 6.6.2.1.2-6: Incidence of skeletal findings in rats

Dose level [mg/kg bw per day]	**0**	**40**	**200**	**1000**
Litters Evaluated	24	24	24	23
Fetuses Evaluated	179	189	186	166
Discrete unossified area in frontal bone				
- Fetal incidence [n]	1	1	1	2
- Fetal incidence [%] *HCD: 0.0-2.4*	0.6	0.5	0.5	1.8
- Litter incidence [No.]	1	1	1	2
- % fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	0.52 ± 3.4	0.59 ± 2.9	0.46±2.3	1.16±3.9
Incomplete ossification of hyoid bone				
- Fetal incidence [n]	7	14	11	16
- Fetal incidence [%] *HCD: 0.0-14.8*	3.9	7.4	5.9	9.6
- Litter incidence [No.]	3	9	10	11
- % fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	3.79 ± 19.8	7.63 ± 13.3	5.77 ± 7.7	9.39 ± 13.4
Ribs 13/14				
- Fetal incidence [n]	21	25	25	28
- Fetal incidence [%] *HCD: 0.0-15.9**	11.7	13.2	13.4	*16.9*
- Litter incidence [No.]	11	18	13	15

[4] Wickramaratne GA. (1988; BASF DocID 1988/1003516) The Post-natal Fate of Supernumerary Ribs in Rat Teratogenicity Studies. Journal of Applied Toxicology 8(2): 91-94

[5] Hood RD. (2006) Developmental and Reproductive Toxicology: A Practical Approach

Dose level [mg/kg bw per day]	**0**	**40**	**200**	**1000**
- % fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*[5]	11.73±54.8	13.58±11.2	12.38±14.2	17.01±16.9
14th rib				
- Fetal incidence [n]	9	17	18	24
- Fetal incidence [%] *HCD: 0-10.4**	5.0	9.0	9.7	*14.5*
- Litter incidence [No.]	3	6	9	9
- % fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	4.97±26.2	8.31±15.7	9.06±16.5	14.58±21.7
14th rib or enlarged rib				
- Fetal incidence [n]	0	1	1	2
- Fetal incidence [%] HCD: 0.0-8.3*	0.0	0.5	0.5	1.2
- Litter incidence [No.]	0	1	1	2
- % fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	0	0.42±2.0	0.38±1.9	1.35±4.5

*Historical background data collected in 16 studies conducted approximately in the time of the present study and in 91 studies conducted between 1991 and 1994.

It is concluded that the observed maternal toxicity could have contributed to marginally increased incidences in skeletal variations at 1000 mg/kg bw per day. All observed effects were developmental variations or signs of delayed development but no treatment related teratogenic effects were observed. The increased incidence (above HCD) of hydronephrosis in the preliminary study at 1250 mg/kg bw per day was not reproduced in this study at 1000 mg/kg bw per day, however, no direct comparison can be made.

Conclusion

In the DAR (2003) the NOAEL for maternal toxicity in this study was considered to be 40 mg/kg bw per day based on slight reduction in body weight gain and food consumption at 1000 mg/kg bw per day, and incidences of pale areas in the liver of two animals each at 200 and 1000 mg/kg bw per day. Foetal survival and growth was not affected in any dose group. However, there was an apparently increase in the incidence of foetuses with an additional 14th rib or pair of ribs at all dose levels (not regarded to be a malformation), but was only outside the historical range at 1000 mg/kg bw per day. Therefore, the foetal NOAEL was set at 200 mg/kg bw per day. There was no teratogenic effect related to the treatment at any dose level.

In the DRAR (2016) RMS carefully re-assessed the findings and proposed to revise the maternal NOAEL from 40 to 200 mg/kg bw per day, based on reduction in body weight gain from GD 12- 16 (85.5% of control value) at 1000 mg/kg bw per day. The only effects observed at 200 mg/kg bw per day (pale areas on surface of median/lateral liver lobes) in two dams were not considered justified to derive a NOAEL at 40 mg/kg bw per day. Regarding foetal effects, RMS confirmed the NOAEL of 200 mg/kg bw per day from DAR (2003), based on the increased incidence of additional 13th and 14th rib at 1000 mg/kg bw per day, slightly above the historical control data. Bilateral hydronephrosis observed in the range finding study above the HCD in animals treated with 1250 mg/kg bw per day was not confirmed in the main study up to 1000 mg/kg bw per day (limit dose) and in contrast to range finding

study, also no dose response was observed in the main study. It cannot be excluded that hydronephrosis, already observed in dams in the range-finding study, also might indicate some genetic prevalence in the animals used in that study.

Based on the available information no classification for developmental effects is justified.

B.6.6.2.2. Rabbit

B.6.6.2.2.1. **Tolerance rabbit study**

Previous evaluation:	No
DRAR (2016)	Although theoretically available at the time of first DAR (2003), the study was not included in the dossier that time. Therefore, the study is evaluated for the first time for the purpose of renewal of triticonazole

Reference:	RPA 400727: Tolerance study in the rabbit
Author(s), year:	[redacted] 1990
Report/Doc. number::	C044414 / -
Guideline(s):	No Guideline for this type of study available
GLP:	Yes
Deviations	-
Acceptability:	Yes; only very limited information available

A tolerance study has been conducted with non-pregnant rats. Triticonazole (batch: BD 1074, purity: 99.7%) was administered by gavage to two non-pregnant New Zealand White rabbits, commencing at an initial dosage of 500 mg/kg bw per dayay for two days. Following marked toxicity, the females were terminated and a further two allocated to receive triticonazole at a dose level of 25 mg/kg bw per dayay. In the absence of any adverse response, the dosage was doubled every two days until a reaction to treatment was elicited at a dose level of 100 mg/kg bw. Two females were then inseminated and received triticonazole at a dosage of 50 mg/kg bw from day 6 to day 12 of gestation. Following completion of treatment (day 13 of gestation), all animals were sacrificed and examined for evidence of reaction to treatment and pregnancy.

Results

Group I (500 mg/kg bw, non-pregnant animals): One female was killed *in extremis* and the second terminated following a marked toxic response, characterized by bodyweight loss and reduced food intake and faecal output, following administration of 500 mg/kg bw. Animal killed *in extremis* had shallow respiration, dark eyes and was prone and unable to rise. Necropsy investigation revealed no differences in animal killed *in extremis* on day 3 and animal terminated on day 3.

Group II (variable dose, non-pregnant animals see above): Bodyweight loss and reduced food intake and faecal output were observed following the first dose at 100 mg/kg bw. Terminal necropsy of both females revealed no macroscopic changes that were attributable to treatment.

Group III (50 mg/kg bw pregnant animals): Both females exhibited timid behavior for varying durations during the study. Both females showed slight transient bodyweight loss following commencement of treatment. No other adverse effects of treatment with triticonazole were observed. Both animals were pregnant. One female had one late resorption, but all remining foetuses from both females appeared grossly normal.

Table 6.6.2.2.1-1: Summary of animal observations at terminal necropsy

Group	Animal number	Summary of necropsy findings
1	23TJ699	Thoracic cavity: NAD Abdominal cavity: stomach contents reduced, caecal contents dark, compacted and dehydrated. Remainder of the intestinal tract generally devoid of content, no faecal pellet formation in rectum
2	23TJ688	Thoracic cavity: NAD Abdominal cavity: NAD
2	23TJ696	Thoracic cavity: NAD Abdominal cavity: NAD
3	23TJ690	Thoracic cavity: NAD Abdominal cavity: NAD Pregnant, all implantations grossly normal
3	23TJ704	Thoracic cavity: NAD Abdominal cavity: NAD Pregnant, one late resorption, all remaining implantations appear grossly normal

NAD No abnormality detected

Table 6.6.2.2.1-2: Summary of mortality

Group	Animal number	Summary of necropsy findings
1	23TJ702	Thoracic cavity: NAD Abdominal cavity: stomach contents reduced, caecal contents dark, compacted and dehydrated. Remainder of the intestinal tract generally devoid of content, no faecal pellet formation in rectum

NAD No abnormality detected

Conclusion

It was concluded from this investigation that dosages of triticonazole for use in a preliminary teratology study in the rabbit should not exceed 50 mg/kg bw.

B.6.6.2.2.2. Range finding rabbit study

Previous evaluation: DRAR (2016)	No Although theoretically available at the time of first DAR (2003), the study was not included in the dossier that time. Therefore, the study is evaluated for the first time for the purpose of renewal

Reference:	RPA 400727: Preliminary teratology study in the rabbit
Author(s), year:	[redacted] 1990
Report/Doc. number::	C019984 / -
Guideline(s):	US EPA guideline 83-3 and OECD 414 (2001)
GLP:	Yes
Deviations from OECD 414 (2001):	According to the most recent OECD TG 414, the recommended administration period is covering the whole gestation period and should continue until the day prior to scheduled caesarean section. While range-finding, lower number of does (7-8) was included in the study.
Acceptability:	Yes; additional information

Material and methods

Before entering the main rabbit prenatal developmental toxicity study of triticonazole (batch: YG 2156/1, purity: 99.5%) a range-finding study had been conducted in seven groups of each 7 - 8 pregnant female New Zealand White (NZW) rabbit of that two groups served as controls and received vehicle only (0.5% w/v methyl cellulose in distilled water), and the other groups received dose levels of 5, 15, 50, 75 and 150 mg Triticonazole/kg bw/d on days 6-19 of gestation via gavage. Animals were sacrificed on day 29 and the uterine contents examined. All foetuses were examined for external findings. The neck and the thoracic and abdominal cavities were dissected and also examined in all foetuses.

Test animals:

Species:	Rabbit
Strain:	New Zealand White
Sex:	female
Age:	ca. 18-26 weeks (at arrival)
Weight:	3.36 to 4.63 kg (at commencement of the study)
Source:	[redacted]
Acclimation period:	at least 7 days
Diet:	S.Q.C. Standard Rabbit Diet (Special Diet Services Limited, Witham, England), ad libitum
Water:	Tap water in polythene bottles and chromium-plated sipper tubes, ad libitum
Housing:	Single housing in suspended stainless steel cages (type RC10/L; 61 x 76 x 46 cm) mounted in batteries (Modular Systems Development Co. Ltd., Woolwich, England) that were fitted with perforated counter-sunk floor panels. An undertray beneath the floor was linked with absorbent crepe-paper wich was changed at least three times per week.

Environmental conditions:

Temperature:	15 – 23°C
Humidity:	40 – 70%
Air changes:	approx. 17-20 air changes/hour
Photo period:	Alternating 14-hour light and 10-hour dark cycles

Three weeks prior to the expected date of insemination, oestrus cyclus was synchronized by the supplier by intravenous injection of 25 i.u. luteinizing hormone (Profasi, Serono). Females were artificially inseminated with pooled semen from New Zealand White males of established fertility. Following insemination, each female was injected intravenously with 25 i.u. of luteinising hormone (Profasi, Serono), to ensure successful ovulation. The day of insemination was designated day 0 of gestation.

All animals were examined on day 6 of gestation (GD) prior to dosing and one female (Study Group 1/RHA/414) considered unsuitable was excluded from the study prior to dosing. No data are presented for this animal.

The seven groups were treated as follows:

Table 6.6.2.2.2-1: Study groups for range-finding rabbit teratology study

Study Group	Treatment	Dose level [mg/kg bw per day]	Animal number per group
1- 1/RHA/346	Control	0	8
2 - 2/RHA/346	Triticonazole	5	8
3 - 3/RHA/346	Triticonazole	15	8
4 - 4/RHA/346	Triticonazole	50	8
5 - 1/RHA/414	Control	0	7
6 - 2/RHA/414	Triticonazole	75	8
7 - 3/RHA/414	Triticonazole	150	8

The test compound was formulated freshly each day in 0.5% w/v aqueous MC. Animals were dosed daily by gavage from day 6 to day 19 of gestation at a volume-dosage of 5 mL/kg. Control animals received the vehicle at the same volume-dosage during the same treatment period. The volume administered daily to each animal was based on the animal's bodyweight on that day and was recorded.

Samples of each concentration of the test substance mixtures were taken during the first and last weeks of treatment.

Where relevant, means and standard deviations of all test groups were calculated. Because of the small sample size in this range-finding assay no statistical evaluation was performed. Biological significance was assessed by reference to historical control data. Regarding historical control data RMS concluded that these can be seen only as indicative values since the time frame when the studies were conducted could not be proven and not provided by the laboratory. Upon the reqest of the RMS to provide the time frame for the HCD the notifier tried to do so, but due to the old age of the study and expiry of the data storage requirement of GLP, the notifier was not able to retrieve additional historical control data from the contract laboratory in which the study was performed.

The animals were examined once daily for any visible signs of reaction to treatment.

Maternal body weight was recorded daily throughout the study period.

Food and water consumption were recorded for each animals for the following time-intervals: days 1-5, 6-12, 13-19, 20-23 and 24-28.

Animals which were terminated prematurely were subjected to a thorough macroscopic examination of the visceral organs with the objective of identifying the cause of their condition. Specimens of abnormal tissues were retained.

The animal that aborted was killed by intravenous injection of pentobarbitone sodium B.P. Vet. (Expiral, Sanofi Animal Health, Watford, Hertfordshire) on the same day that the abortion was detected. The female was subjected to a detailed macroscopic examination and the numbers of corpora lutea and implantation sites were recorded.

On GD 29, the does were sacrificed by intravenous injection of pentobarbitone sodium for examination of their uterine contents. Each animal was first examined macroscopically for evidence of disease or adverse reaction to treatment and specimens of tissues considered abnormal were retained in an appropriate fixative.

The reproductive tract, complete with ovaries, was dissected out and the following parameter were recorded:

- Number of corpora lutea
- Number of implantation sites (In apparently non-pregnant animals, presence of implantation sites was checked using the Salewski staining technique)
- Number of resorption sites (classified as early or late)
- Number and distribution of live and dead foetuses in each uterine horn
- Weight and eternal abnormalities of individual placentae

Based on the above the following parameters were calculated:

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

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

Post-implantation loss covered only the period between days 10 and 29 of gestation; ir did not include the first 3-4 days post-implantation as any death that occurred in thisphase would leave no remainsvisible on Day 29.

All foetuses were killed by subcutaneous injection of pentobarbitone sodium. The following examinations were performed with the foetuses:

- Weight and sex of individual foetuses
- External abnormalities of individual foetuses
- The neck and the thoracic and abdominal cavities of all foetuses from each litter were dissected and examined. Following examination and evisceration the one third of the foetuses in each litter were decapitated and the heads stored in Boulin's fluid.
- Torsos and the remaining intact foetuses were stored in industrial methylated spirit (74°o.p.)

Based on the above the following parameters were calculated:

$$\text{Foetal observations [\%]: } \frac{\text{Number of foetuses with particular observation}}{\text{Number of foetuses examined}} \times 100$$

The number of litters in which a particular observation occurred has also been presented for each group.

Results

The actual test-item concentrations were in the range of 97.7% to 108.4% of the nominal concentrations, verifying the correctness of the applied doses. No test item was detected in the control application.

Mortality

All females of the top dose (150 mg/kg bw per dayay) were terminated prematurely on GD 8/9 after insemination due to animal welfare reasons.

No deaths occurred during the study in all other dose groups.

Necropsy observations

The animals of the 150 mg/kg bw dose group, which were prematurely sacrificed at GD 8 or 9 displayed weight losses (8/8 animals), reduced food and water intakes (8/8 animals) and reduced faecal output (7/8 animals). Compacted and/or gaseous (one incidence) stomach contents and reduced GI-tract or caecum contents were seen at necropsy in some sacrificed animals. At terminal necropsy on GD 29, no macroscopic changes in maternal condition were recorded that could be attributed to treatment with triticonazole at 75 mg/kg bw.

Table 6.6.2.2.2-2: Individual observations at necropsy

Group (mg/kg bw per day)	Animal number	History and circumstances of death	Summary of necropsy findings
150	89FR199	Weight loss 380 g from day 6. Reduced food and water intakes and faecal output. Apparent reduction in body temperature. Terminated Day 9 after insemination.	Thoracic cavity: NAD Abdominal cavity: NAD Pregnant, all implantationsappeared grossly normal
150	89FR209	Weight loss 390 g from day 6. Reduced food and water intakes and faecal output. Apparent reduction in body temperature. Terminated Day 9 after insemination	Thoracic cavity: NAD Abdominal cavity: Stomach contents compacted. Reminder of intestinal tract generally devoid of content. Numerous small indentations on the surface of both kidneys. Pregnant, all implantations appeared grossly normal
150	89FR226	Weight loss 370 g from day 6. Reduced food and water intakes and faecal output. Terminated Day 9 after insemination	Thoracic cavity: NAD Abdominal cavity: Pale raised area on surface of median liver lobe, cut section reveals prulent material. Pregnant, all implantations grossly normal
150	89FR229	Weight loss 460 g from day 6. Reduced food and water intakes and faecal output. Apparent reduction in body temperature. Terminated Day 9 after insemination	Thoracic cavity: NAD Abdominal cavity: Stomach contents gaseous. Gall bladder reduces. Numerous hard pellets in large intestine, rectum devoid of content. Pregnant, one early resorption, all other implantations appeared grossly normal
150	89FR232	Weight loss 410 g from day 6. Reduced food and water intakes and faecal output. Apparent reduction in body temperature. Terminated Day 9 after insemination	Thoracic cavity: NAD Abdominal cavity: Stomach contents slightly compacted, caecal contents reduced. Numerous soft fecal pellets in rectum. Pregnant, all implantations appear grossly normal
150	89FR238	Weight loss 490 g from day 6. Reduced food and water intakes and faecal output. Apparent reduction in body temperature. Terminated Day 9 after insemination	Thoracic cavity: NAD Abdominal cavity: Gastro-intestinal tract contents dehydrated. Pregnant, all implantations appear grossly normal
150	89FR247	Weight loss 70 g from day 6. Reduced food and water intakes Days 7 and 8. Terminated Day 9 after insemination	Thoracic cavity: NAD Abdominal cavity: Gall bladder contents pale. Pregnancy not confirmed.

150	89FR250	Weight loss 330 g from day 6. Reduced food and water intakes and faecal output. Difficult to dose. Increased respiration. Terminated Day 8 after insemination	Thoracic cavity: NAD Abdominal cavity: Extensive haemorrhage in muscle tissue and fat in the lumbar region. Pregnant, all implantations appear grossly normal

NAD No abnormality detected

Clinical signs of toxicity

Top dose females receiving 150 mg/kg bw per dayay exhibited a marked adverse response to treatment as evidenced by marked body weight loss, reduced food and water consumption as well as reduced defecation and apparent reduction in body temperature.

Does receiving 75 mg/kg bw per dayay showed similar responses, however with a reduced severity.

All other females receiving triticonazole did not show any clinical signs of toxicity that could be attributed to the treatment.

Table 6.6.2.2.2-3: Observations and clinical signs of toxicity in does administered Triticonazole on GD 6 -19

Parameter	**Dose level [mg/kg bw per day]**						
	0	**5**	**15**	**50**	**0**	**75**	**150**
Total Nr. Inseminated	8	8	8	8	7	8	8
Total Nr. pregnant*	7	7	6	6	5	7	8
Abortions	1	0	0	0	0	0	0
Total litter loss	1	0	0	0	0	0	0
Clinical signs of toxicity							
Reduced food consumption	0	0	0	0	1	5	8
Reduced water consumption	0	0	0	0	0	4	8
Reduced defecation	0	0	0	0	0	4	7
Apparent reduction in body temperature	0	0	0	0	0	2	5

* = observations are given for the pregnant females only

Food and water consumption

Top dose females receiving 150 mg/kg/day revealed a markedly reduced food and water consumption from commencement of treatment until premature termination of this dose group on GD 8 or 9.

In the 75 mg/kg bw dose group reduced food consumption was noticed during the treatment period (GD 6 – 19). No relevant treatment related changes were observed in the other groups.

Water consumption was highly variable, and thus no relevant dose-related trend was observed that could clearly be attributed to treatment.

Table 6.6.2.2.2-4: Food and water consumption of does administered Triticonazole on GD 6 -19

Parameter		**Dose level [mg/kg bw per day]**						
		0*	**5**	**15**	**50**	**0***	**75**	**150**
Animals evaluated	[n]	5	7	6	6	5	7	8
Food consumption [g/animal/day]								
Day 1 - 5	mean ± SD	149 ± 44	172 ± 28	178 ± 25	174 ± 25	201 ± 32	202 ± 15	188 ± 28
	Δ%	-25.9	+15.4	+19.5	+16.8	+34.9	+0.5	-6.3
Day 6 - 19	mean ± SD	**162 ± 29**	**176 ± 24**	**174 ± 18**	**174 ± 38**	**188 ± 30**	**147 ± 33**	-
	Δ%	**-13.9**	**+9.0**	**+7.7**	**+7.4**	**+16.1**	**-21.9**	

Parameter		Dose level [mg/kg bw per day]						
		0*	**5**	**15**	**50**	**0***	**75**	**150**
Day 6 - 28	mean ± SD	156 ± 32	149 ± 22	155 ± 28	159 ± 28	169 ± 35	160 ± 40	-
	Δ%	-7.7	-4.5	-0.6	+2.2	+8.3	-5.3	
Day 0 - 28	mean ± SD	154 ± 34	153 ± 23	159 ± 28	162 ± 27	175 ± 34	168 ± 35	-
	Δ%	-11.9	-0.6	+3.2	+5.1	+13.5	-4.0	
Water consumption [mL/animal/day]								
Day 1 - 5	mean ± SD	444 ± 105	468 ± 148	417 ± 73	464 ± 100	523 ± 98	507 ± 72	426 ± 76
	Δ%	-15.1	+5.4	-6.1	+4.5	+17.8	-3.1	-18.5
Day 6 - 19	mean ± SD	540 ± 209	457 ± 197	428 ± 105	449 ± 127	504 ± 81	395 ± 115	-
	Δ%	-7.1	-15.4	-20.8	-16.9	-6.7	-21.7	
Day 6 - 28	mean ± SD	524 ± 210	415 ± 181	428 ± 131	435 ± 143	461 ± 95	442 ± 97	-
	Δ%	+13.7	-20.7	-18.7	-17.0	-12.1	-4.0	
Day 0 - 28	mean ± SD	508 ± 189	426 ± 175	426 ± 119	441 ± 134	473 ± 96	455 ± 92	-
	Δ%	+7.4	-16.1	-16.1	-13.2	-6.9	-3.8	

* = both controls were related to each other, respectively

Body weight development

Top dose females receiving 150 mg/kg/day showed marked and persistent body weight losses from commencement of treatment until premature termination of this dose group on GD 8 or 9.

Females receiving 75 mg/kg/day exhibited a marked, but transient loss in body weight following commencement of treatment. However, their subsequent body weight development was essentially similar to that of the respective controls, although the initial deficit was not recouped.

With the exception of a transient body weight loss in females receiving 15 and 50 mg/kg/day during the first two days of the treatment period, the body weight development of females treated up to 50 mg/kg bw per day was similar or slightly superior to the respective controls.

Table 6.6.2.2.2-5: Body weight development of does administered Triticonazole on GD 6 -19

Parameter		Dose level [mg/kg bw per day]						
		0*	**5**	**15**	**50**	**0***	**75**	**150**
Animals evaluated	[n]	5	7	6	6	5	7	8
Body weight [kg]								
Day 0	mean ± SD	3.51 ± 0.1	3.84 ± 0.2	3.83 ± 0.4	3.63 ± 0.2	3.71 ± 0.2	3.87 ± 0.3	3.70 ± 0.2
	Δ%	-5.4	+9.4	+9.1	+3.4	+5.7	+4.3	-0.2
Day 6	mean ± SD	3.67 ±0.2	3.98 ± 0.2	4.03 ± 0.4	3.82 ± 0.2	3.89 ± 0.2	4.02 ± 0.3	3.85 ± 0.1
	Δ%	-5.7	+8.4	+9.8	+4.4	+6.0	+3.3	-1.1
Day 8	mean ± SD	3.67 ± 0.2	4.00 ± 0.2	4.01 ± 0.4	3.73 ± 0.2	3.90 ± 0.2	3.86 ± 0.3	3.55 ± 0.1
	Δ%	-5.9	+9.0	+9.3	+1.6	+6.3	-1.0	-8.9
Day 18	mean ± SD	3.86 ± 0.2	4.21 ± 0.3	4.24 ± 0.4	4.06 ± 0.3	4.13 ± 0.2	4.11 ± 0.4	-
	Δ%							
Day 28	mean ± SD	4.05 ± 0.2	4.33 ± 0.2	4.41 ± 0.3	4.23 ± 0.2	4.27 ± 0.2	4.28 ± 0.3	-
	Δ%	-5.2	+6.9	+8.9	+4.4	+5.4	+0.2	
Body weight gain [kg]								
Day 0 - 6	mean	0.16	0.14	0.20	0.20	0.18	0.15	0.15
	Δ%	-11.1	-12.5	+25	+25	+12.5	-16.7	-19.4
Day 6 - 8	mean	0.0	+0.02	-0.02	-0.09	0.01	-0.16	-0.3
	Δ%	0	-	-	-	0	**-**	**-**
Day 6 - 18	mean	0.19	0.23	0.21	0.23	0.24	**0.09**	**-0.29#**
	Δ%	-20.8	+21.1	+10.5	+21.1	+26.3	**-62.5**	**- >100%**
Day 6 - 28	mean	0.38	0.35	0.38	0.40	0.38	0.26	-

Parameter		Dose level [mg/kg bw per day]						
		0*	**5**	**15**	**50**	**0***	**75**	**150**
	Δ%	0.0	-7.9	0.0	+5.2	0.0	-31.6	
Day 0 - 28	mean	0.54	0.49	0.58	0.60	0.56	0.41	-
	Δ%	-3.6	-9.3	+7.4	+11.1	+3.7	-26.8	

* = both controls were related to each other, respectively
# = for the top dose (150 mg/kg bw per dayay) ony data for days 0-8 are available, due to preterminal sacrifice on GD 8 or 9
- = Δ% not calculated

The mean body weight development of the controls compared to the treatment groups is shown in the Figure 6.6.2.2.2.-1 for the control, 5, 15, and 50 mg/kg bw groups and for the control, and 75 mg/kg bw group. There is a significant body weight decrease seen in the 15 and the 50 mg/kg bw dose group after beginning of treatment. In the 75 mg/kg bw group, decreased body weight lasted over the whole treatment period.

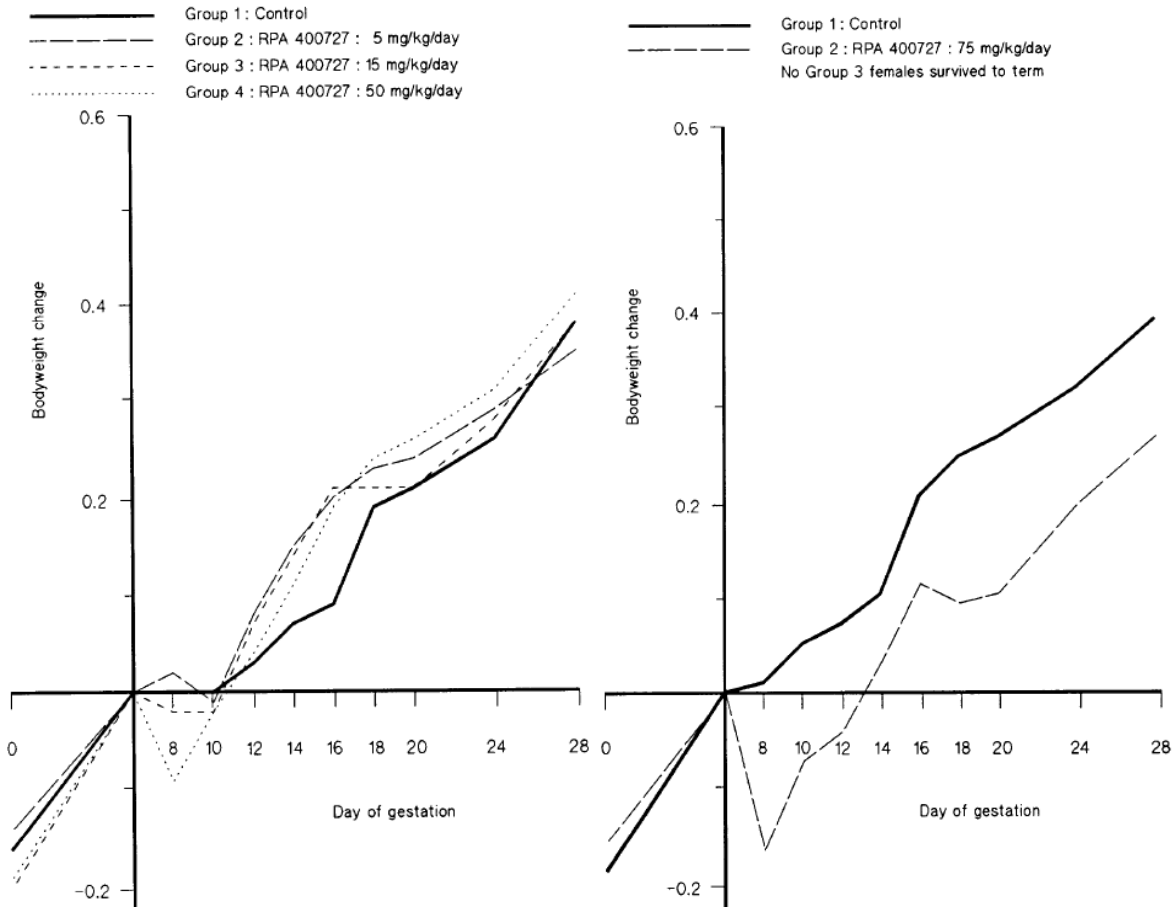


Figure 6.6.2.2.2-1: Group mean body weight change [kg] of females during gestation; left side: controls and 5, 15, and 50 mg/kg bw dose groups; right side: controls, 75 mg/kg bw dose group

Litter responses

One female in control group aborted and a second female in this group exhibited total litter resorption at terminal necropsy on day 29 of gestation. However, these findings were cleary incidental in nature.

At 75 mg/kg bw per dayay, a marked increase in post-implantation loss, predominantly early resorptions, with a consequent reduction in the number of live fetuses was observed. The percentage of early resorptions was 3.4% in the 75 mg/kg bw group and 1.5% in the 50 mg/kg bw dose group, thereby exceeding the historical control data range (Table 6.6.2.2.2-6). At 50 mg/kg bw per day the number of total resorptions was however within the historical control data range.

These findings are considered to be a consequence of general toxicity of triticonazole, taking into account the considerable body weight losses /decreased body weigh gain seen in the early phase of treatment (between GD 6 and 9). With this exception, litter parameters were essentially similar to those of the respective control group.

Regarding all other effects, foetal weight in all treated groups was marginally lower than in the control group and the number of small (less than 32.0 g) foetuses was higher (Table 6.6.2.2.2-7). In general, the lighter foetuses were associated with larger litters (especially seen in the 5 and the 50 mg/kg bw dose groups) and it was, therefore, considered that the apparent weight reduction reflected the slightly larger litter size in the treated groups rather than an effect of treatment. In addition, no effect on foetal weights were seen in the higher dose group of 75 mg/kg bw.

All other litter parameters were essentially similar in all groups.

Table 6.6.2.2.2-6: Group mean litter data – females sacrificed on day 29 of gestation

Dose level [mg/kg bw per dayay]	**0**	**5**	**15**	**50**	**0**	**75**	**150**
Animals pregnant [n]	7	7	6	6	5	7	8
Animals with viable youngth [n]	5	7	6	6	5	7	0
Pregnancy status (mean ± SD) [n]							
- viable young (male) HC: 4.4 (2.8-5.4)	4.6 ± 2.1	4.4 ± 1.7	4.3 ± 2.7	4.7 ± 2.0	3.2 ± 1.8	**2.6** ± 0.5	-
- viable young (female) HC: 3.8 (2.6-5.1)	2.8 ± 1.1	5.6 ± 2.6	5.2 ± 3.1	5.3 ± 2.4	5.6 ± 2.3	3.6 ± 1.4	-
- viable young (total) HC: 8.2 (5.7-9.7)	7.4 ± 3.0	**10.0** ± 2.9	9.5 ± 4.8	**10.0** ± 3.2	8.8 ± 4.0	6.1 ± 1.8	-
- abortion and total litter loss [%] HC: 5.4 (0.0-15.4)	28.6	0	0	0	0	0	-
Cesarean section data							
- Corpora lutea [mean ± SD] HC: 11.6 (9.3-13.2)	11.8 ± 0.8	12.9 ± 3.5	13.3 ± 2.0	13.2 ± 2.1	11.4 ± 1.9	11.4 ± 2.9	-
- Implantations [mean ± SD] HC: 9.3 (6.5-11.5)	8.6 ± 3.4	10.7 ± 3.7	10.3 ± 5.2	12.0 ± 3.0	9.8 ± 4.0	9.6 ± 2.5	-
- Pre-implantation loss [mean %] HC: 19.4 (8.9-43.0)	27.1	16.7	22.5	8.9	14.0	17.3	-
- Post-implantation loss [mean %] HC: 12.4 (4.8-21.7)	14.0	6.7	8.1	16.7	10.2	**35.8**	-
- Resorptions [mean ± SD] HC: 1.2 (0.5-2.5)	1.2 ± 1.1	0.7 ± 0.8	0.8 ± 0.9	2.0 ± 1.4	1.0 ± 1.0	**3.4** ± 1.9	-
- Early resorptions [mean ± SD] HC: 0.5 (0.1-1.4)	1.0 ± 1.0	0.3 ± 0.5	0.3 ± 0.6	**1.5** ± 1.2	0.4 ± 0.6	**3.1** ± 1.8	-
- Late resorptions [mean ± SD] HC: 0.6 (0.2-1.6)	0.2 ± 0.4	0.4 ± 0.7	0.5 ± 0.7	0.5 ± 0.7	0.6 ± 0.8	0.3 ± 0.5	-
Weight parameters (mean ± SD)							
Placental weight [g] HC: 5.6 (4.7-6.6)	5.5 ± 0.4	5.2 ± 0.3	5.4 ± 0.2	5.4 ± 0.2	**7.2** ± 0.4	6.2 ± 0.3	-
Foetal weight [g] HC: 41.1 (38.5-45.0)	41.5 ± 1.3	**38.4** ± 1.7	38.9 ± 1.3	**37.1** ± 1.4	44.5 ± 1.8	43.2 ± 1.8	-

HC: Historical control data from 18 studies [mean (range)]; values exceeding the historical control data range are marked in bold
Remark to HCD by RMS: the time frame of HCD could not be definitely determined (the data were stated in the study report), however, RMS does not consider them as pivotal but only as indicative
The means are derived only from animals that survived to term and born viable youngs

Foetal observations at necropsy

External examination

One control foetus of the first control group (1/RHA/346) exhibited complex malformations of the abdominal viscera, including umbilical hernia.

Two foetuses of the same litter in the 75 mg/kg/day dose group had complex malformations, one with cranial abnormalities and umbilical hernia, and the other with gastroschisis, cardiovascular and limb abnormalities. Both of these fetuses with multiple malformations were small fetuses (< 32.0 g). Foetuses in the remaining litters showed a low incidence of anomalies of types seen previously in this strain of rabbit in these laboratories.

Examination of foetuses of other treatment groups revealed a number of anomalies in all groups which were of isolated nature. Lacking dose-response relationship and/or occurred at incidences previously recorded in this strain of rabbits in these laboratories, and thus, showed no association with the test item treatment.

The observation of two multiply malformed fetuses in one litter out of the 75 mg/kg bw group gives no evidence for a specific treatment-related pattern of toxicity, also when taking into account the isolated findings, occurring within normal background incidences as shown in the table below.

Table 6.6.2.2.2-7: Results from external examination of foetuses

Dose level [mg/kg bw per day]	**0**	**5**	**15**	**50**	**0**	**75**	**150**
Litters Evaluated	5	7	6	6	5	7	-
Foetuses Evaluated	37	70	55	60	44	43	-
Observations [% incidence] (no. of litters)							
- Abnormal foetus	**2.7**	0.0	0.0	0.0	0.0	**4.7**	-
HC: 0.2 (0.0-1.4)	(1)	(0)	(0)	(0)	(0)	(1)	
- Small foetus (less than 32.0 g)	2.7	27.1	18.2	**31.7**	9.1	4.7	-
HC: 15.99 (2.7-28.6)	(1)	(5)	(49	(4)	(2)	(1)	
- Unilateral/bilateral fore-limb flexure	0.0	0.0	0.0	0.0	0.0	**4.7**	-
HC: no data	(0)	(0)	(0)	(0)	(0)	(1)	
- Bilateral hind-limb flexure	0.0	0.0	0.0	0.0	0.0	**2.3**	-
HC: 0.05 (0.0-0.9)	(0)	(0)	(0)	(0)	(0)	(1)	
- Discolouration of placenta	0.0	0.0	0.0	**1.7**	**4.5**	0.0	-
HC: no data	(0)	(0)	(0)	(1)	(1)	(0)	
- Amniotic fluid: dark yellow	0.0	0.0	0.0	0.0	0.0	**2.3**	-
HC: 0.05 (0.0-1.4)	(0)	(0)	(0)	(0)	(0)	(1)	
- Amniotic sac: white precipitate	0.0	0.0	0.0	0.0	**2.3**	0.0	-
HC: 0.10 (0.0-1.3)	(0)	(0)	(0)	(0)	(1)	(0)	

HC: Historical control data from 18 studies including 2007 foetuses [mean (range)]
Remark to HCD by RMS: the time frame of HCD could not be definitely determined (the data were stated in the study report), however, RMS does not consider them as pivotal but only as indicative

Internal examination

One control foetus of the first control group (1/RHA/346) that also revealed external findings exhibited fusion of liver lobes and intestinal abnormalities.

One of the multiply malformed foetuses of the 75 mg/kg/day dose group revealed abnormal heart and major vessels, enlarged left and reduced right ventricles, inter-ventricular septal defects and malpositioned kidney. Foetuses in the remaining litters showed a low incidence of anomalies of types seen previously in this strain of rabbit in these laboratories.

Examination of foetuses of other treatment groups revealed a number of anomalies in all groups which were of isolated nature. Lacking dose-response relationship and/or occurred at incidences previously recorded in this strain of rabbits in these laboratories, and thus, showed no association with the test item treatment.

Table 6.6.2.2.2-8: Results from internal examination of foetuses

Dose level [mg/kg bw per day]	**0**	**5**	**15**	**50**	**0**	**75**	**150**
Litters Evaluated	5	7	6	6	5	7	-
Foetuses Evaluated	37	70	55	60	44	43	-
Observations [% incidence] (no. of litters)							
- Abnormal foetus	**2.7**	0.0	0.0	0.0	0.0	**2.3**	-
HC: 0.2 (0.0-1.4)	(1)	(0)	(0)	(0)	(0)	(1)	
- Thymus gland: haemorrhagic	**2.7**	0.0	0.0	0.0	0.0	0.0	-
HC: no data	(1)	(0)	(0)	(0)	(0)	(0)	
- Pulmonary artery wall: haemorrhage	0.0	**1.4**	0.0	0.0	0.0	0.0	-
HC: no data	(0)	(1)	(0)	(0)	(0)	(0)	
- Possible abnormal vessels	0.0	0.0	0.0	**1.7**	0.0	0.0	-
HC: 0.15 (0.0-1.4)	(0)	(0)	(0)	(1)	(0)	(0)	
- Median lung lobe: agenesis	0.0	0.0	0.0	**3.3**	0.0	0.0	-
HC: 0.10 (0.0-1.4)	(0)	(0)	(0)	(1)	(0)	(0)	
- Posterior cardinal vein: persisten	0.0	0.0	0.0	**1.7**	0.0	0.0	-
HC: no data	(0)	(0)	(0)	(1)	(0)	(0)	
- Abdomen: free/clotted blood	0.0	1.4	**3.6**	1.7	0.0	0.0	-
HC: 0.30 (0.0-1.7)	(0)	(1)	(1)	(1)	(0)	(0)	
- Gall bladder: variants	8.1	5.7	**18.2**	6.7	9.1	**14.0**	-
HC: 5.88 (0.0-13.5)	(2)	(2)	(3)	(2)	(3)	(4)	
-Stomach: dark content	0.0	1.4	0.0	0.0	0.0	0.0	-
HC: 0.25 (0.0-2.7)	(0)	(1)	(0)	(0)	(0)	(0)	
- Stomach: gaseous content	5.4	1.4	0.0	0.0	0.0	2.3	-
HC:1.69 (0.0-6.7)	(2)	(1)	(0)	(0)	(0)	(1)	
- Clear cyst on right ovary, abnormal left uterine horn	**7.1**	0.0	0.0	0.0	0.0	0.0	-
HC: no data	(1)	(0)	(0)	(0)	(0)	(0)	
- Urinary bladder: clotted blood around	0.0	4.3	5.5	0.0	0.0	2.3	-
HC: 0.40 (0.0-2.8)	(0)	(2)	(2)	(0)	(0)	(1)	

HC: Historical control data from 18 studies including 2007 foetuses [mean (range)]
Remark to HCD by RMS: the time frame of HCD could not be definitely determined (the data were stated in the study report), however, RMS does not consider them as pivotal but only as indicative

Conclusion:

Based on the toxicological response of dams and foetuses in utero to a dosage of 75 mg/kg bw per dayay, this dosage was considered to be a suitable top dose for the main development toxicity study on rabbits.

If a NOAEL is to be derived for this study, the maternal NOAEL is proposed at 5 mg/kg bw per day, based on dose-related (slight) body weight loss (days 6 to 8) at ≥ 15 mg/kg bw per day. Fetal NOAEL is proposed at 50 mg/kg bw per day, based on increased post-implantation loss, early resorptions and increased limb flexures (external examination) at 75 mg/kg bw per day.

B.6.6.2.2.3. Main rabbit study

Previous evaluation: DRAR (2016)	DAR (2003) Additional information/tables added in the results Conclusion on the foetal and the maternal NOAEL from DAR 2003 (5 mg/kg bw per day) confirmed

Reference:	RPA 400727: Teratology study in the rabbit
Author(s), year:	[redacted] 1991
Report/Doc. number::	C018959 / -
Guideline(s):	US EPA guideline 83-3 and OECD 414 (1981)
GLP:	Yes
Deviations from OECD 414 (2001):	According to the most recent OECD TG 414, the recommended administration period is covering the whole gestation period and should continue until the day prior to scheduled caesarean section. Otherwise the study conducted in 1991 broadly met the requirements of the 2001 version of the guideline. The study protocol is considered to be able to identify teratogenicity
Acceptability:	Yes; since one of the effects of triazoles is possible cleft palate formation, the duration of administration is considered acceptable (hard palate closure is finalised at end of organogenesis)

Material and methods

Groups of 18 pregnant does/dose (strain: New Zealand White; source: [redacted]) were treated with triticonazole (batch no.YG2156/1; purity 99.51 %; suspended in 0.5 % w/v methyl cellulose in distilled water) from day 6 to 19 of pregnancy by oral gavage. The dose levels of 0 (vehicle control), 5, 25, 50 and 75 mg/kg bw per day (with a dosing volume of 5 ml/kg) were selected following examination of results in a preliminary range-finding study (not included in the submission). Stability and homogenicity of the test formulation was confirmed periodically by analysis.

All animals were examined daily throughout the study for clinical signs and mortality. Body weights of the does were recorded daily and food consumption was recorded over 5-day intervals from day 1 to day 28 of gestation. On day 29 of pregnancy, all females were examined macroscopically for abnormalities, for the presence and number of corpora lutea, implantation sites, resorption sites (early or late) and of live or dead foetuses. Live foetuses were weighed, sexed and examined for external abnormalities in the neck, thorax and abdominal cavities. Following this examination, the foetuses were eviscerated and the heads of one-third of the foetuses in each litter were removed for subsequent examination following random serial sectioning. Torsos and the remaining intact foetuses were processed for skeletal examination.

Results

Maternal effects: There were seven deaths during the study considered treatment-related. One female at 50 mg/kg and 6 females at 75 mg/kg were sacrificed in extremis between day 13 to 18 following marked weight loss, reduced food intake and reduced faecal output, reduced body temperature and red staining in the cage undertray. In addition, abortion in one female receiving 75 mg/kg was also considered treatment-related.

Table 6.6.2.2.3-1: Disposition of animals

Dose level [mg/kg bw per day]	0	5	25	50	75
Total number inseminated	**20**	**20**	**20**	**20**	**20**
Deaths	0	1 (incidental dosing trauma)	0	2 (1 death treatment related, 1 following incidental dosing trauma)	6
Not pregnant	0	2	0	2	0
Abortion	0	1	0	0	1
Total litter loss	0	0	2	0	0
Disposition	20	16	18	16	13

Other clinical signs of maternal toxicity (increased respiration rate, reduced faecal output) were noted at 50 and 75 mg/kg (table 6.6.2.2.3-2).

Table 6.6.2.2.3-2: Clinical observations in rabbits administered triticonazole during gestation days 6 to 28

Dose level [mg/kg]	Animal #	Gestation Day	Observation
Control	2 (#472, 485)	9-29	Increased respiration
5 mg/kg	4 (#439, 471, 493, 508)	8, 16-29	Increased respiration
	3 (#442, 473, 508)	16-25, 28-29	Few faeces in undertray
25 mg/kg	2 (#468, 504)	11-15, 19-29	Increased respiration
	6 (#459, 465, 509, 534, 440, 511)	18-29	Few faeces in undertray
50 mg/kg	5 (#425, 443, 467, 470, 499)	11-29	Increased respiration
	7 (#414, 443, 467, 470, 497, 515)	9-19	Few faeces in undertray
75 mg/kg	5 (#257, 423, 494, 502, 488)	7-29	Increased respiration
	15 (257, 423, 444, 466, 479, 494, 502, 505, 516, 445, 448, 455, 488, 521, 541)	7-19	Few faeces in undertray

Table 6.6.2.2.3-3: Individual observations at necropsy (animals killed *in extremis*)

Group (mg/kg bw per day)	Animal number	History and circumstances of death	Summary of necropsy findings
50	89FR515	Weight loss 660 g from Day 6. Reduced food intake and faecal output. Animal thin with apparent reduction in	Thoracic cavity: NAD Abdominal cavity: Stomach contents reduced. Large amount of yellow mucoid

		body temperature. Red staining in undertray. Killed *in extremis* Day 17 after insemination.	material in small intestine. Pregnant, all implantations resorbing.
75	89FR445	Weight loss 410 g from day 6. Reduced food intake and faecal output. Apparent reduction in body temperature; hunched posture and thin. Red liquid in undertray. Killed *in extremis* Day 13 after insemination	Thoracic cavity: NAD Abdominal cavity: Liver friable with pale areas and lobular pattern accentuated. Stomach contents reduced, gastro-intestinal tract gaseous, caecal contents dehydrated. Pregnant, all implantations early resorptions.
75	89FR448	Weight loss 500 g from Day 6. Reduced food intake and faecal output. Lethargic, apparent reduction in body temperature. Red liquid in undertray. Killed *in extremis* Day 13 after insemination	Thoracic cavity: NAD Abdominal cavity: NAD Pregnant, all implantations early resorptions
75	89FR455	Weight loss 530 g from Day 6. Reduced food intake and faecal output. Apparent reduction in body temperature. Killed *in extremis* Day 14 after insemination	Thoracic cavity: NAD Abdominal cavity: Liver lobular pattern slightly accentuated. Stomach contents compacted. Pregnant, one early resorption, remainder of implantations grossly normal
75	89FR488	Weight loss 680 g from day 6. Reduced food intake and faecal output. Increased respiration rate and apparent reduction in body temperature. Red liquid in undertray. Killed *in extremis* Day 13 after insemination	Thoracic cavity: NAD Abdominal cavity: Liver lobular pattern slightly accentuated, irregular dark striations on periphery of left liver lobe. Stomach contents compacted with fur and food material. Caecal contents dark and reduced. Remainder of gastro-intestinal tract generally devoid of content. No fecal pellet formation in rectum. Pregnant, all implantations resorbing
75	89FR521	Weight loss 590 g from day 6. Reduced food intake and faecal output. Red liquid in undertray. Killed *in extremis* Day 15after insemination	Thoracic cavity: NAD Abdominal cavity: Stomach contents compacted Pregnant, all implantations grossly normal
75	89FR541	Weight loss 710 g Days 6-15. Reduced food intake and fecal output. Red staining in undertray. Apparent reduction in body temperature. Killed *in extremis* Day 16 after insemination	External: animal thin Thoracic cavity: NAD Abdominal cavity: Stomach contents compacted with fur and food material. Caecal contents reduced and dehydrated. Remainder of gastro-intestinal tract contents reduced. Pregnant, all implantations resorbing

NAD No abnormality detected

Dose-related body weight loss was observed in dams receiving 25, 50 and 75 mg/kg bw per day during the first two days of treatment (table 6.6.2.2.3-4 and figure 6.6.2.2.3-1) Thereafter, body weight gain was essentially similar to controls in all dose groups. Food intake was reduced throughout the treatment period at ≥ 50 mg/kg, and to a marginal extent also at 25 mg/kg.

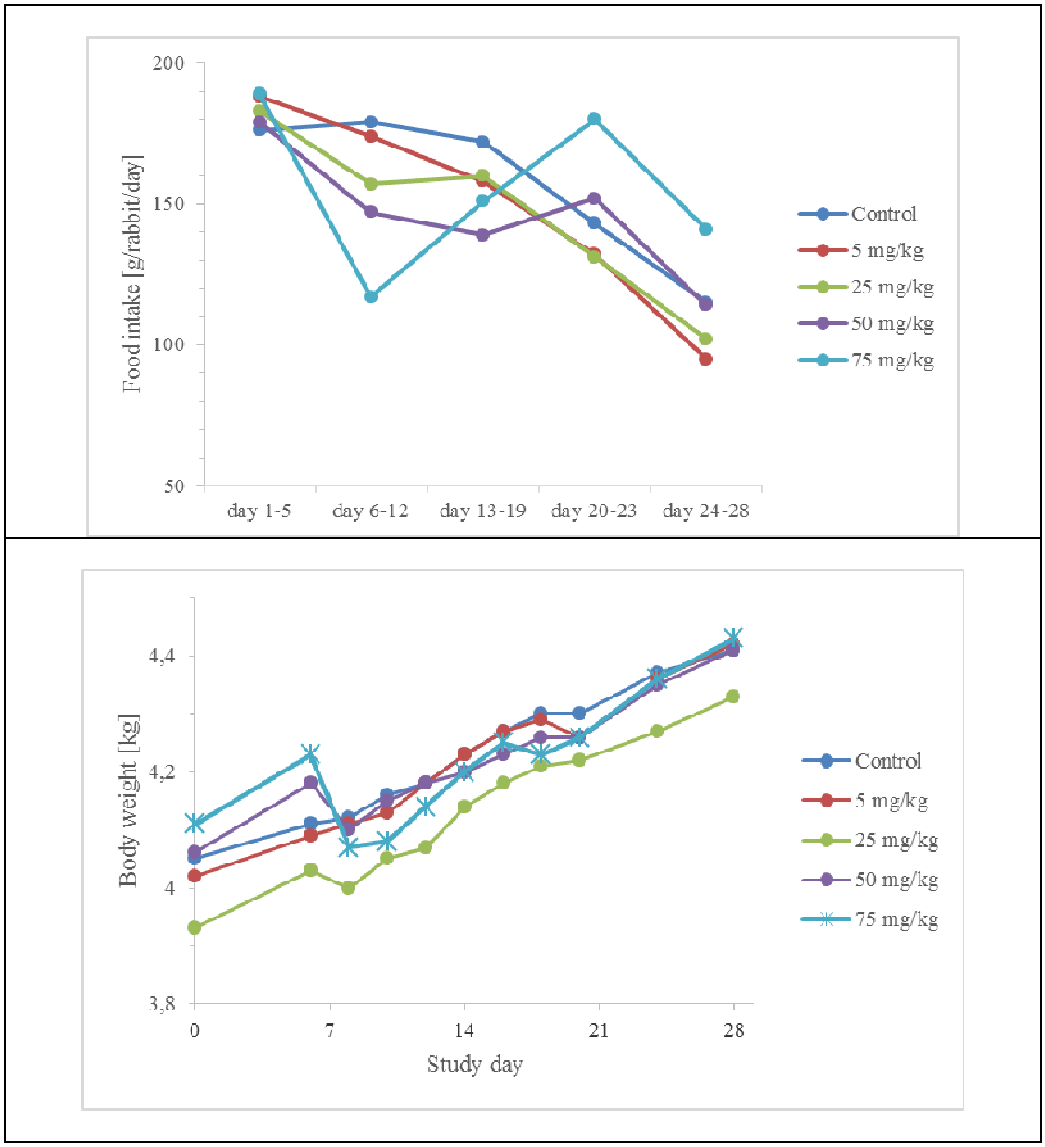


Figure 6.6.2.2.3-1: Food consumption and body weight development in rabbits administered triticonazole

Table 6.6.2.2.3-4: Food consumption and body weight development in rabbits administered triticonazole

Dose level [mg/kg bw per day]	**0**	**5**	**25**	**50**	**75**
Food consumption [g/animal/day] (% control)					
Day 1-5	176±20	188±23 (106.8)	183±32 (104.0)	179±26 (101.7)	189±30 (107.4)
Day 6-12	179±33	174±22 (97.2)	**157**±28 **(87.7)**	**147**±34 **(82.1)**	**117**±39 **(65.4)**
Day 13-19	172±33	158±34 (91.9)	160±44 (93.0)	**139**±51 **(80.8)**	**151**±61 **(87.8)**
Day 20-23	143±43	132±29 (92.3)	131±43 (91.6)	152±54 (106.3)	180±34 (125.9)
Day 24-29	115±39	95±38 (82.6)	102±36 (88.7)	114±37 (99.1)	141±42 (122.6)
Body weight gain [g] (% control)					
Day 0-6	0.06	0.07	0.1	0.12	0.12

Dose level [mg/kg bw per day]	**0**	**5**	**25**	**50**	**75**
		(116.7)	(166.7)	(200.0)	(200.0)
Day 6-20	0.19	0.17 (89.5)	0.19 (100.0)	**0.08** **(42.1)**	**0.03** **(15.8)**
Day 20-28	0.11	0.16 (145.5)	0.11 (100.0)	0.15 (136.4)	0.17 (154.5)
Maternal body weight [g] **(% control)**					
day 0 p.c	4.05±0.27	4.02±0.36	3.93±0.23	4.06±0.24	4.11±0.35
day 6p.c.	4.11±0.23	4.09±0.35	4.03±0.24	4.18±0.28	4.23±0.32
day 8 p.c. *(% bw from day 6 to 8)*	4.12±0.25	4.11±0.34	**4.00±0.25** *(99.3)*	**4.10±0.27***** *(98.1)*	**4.07±0.33***** *(96.2)*
day 18 p.c	4.30±0.27	4.29±0.35	4.21±0.27	4.26±0.3	4.23±0.35
day 28 p.c.	4.41±0.26	4.42±0.26	4.33±0.25	4.41±0.29	4.43±0.28

*** $p \leq 0.001$ (Dunnett-test, two sided)

At necropsy on day 29 of gestation, no macroscopic changes considered to be treatment-related were observed in the dams.

Litter data/foetal parameters: A slight, but not statistically significant increase in pre- and post-implantation losses was observed at 75 mg/kg, but was within the historical control data. Furthermore, early resorptions were slightly increased in the 50 and 75 mg/kg bw test group, only slightly exceeding historical control data of 18 studies from the laboratory. The effects were considered to be related to maternal toxicity (food consumption reduced at days 6 to 12; body weight loss between days 6 and 12) and not an indication of a specific developmental toxicity.

Table 6.6.2.2.3-5: Pregnancy status and caesarean section data of dams treated with triticonazole

Parameter **Dose level [mg/kg bw per day]**	**0**	**5**	**25**	**50**	**75**
Pregnancy status					
- mated [n]	20	20	20	20	20
- pregnant [n]	20	18	20	18	20
conception rate [%]	100	90	100	90	100
-Maternal mortality	0	1	0	2	6
- aborted [n]	0	1	0	0	1
- dams with all resorptions [n]	0	0	2	0	0
Cesarean section data					
- Corpora lutea/doe [n]	12.7±2.2	12.8±2.1	12.1±2.7	13.6±2.9	12.5±2.3
- Implantation sites/doe [n]	10.3±3.2	11.6±2.2	10.4±2.7	11.2±3.5	8.9±2.4
- Pre-implantation loss [%] *HCD: 8.9-43.0*	18.9	9.7	14.2	17.9	28.8
- Post-implantation loss [%] *HCD: 4.8-21.7*	13.6	8.1	7.4	16.2	20.7
- Early resorptions [n] *HCD: 0.1-1.4*	1.0±1.0	0.4±0.6	0.6±0.7	***1.6**±1.3*	***1.5**±1.2*
- Late resorptions [n]	0.5±0.7	0.4±0.6	0.7±0.8	0.3±0.5	0.3±0.6

Parameter Dose level [mg/kg bw per day]	0	5	25	50	75
- Dead fetuses/litter [n]	0.0±0.0	0.2±0.4	0.1±0.3	0.0±0.0	0.0±0.0
- Live fetuses/litter [n]	8.9±2.7	10.7±2.1	9.7±2.9	9.4±3.9	7.1±3.0
- Sex ratio [% male]	58.4	57.3	55.4	51.3	50.0
Placental weights [g]	5.1	5.1	5.3	5.2	5.2
Mean fetal weight [g]	39.8±2.1	39.8±1.5	39.4±1.4	39.1±1.6	39.7±2.7
- males [g]	40.7±2.5	40.0±1.9	39.3±1.8	39.5±2.2	40.5±2.4
- females [g]	36.3±2.8	39.3±2.4	39.3±2.0	37.9±3.0	39.4±2.5

All other litter parameters were similar in all groups. In addition, the pattern of incidences of gross abnormalities and visceral anomalies did not indicate any treatment-related effect.

Skeletal findings

The percentage of foetuses affected per litter (as litter is the experimental unit) has been calculated according to Hood RD, 2006 (Developmental and Reproductive Toxicology: A Practical Approach) as sum of percent of affected foetuses per litter divided by total number of litters. Since this parameter has been calculated by RMS, no historical control data are available. Historical control data are however available for percentage incidence of foetuses. Due to the fact that in the high dose group only 13 dams with their foetuses were investigated (due to mortality and abortion) and the number of investigated foetuses was therefore markedly lower, the percentage of foetal incidences in this dose group may represent an overestimation (foetuses in dead dams could be healthy) but also an underestimation (foetuses in dead dams could be affected) of findings, also compared to historical control data.

Head

Regarding skeletal findings of head, only those observed at 75 mg/kg bw per day were considered to be related to the treatment. The dose of 75 mg/kg bw per day was excessively toxic to the does, leading to 58% and 84% lower body weight gains during days 6-20 of gestation (see table 6.6.2.2.3-4) and even to maternal deaths.

The high dose finding (malformation) “frontal bone fusion and other major cranial anomalies” (see table 6.6.2.2.3-6) was not considered by study author to be indicative for a teratogenic response of triticonazole. One fetus from doe no. 89FR444, which showed “frontal bone fusion and other major cranial anomalies” was multiply malformed and was therefore taken out of the calculation for this specific finding. In that case the litter incidence reduces to one and the fetal incidence to 1.6, which is covered by historical controls, since the given percentages indicates that there were studies, where at least one fetus was affected.

Besides the finding “frontal bone fusion and other major cranial anomalies” also findings like “anterior fontanelle extended anteriorly”, “additional suture in nasal bone”, “irregular ossification of frontal suture”, “frontal suture enlarged at fronto-nasal junction”, “additional plaque of bone in nasal structure” and “lachrymal fossa enlarged” were observed in 75 mg/kg bw per day group fetuses above HCD of the laboratory. The higher incidence of these findings is considered to be treatment-related but attributable to the excessive maternal toxicity observed at 75 mg/kg bw per day.

The notifier stated that maternal circulation might have been severely affected during specific phases of gestation leading to insufficient oxygen or nutrient supply to the embryo and cited the publication by Danielson B.R, 2013[6]. In Danielson B.R, 2013 embryonic hypoxia was also a consequence of the inhibition of hERG channel (the human Ether-à-go-go-Related Gene) which contributes to the electrical activity of the heart. Although it is stated that more than 200 compounds (both drugs and chemical, such as "conazole") inhibit this channel, no further details are given and therefore no conclusion on potential insufficient oxygen supply of foetuses during gestation in case of triticonazole can be made.

Sternebrae and ribs

The foetal incidence of variation "rudimentary floating 13th rib or ribs" (10.9%) was only very slightly above HCD (0-7-8.5%) in 75 mg/kg bw per day group. Based on the excessive maternal toxicity observed at 75 mg/kg bw, it is not unlikely that the maternal toxicity has contributed to the observed increased incidences of this variation.

Vertebrae, limbs and girdles

Foetal incidence of "two or more caudal vertebrae fused and/or reduced, short kinky tail" (2.2%) was observed only at 75 mg/kg bw per day and exceeded historical control data (0 – 0.8%); two litters were affected. According to DevTox[7] "short tail" is considered as a malformation.

With regard to the finding "metacarpals and phalanges incomplete ossified or unossified" the litter incidences do not indicate a relationship to treatment, however the foetal incidences were slightly increased (above HCD) at ≥ 50 mg/kg bw. The fact, that there is no correlation seen to any potentially corresponding external malformations on the fore- or hind-limbs, or digits in this study, gives further evidence that this is only a variation with no impact on development. The notifier cited a publication[8] which studied effects of feed restriction (and maternal reduced body weight gain) on development of rabbit foetuses. One of the effects following maternal reduced body weight gain was also the increase in un-ossified metatarsals and metacarpals in foetuses. At 50 mg/kg bw per day maternal death, reduced food consumption (80% of control value from day 6 to 19), reduced body weight gain (42% of control value from days 6 to 20) and body weight loss (2%) from day 6 to 8 was observed, which is considered to have contributed to this delayed development in foetuses.

When compared with control group, a dose-related increase of elongated acromion process of the scapula was also evident at ≥ 25 mg/kg bw. Concerning the findings of the acromion process of the scapula at the dose levels of 25 and 50 mg/kg, it was stated in the report that the incidences at these dose levels, when compared with concurrent controls, did not approach statistical significance, and considered doubtful whether these differences were the result of treatment with triticonazole. However, considering the clear dose-response in increase with incidences exceeding both concurrent control and historical control range values, relation to treatment can be assumed also at 25 and 50 mg/kg dose levels. The acromion is the lateral processus on the head of the spine of the scapula, which is normally cartilaginous (see Figure 6.6.2.2.3-2). The precocious ossification of the acromion process is of low severity, as this part of the scapula is ossified during development of the offspring and an earlier ossification has no impact on survival or quality of life.

[6] Danielson, B.R.(2013; DocID 2013/1420380) Maternal Toxicity. Methods in Molecular Biology 947: 311-25

[7] http://www.devtox.org/nomenclature/index.php

[8] Cappon at al., 2005: Effects of Feed Restriction During Organogenesis on Embryo-Foetal Development in Rabbit. Birth defects Research (Part B), 74:424-430, 2005

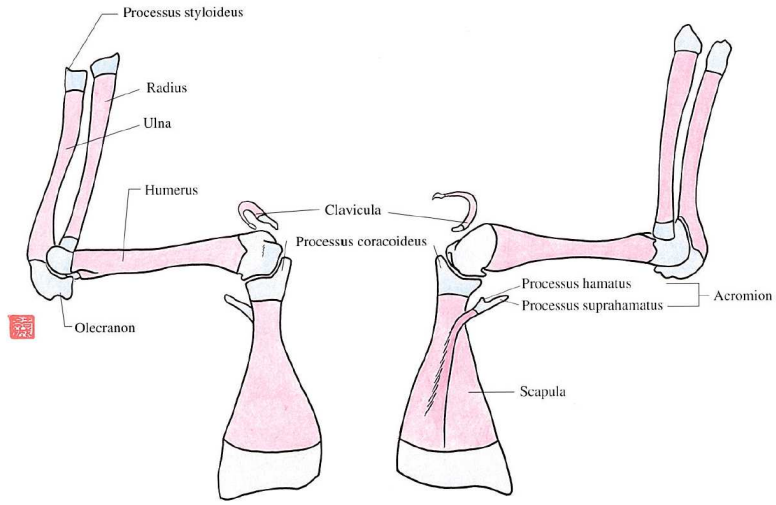


Figure 6.6.2.2.3-2: Schematic picture of a rabbit´s scapula (ossified parts are coloured in red, cartilaginous parts are coloured in blue). Yasuda M, Yuki T (1997): Color atlas of fetal skeleton of the mouse, rat and rabbit

If only the ossified parts of the skeleton is stained – as is the case in this study – the acromion process seems to be elongated, however this has been more accurately be described in the results section of the study as a "precocious ossification of the acromion process". As this part of the skeleton is ossified later in development of the young animals, the increased incidences of precocious ossification of acromion process seen at ≥ 25 mg/kg bw are not considered to affect development or survival of the animals.

During the evaluation of the dossier the notifier provided a statement on the "precocious ossification of acromion process", elaborated by the expert from "Exponent". The statement can be basically summarised as follows:

- By using only alizarin red S (and not alcian blue in addition) only mineralised tissues were clearly discernible and therefore not the entire acromion process could be visualised; based on this, only early ossification centers can be seen and the size of the acromion process cannot be accurately measured
- The large number of findings reported in the control animals suggests that the study investigators were hypervigilant in their examinations, calling changes that most other laboratories would have considered to be within the spectrum of normal
- Cases in the clinical literature of elongated acromion are rare; in humans who have been diagnosed with this rare condition, swinging the arms back and forth has not been compromised
- Since rabbits do not abduct ther upper limbs (do not move the limbs laterally), normal mobility would not be compromised
- Study investigators did not consider this finding as relevant for setting the NOAEL
- No comparable finding has been observed in rat developmental or multigeneration reproductive study.

RMS concluded that elongated acromion might be of no relevance for wildlife not moving limbs laterally, but that the human relevance cannot be compeletely excluded. Although this finding might have a very low impact in human developmental processes, a dose response has been observed for fetal incidence and therefore, elongated acromion

has been considered for setting of NOAEL. Regarding litter as functional unit, 4, 5 and 4 litters were effected at 25, 50 and 75 mg/kg bw per day comparing to one litter in control so that treatment-relation cannot be excluded.

Table 6.6.2.2.3-6: Incidence of skeletal findings[1)]

Dose level [mg/kg]	**0**	**5**	**25**	**50**	**75**
Individual skeletal observations					
Head					
Litters Evaluated[1)] Fetuses Evaluated[1)]	20 124	16 118	18 121	16 103	13 63
- Frontal bone fusion and other major cranial anomalies - Fetal incidence (N) - Fetal incidence [(%)] *HCD*: 0.0-1.5* - Litter incidence [N] -% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	 0 0.0 0 0	 0 0.0 0 0	 0 0.0 0 0	 1 1.0 1 0.78 ± 3.1	 2 3.2 **or 1.6** 2 or **1**[2)] **0.77 ± 2.8**[2)]
Anterior fontanelle extended anteriorly - Fetal incidence (N) - Fetal incidence [(%)] *HCD*: 0.0-1.9* - Litter incidence [N] -% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	 1 0.8 1 1 ± 4.5	 0 0.0 0 0	 0 0.0 0 0	 1 1.0 1 0.78 ± 3.1	 4 **6.3** 2 4.94 ± 14.1
Additional suture in nasal bone - Fetal incidence (N) - Fetal incidence [(%)] *HCD*: 0.0-1.2* - Litter incidence [N] -% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	 0 0.0 0 0	 0 0.0 0 0	 1 0.8 1 0.62 ± 2.6	 0 0.0 0 0	 1 **1.6** 1 1.28 ± 4.6
- Irregular ossification of frontal suture - Fetal incidence (N) - Fetal incidence [(%)] *HCD*: 0.0-9.1* - Litter incidence [N] -% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	 7 5.6 5 3.91 ± 7.8	 3 2.5 3 1.77 ± 3.8	 2 1.7 2 0.95 ± 2.8	 6 5.8 4 4.74 ± 9.7	 10 **15.9** 8 11.33 ± 11.3
- Frontal suture enlarged at fronto-nasal junction - Fetal incidence (N) - Fetal incidence [(%)] *HCD*: 0.0-1.9* - Litter incidence [N] -% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD) *No HCD available for this parameter (parameter calculated by RMS)*	 0 0.0 0 0	 0 0.0 0 0	 0 0.0 0 0	 0 0.0 0 0	 2 **3.2** 1 2.56 ± 9.2
- Additional plaque of bone in nasal structure - Fetal incidence (N) - Fetal incidence [(%)]	 0 0.0	 0 0.0	 0 0.0	 0 0.0	 4 **6.3**

Dose level [mg/kg]	**0**	**5**	**25**	**50**	**75**
HCD: 0.0-1.5*					
- Litter incidence [N]	0	0	0	0	4
-% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD)	0	0	0	0	4.26 ± 7.0
No HCD available for this parameter (parameter calculated by RMS)					
- Lachrymal fossa enlarged					
- Fetal incidence (N)	1	0	0	1	3[2)]
- Fetal incidence [(%)]	0.8	0.0	0.0	1.0	**4.8 or 3.2**
HCD: 0.0-3.8*					
- Litter incidence [N]	1	0	0	1	3 or 2[2)]
-% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD)	0.56 ± 2.5	0	0	0.45 ± 1.8	2.82 ± 6.9
No HCD available for this parameter (parameter calculated by RMS)					
Sternebrae and ribs					
Litters Evaluated[1)]	20	16	18	16	13
Fetuses Evaluated[1)]	178	171	174	150	92
- Rudimentary floating 13th rib or ribs					
- Fetal incidence (N)	7	4	11	5	9
- Fetal incidence [(%)]	3.9	2.3	6.3	3.3	**9.8**
HCD: 0.7-8.5*					
- Litter incidence [N]	5	3	9	5	6
-% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD)	3.39 ± 6.9	2.37 ± 5.1	5.87 ± 7.0	3.18 ± 5.3	9.36 ± 12.1
No HCD available for this parameter (parameter calculated by RMS)					
Vertebrae, limbs and girdles					
Litters Evaluated[1)]	20	16	18	16	13
Fetuses Evaluated[1)]	178	171	174	150	92
- Two or more caudal vertebrae fused and/or reduced, short kinky tail					
- Fetal incidence (N)	0	0	0	0	2
- Fetal incidence [(%)]	0.0	0.0	0.0	0.0	**2.2**
HCD: 0.0-0.8*					
- Litter incidence [N]	0	0	0	0	2
-% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD)	0	0	0	0	1.95 ± 4.8
No HCD available for this parameter (parameter calculated by RMS)					
- One or both acromion process elongated (=precocious ossification of acromion process)					
- Fetal incidence (N)	3	0	6	8	10
- Fetal incidence [(%)]	**1.7**	0.0	**3.4**	**5.3**	**10.9***
HCD: 0.0-1.0*					
- Litter incidence [N]	1	0	4	5	4
-% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD)	1.15 ± 5.1	0	4.56 ± 9.9	5.62 ± 11.4	14.10 ± 29.6
No HCD available for this parameter (parameter calculated by RMS)					
- Metacarpals and phalanges incomplete ossified or unossified					
- Fetal incidence (N)	41	22	24	52	**36**
- Fetal incidence [(%)]	23.0	12.9	13.8	**34.7**	**39.1**
HCD: 2.8-26.0*					
- Litter incidence [N]	13	7	10	11	11
-% fetuses affected per litter (sum of percent affected per litter/ No. of litters ± SD)	23.25 ± 27.5	11.88 ± 20.3	14.52 ± 16.9	34.49 ± 34.9	42.54 ± 31.8

Dose level [mg/kg]	0	5	25	50	75
No HCD available for this parameter (parameter calculated by RMS)					

*p<0.05

1)only two-third of foetuses have been evaluated in the skeletal examinations of the heads (as one-third of the heads underwent a subsequent examination following random serial sectioning), for the other parts of the body, all foetuses were investigated

2) one out of two foetuses of the high dose group (#89FR444) showing the malformation "frontal bone fusion and other major cranial anomalies" and the variation "lachrymal fossa enlarged" was multiply malformed and was therefore not included in the calculation of the affected foetuses/litter percentage

*Historical background data of the laboratory collected in 18 studies 5 years near the time of the present study.

The foetal incidence of 1% for cleft palate (falling within the HCD (0-1.5%)) was only recorded at 50 mg/kg bw per day, was not observed at any other dose and was therefore judged to be not-treatment related.

Conclusion

The NOAEL for maternal toxicity in this study can be confirmed from DAR 2003 to be 5 mg/kg bw per day based on a dose-related (slight) body weight loss (days 6 to 8) and reduced food intake (days 6 to 12) at ≥ 25 mg/kg bw per day. Both top doses (50 and 75 mg/kg bw) caused excessive maternal toxicity indicated by deaths, abortions, reduced food consumption, reduced body weight gain (even body weight loss from days 6 to 8), decreased faeces and an increased respiration rate. Additionally, the MTD was clearly exceeded in the top dose group with > 10 % maternal mortality.

Concerning foetal findings, a slight increase in both pre- and post-implantation losses was observed at 75 mg/kg bw per day, which is attributed to excessive maternal toxicity at this dose. Increased incidences of different skeletal findings (midline anterior cranial bones, rudimentary floating 13th rib, reduced/incomplete ossification of metacarpals and phalanges, etc.) were observed mostly in 75 mg/kg bw per day group. Only one skeletal anomaly (precocious ossification of acromion process) was noted at ≥ 25 mg/kg bw per day above historical control data (but was statistically significant only at 75 mg/kg bw per day). There was no correlation between foetal body weight (unaffected by the treatment) and observed foetal variations/delayed development. Exept the increased incidence of "two or more caudal vertebrae fused and/or reduced, short kinky tail ","Frontal bone fusion and other major cranial anomalies" in high dose group and one cleft palate finding in the 50 mg/kg bw per day group there was no other malformations observed at any dose level. These malformations were considered either to be spontaneous or to be the consequence of extensive maternal toxicity. The foetal NOAEL from DAR 2003 can be confirmed at 5 mg/kg bw per day.

Regarding **classification for developmental effects**, the Guidance on the Application of the CLP Criteria (July 2017) states that "*an increased incidence of mortality among the treated dams over the controls shall be considered evidence of maternal toxicity if the increase occurs in a dose-related manner and can be attributed to the systemic toxicity of the test material. Maternal mortality greater than 10 % is considered excessive and the data for that dose level shall not normally be considered for further evaluation*." The two observed malformations "two or more caudal vertebrae fused and/or reduced, short kinky tail " and "frontal bone fusion and other major cranial anomalies" were observed above the historical control data only at 75 mg/kg bw per day where high mortality (30%) of dams was recorded. Therefore this dose should not be considered for classification purposes. At lower doses, no

malformations were observed. The foetal effects recorded at 50 mg/kg bw per day were all regarded as effects on delayed development also attributable to excessive maternal toxicity, obvious as reduced food consumption (about 82% of control from days 6 to 19), reduced body weight gain (42% of control from days 6 to 20), body weight loss from days 6 to 8 (2%), decreased faeces and an increased respiration rate. At 25 mg/kg bw per day only precocious ossification of acromion process (variation) was recorded (not statistically significant) above historical control data (0.0 – 1.0%) but in this study also the incidence of this effect in control animals (1.7% foetal incidence) exceeded HCD. Summarising all observed effects the RMS concluded that there is no justification for classification of triticonazole for developmental effects based on the results observed in main rabbit developmental study.

B.6.6.2.3. Other studies

One study (Chicken embryotoxicity screening test (CHEST)) and several open literature data on embryotoxicity were provided for purpose of renewal.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	BAS 595 F (Triticonazole) - Chicken embryotoxicity screening test (CHEST)
Author(s), year:	[redacted]., 2010
Report/Doc. number::	2010/1177161 / -
Guideline(s):	No
GLP:	No
Deviations from OECD Guideline	No Guideline study
Acceptability:	Supplemental information

The chicken embryotoxicity screening test (CHEST) is performed by BASF as an in house screening study. Based on an in house validation study assessing 53 compounds, BASF stated that it was demonstrated that the CHEST assay could be used as an indicator test for embryotoxicity with an overall concordance of 85%. In a post-validation process, conclusive results were obtained for 68 non-corrosive and non-acute toxic compounds out of 88 compounds tested. In conclusion, the overall concordance of the CHEST assay is 87%.

Material and methods

Test Material	Triticonazole (BAS 595 F)
Description:	solid / white
Lot/Batch #:	COD-000601
Purity:	90.3%
Stability of test compound:	not specified
Solvent:	DMSO

Fertilized white leghorn chicken eggs (SPF) were pre-incubated for about 48 hours and synchronized for development. The eggs were windowed and 10 µL of the test substance preparation were injected directly below the embryo (*area pellucida*). The study was performed with 3 test substance concentrations (0.1, 1 and 10 mg/mL) and a vehicle control (2% DMSO). Each concentration and the vehicle control were applied to 8 embryos in 3 independent experiments (24 eggs per concentration). The eggs were sealed with tape and re-incubated for 24 hours.

The endpoints mortality, blood vessel and embryo development, blood vessel coloration and malformation were assessed. For the qualitative examination of endpoints the findings were compared to the vehicle control data.

Results

In comparison to the incidence in vehicle treated embryos, the effects of the test substance on mortality, blood vessel development, and blood vessels discoloration were within the laboratory historical control data for all concentration tested. Only reduced embryo development (incidence of 4% compared to vehicle control) was slightly above the laboratory historical control data (2%) for animals treated with 10 mg/mL.

Table 6.6.2.3-1: Mean results of the CHEST endpoints

Endpoint	Dose group [mg/mL]			
	0.1	1	10	Historical control
Mortality				
Finding (%)	0	0	0	4
SD	0	0	0	7
Evaluation				
Run 1	-	-	-	
Run 2	-	-	-	
Run 3	-	-	-	
All runs	-	-	-	
Reduced vessel development				
Finding (%)	4	0	8	14
SD	7	0	7	12
Evaluation				
Run 1	-	-	-	
Run 2	-	-	-	
Run 3	-	-	-	
All runs	-	-	-	
Reduced embryo development				
Finding (%)	0	0	4	2
SD	0	0	7	6
Evaluation				
Run 1	-	-	-	
Run 2	-	-	-	
Run 3	-	-	-	
All runs	-	-	-	
Discolored blood vessel				
Finding (%)	13	0	8	21
SD	13	0	7	14
Evaluation				
Run 1	-	-	-	
Run 2	-	-	-	
Run 3	-	-	-	
All runs	-	-	-	

Conclusion

According to the results of the present study, the test substance triticonazole showed no effects on mortality, blood vessel development, and blood vessels discoloration in the Chicken Embryotoxicity Screening Test (CHEST) under the conditions reported. Reduced embryo development (incidence of 4% compared to vehicle control) was slightly above the laboratory historical control data (2%) for animals treated with the highest dose.

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Zebrafish developmental screening of the ToxCast Phase I chemical library
Author(s), year:	Padilla S. et al., 2011
Report/Doc. number::	2012/1368722/ -
Guideline(s):	No
GLP:	No
Deviations from OECD Guideline	No Guideline study
Acceptability:	Yes; additional information

Executive Summary

Zebrafish (Danio rerio) is an emerging toxicity screening model for both human health and ecology. As part of the Computational Toxicology Research Program of the U.S. EPA, the toxicity of the 309 ToxCast™ Phase I chemicals was assessed using a zebrafish screen for developmental toxicity. All exposures were by immersion from 6–8 h post fertilization (hpf) to 5 days post fertilization (dpf); nominal concentration range of 1 nM–80µM. On 6 dpf larvae were assessed for death and overt structural defects. Results revealed that the majority (62%) of chemicals was toxic to the developing zebrafish; both toxicity incidence and potency was correlated with chemical class and hydrophobicity (logP); and inter-and intra-plate replicates showed good agreement. The numerical score groups into lethality (40), non-hatching (20) and malformation index (<20).

A toxicity score of 7 (mean toxicity score for the controls was 2.24 $\pm$ 9.53) was calculated for triticonazole based on the single concentration study, indicating a positive response. An AC_{50} of 13.05 µM was derived for triticonazole in the dose-response experiment. In conclusion, equivocal effects were observed at high concentrations because of a wide variation of individual scores.

Materials and methods

Test Material:	The chemical library consisted of EPA´s Phase I ToxCAST library of 320 substances, among them was triticonazole (CAS No. 131983-72-7)
Vehicle and/or positive control:	DMSO
Test animals:	
Species:	Zebrafish (Danio rerio) embryos
Strain:	undefined outbred stock
Sex:	Male and female
Age:	6-8 h after fertilization the exposure of the embryos was started
Source:	Aquatic Research Organisms, Hampton, NH, 03842
Housing:	2-3 females per male and 15-20 adults per tank were kept in one of several 9-L flow-through colony tanks (Aquatic Habitats, Apopka, FL). One hour prior to light onset all adults in a colony tank were placed in a 2 L (static) breeding tank (Aquatic Habitats, Apopka, FL). Two hours after light onset the adults were returned to the colony tank.
Environmental conditions:	
Temperature:	28°C (adults), 26 ± 0.1°C (embryos and larvae)

Photo period: 14:10 h light:dark cycle (lights on at 08:30 h)

Zebrafish husbandry and exposure:

Adult zebrafish (2–3 females per male; density = 15–20 adults per tank) were kept in one of several 9-liter flow-through colony tanks. All adults in a colony tank were placed in a 2 L (static) breeding tank one hour prior to light onset. Typically, adults from two to three colony tanks were mated on the same day. Two hours after light onset the adults were returned to the colony tank.

All embryos were gathered from each breeder tank, pooled, and placed in a 28 °C water bath for 2 h, followed by two washes with 0.06% bleach (v/v) in 10% Hanks' Balanced Salt Solution (13.7 mM NaCl, 0.54 mM KCl, 25 µM Na2HPO4, 130 µM $CaCl_2$ 100 µm MgSO4 and 420 µm $NaHCO_3$), hereafter referred to as Hanks' solution, for 5 min in order to remove any residual bacteria or fungi.

Zebrafish embryos were exposed in 96-well plates. On day 0, approximately 6–8 h after fertilization, zebrafish embryos were placed 1 embryo per well in Millipore Multiscreen Nylon mesh plates and exposed to nominal concentrations of the chemicals. In each well, 1µL of the chemical in DMSO from the stock plate was diluted with 250 µL of 10% Hanks' solution; the final DMSO concentration was 0.4% (v/v) in all wells; vehicle controls receives DMSO only. Each plate was sealed with a non-adhesive material, covered with a lid, and wrapped in Parafilm® to minimize evaporation. All embryos and larvae were kept in a 26 ± 0.1°C incubator with a 14:10 h light-dark cycle (with lights on at 08:30 h and off at 22:30 h).

Embryos were exposed to the chemicals for 5 days post fertilization (dpf) (i.e., 120 h post fertilization) with daily dosing (i.e., complete solution change with chemical renewal every 24 h), followed by a wash-out in Hanks' buffer for 1 day prior to the lethality, hatching, and malformation assessments performed on 6 dpf.

Single concentration study:

The embryos were exposed to the chemicals (80 µM final concentration, renewed daily) by immersion from 0 dpf until 5 dpf. Eighty micromolar was chosen as the highest concentration for two reasons (1) because the stock solutions were prepared at 20 mM in 100% DMSO. Therefore to limit the amount of DMSO in the rearing solution, the smallest accurate amount possible was used: 1 µL diluted in 250 µL of 10% Hanks' which gave a final, highest concentration of 80 µM: and (2) concentrations above 80 µM would be beyond environmentally or pharmacologically relevant concentrations.

On 6 dpf, each embryo/larva was assessed for viability, hatching status and malformations. There were 4 embryos per concentration per chemical (each embryo on a separate microtiter plate). If more than two controls on a plate (i.e.,≥2/16 = 12.5%) showed lethality or significant malformations, the data from that entire plate were rejected, and the experiment was repeated. Less than 4% of the plates were rejected.

Concentration response study:

The chemical was arrayed in an 11-point (semi-log) concentration-response with the highest stock concentration (20 mM), the same as in the Single Concentration Study. Adding 1 µL of the chemical solution from the stock plate to the treatment plate and diluting with 250 µL of Hanks' buffer resulted in a descending concentration-response curve

spanning 5 orders of magnitude (80.000, 26.600, 8.800, 2.960, 1.000, 0.320, 0.110, 0.030, 0.012, 0.004, and 0.001 µM). The vehicle control was DMSO at a final concentration of 0.4%). A positive reference chemical (chlorpyrifos ethyl) was included. The embryos were exposed to the chemicals (renewed daily) by immersion from 0 dpf until 5 dpf, and on 6 dpf, each embryo/larva was assessed for viability, hatching status and malformations. Usually, there were 2 embryos per concentration per chemical (each embryo on a separate microtiter plate). If more than one control on a plate (≥12.5%, i.e., ≥1/8) showed lethality or significant malformations, the data from that entire plate were rejected, and the experiment was repeated. Less than 4% of the plates were rejected.

Embryo/larval assessments:

On 6 dpf (144 h post fertilization), each larva was assessed by visual inspection under a dissection microscope. If a larva was dead, no more assessments were made. If a larva was viable, it was then determined if it had hatched or not. If the larva had not hatched, then that information was recorded as an endpoint. If a larva was alive and hatched, an assessment of the degree of malformation was made.

Embryos/larvae were considered dead at 6 dpf if there were signs of coagulation, decay, or no visible heartbeat. Embryos/larvae were considered not hatched if they remained encased in the chorion. If a larva was alive and hatched it was assessed by an observer, blinded to the treatment. Larva was assessed for malformations of general categories. This involved the following assessments: (1) spine (e.g., stunted skeletal growth, curved spine, kink in tail), (2) fins (e.g., malformed or stunted fins), (3) cranial/facial (e.g., abnormal head, eyes, or otoliths), (4) thorax (e.g., distension, heart malformations), (5) abdomen (e.g., edema, emaciation), and (6) position in the water column (e.g., floating, lying on side). These features were scored for each of the categories, which thus may contain a number of possible malformations that could occur. Some malformations were scored in binary fashion (1, 0 for present or not) while others were scored by relative degree, from not present (0) through severe (4). The aggregated scores across all categories of malformations were then summed for each condition and defined as the "Malformation Index". Higher Malformation Indices denote more severely malformed fish, and the indices for the present study went as high as 34, with the historical control values normally between 0 and 3. The Malformation Index mean (± SEM; standard error of the mean) for the controls in the Single Concentration Study was 0.51 ± 0.10 (n = 217 embryos that were alive and hatched), and for the Concentration-Response Study the mean was 0.66 ± 0.09 (n = 706 embryos that were alive and hatched).

Toxicity score:

In order to formalize the descriptive data (lethality and hatching status) with the numerical data (Malformation Index), a numerical score was assigned to the descriptive data: 40 for lethality and 20 for non-hatching, and if the larva was alive and hatched, then the Toxicity Score was equal to the Malformation Index. A chemical was considered active in the Single Concentration Study if the mean Toxicity Score of the four technical repeats for each chemical was greater than the overall mean Toxicity Score of the control fish in the study. For example, in the Single Concentration Study there were 228 controls; the overall mean Toxicity Score for the controls was 2.24 ± 9.53 (SD; standard deviation). Therefore any chemical with a mean Toxicity Score above 2.24 was considered active in the Single Concentration Study.

Chemical potencies were estimated for each compound in the Concentration-Response Study as half-maximal activity concentrations (AC_{50}). The "response" was the combined Toxicity Score, which ranged from 0 to a maximum imputed value of 40.

Standard sigmoidal curves were fit using a 4-parameter Hill model, where the response was defined in terms of the four parameters {T, B, AC50,W} given in Eq. (1):

$$f(X) = T - \frac{T - B}{1 + (X/AC_{50})}$$

The parameters T and B are the upper ("top") and lower ("bottom") asymptotes of each assay response, respectively. The W parameter, or "Hillslope", dictates the curve slope (change in response relative to concentration) between B and T, where higher numbers indicate steeper curves. The parameters were fit using a custom R implementation (R Development Core Team, Vienna, 2011) of the Evolutionary Algorithm Dose Response Modeling (EADRM) algorithm. The EADRM algorithm has been shown to handle complex concentration-response spaces and can overcome the challenge of highly variable initial parameter values. Using such a flexible approach to computing the AC_{50} value for each response provided a systematic way to compare compound potencies, even in the face of heterogeneous response patterns. Positive AC_{50} "hit" acceptance criteria were applied as a combination of efficacy (i.e., response at the top asymptote of the sigmoidal fit), and goodness-of-fit (R2). The minimum R2 cutoff was 0.4, and the minimum efficacy cutoff was 6.5 (calculated as one standard deviation above the mean of the vehicle control response values).

For chemicals where Toxicity Score responses were significant at the lowest concentrations, thus precluding curve fits, AC_{50} values were heuristically set to the minimum concentration tested. AC_{10} values were estimated using the same curve-fitting procedure, except that the equation was solved to find the concentration at which the response was 10% of the "top" activity.

Results

Single concentration study

A Single Concentration Study was conducted as a preliminary to identify any shortcomings in the design of the experimental protocol, and to determine how well the single concentration results predicted the concentration-response results.

A toxicity score (Mean of n = 4) of 7.00 was observed for triticonazole indicating a positive response.

Concentration response study

An AC_{50} of 13.05 µM was observed for triticonazole in the concentration response study. In the following figure green circles represent larvae that were within the normal range, red circles represent nonviable larvae, purple circles represent larvae that did not hatch, and orange circles represent larvae that showed significant malformations. Based on the results of the concentration response study, no indication of malformations was observed after treatment of Zebrafish with triticonazole (absence of orange circles). The increase of the Terata Score above the control value of 0.66 ± 0.09 (n = 706 embryos) was obviously due to excessive toxicity leading to nonviable larvae or larvae that did not hatch. In conclusion, results with triticonazole in the Zebrafish developmental screening assay indicate a toxic effect of triticonazole to the zebrafish larvae, however without giving rise to malformations.

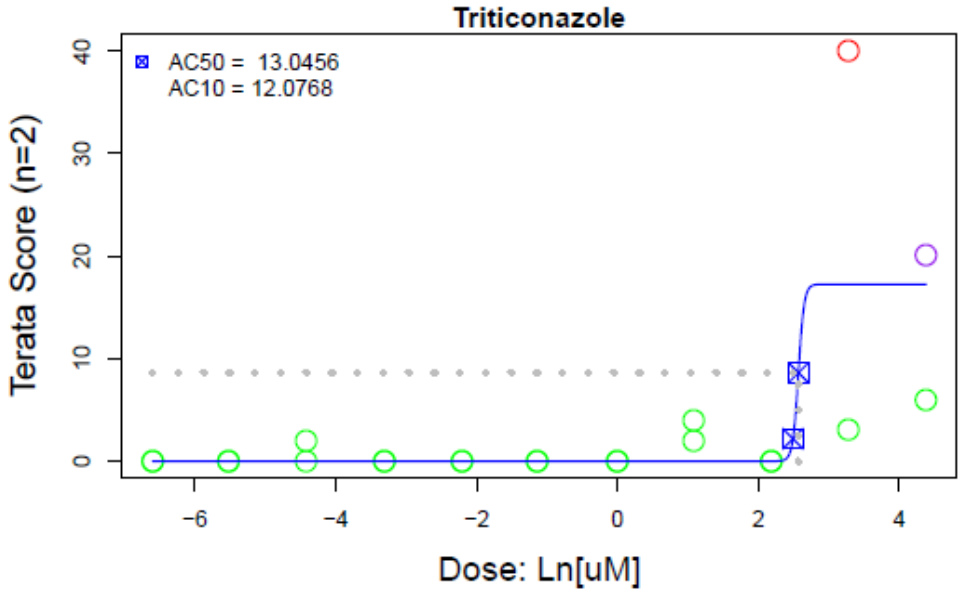


Figure 6.6.2.3-1: Results of the concentration response study

Conclusion

Triticonazole (BAS 595 F) was tested in a Zebrafish developmental screening assay up to 80 µM. The results indicate that triticonazole had a toxic potential on the embryo/larvae indicated by nonviable larvae or larvae that did not hatch at concentrations >20 µM. However, no malformations were observed in the larvae after exposure to triticonazole. The calculated AC_{50} was 13.05 µM.

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies
Author(s), year:	Hermsen S.A.B. et al., 2011
Report/Doc. number::	2011/1297791/ -
Guideline(s):	No
GLP:	No
Deviations from OECD Guideline	No Guideline study
Acceptability:	Yes; additional information

Executive Summary

The zebrafish embryotoxicity test (ZET) is a fast and simple method to study chemical toxicity after exposure of the complete vertebrate embryo during embryogenesis in ovo. A novel quantitative evaluation method to assess the development of the zebrafish embryo based on specific endpoints in time, the general morphology score (GMS) system was used. Morphological evaluation of the embryos was performed at 72 h post fertilization (hpf). The GMS scoring system used is similar to the one used for rat Whole-embryo-cultures (WEC) and comprises the normal development of a zebrafish embryo up to 72 hpf (detachment of tail, somite formation, eye development, movement, heartbeat, blood circulation, pigmentation head-body, pigmentation tail, pectoral fin, protruding mouth, hatching).

The semi-quantitative assessment of specific developmental endpoints supports standardization of the evaluation. An experimental embryo is compared to the reference embryo in the scoring matrix and receives points for each developmental hallmark dependent on its stage of development. All deviations, for instance incomplete detachment of the tail, will result in a lower point score, which corresponds to a certain extent of developmental retardation.

Malformations and other teratogenic effects are separately recorded as present or absent according to a list provided (pericardial edema, yolk sac edema, eye edema, malformation of the head, malformation of saccule/otoliths, malformation of tail, malformation of heart, modified chorda structure, scoliosis, rachischisis, yolk deformation) with the effects scored as present or absent.
In this study triticonazole (TTC) has been tested and compared with other 1,2,4-triazoles (flusilazole (FLU), hexaconazole (HEX), cyproconazole (CYP), triadimefon (TDF), myclobutanil (MYC)).

For triticonazole benchmark concentrations of 80.5 µM and 40 µM were derived for general morphology and teratogenicity. Triticonazole showed minor effects only in the highest concentration tested and was indicated as the least potent triazole tested for general developmental toxicity. With regard to specific teratogenicity in this test system, triticonazole did not cause 100% teratogenicity at the highest concentration tested in contrast to the other compounds. Furthermore, comparison of in vitro data with in vivo data demonstrated a good correlation, indicating that the ZET may be a reliable (screening) tool in assessing teratogenic effects.

Material and methods

Test Material:	Triticonazole (CAS No. 131983-72-7, TTC), Hexaconazole (CAS No. 79983-71-4, HEX), Cyproconazole (CAS No. 94361-06-5, CYP), Triadimefon (CAS No. 43121-43-3, TDF), Myclobutanil (CAS No. 88671-89-0, MYC) Further chemicals were tested during the study but were not mentioned here. Only TTC and triazole derivatives were included in this summary.

Vehicle and/or positive control: DMSO (vehicle control), 3,4-dichloroaniline (positive control)

Test animals:

Species:	Zebrafish (Danio rerio) embryos
Strain:	Singapore wild type stock
Sex:	Male and female
Diet:	Feeding twice daily with dry flakes (Special Diet Services, Tecnilab-BMI BV, The Netherlands) and once daily with defrosted *Artemia* (Landman BV, The Netherlands) in a quantity that was consumed within 5 min. Three days before spawning, females were separately housed and fed only thawed *Artemia*, both to optimize egg production.
Source:	[redacted]
Environmental conditions:	
Temperature:	27 ± 1°C
Photo period:	14:10 h light:dark cycle

Zebrafish husbandry and exposure:

Adult zebrafish were kept in 7.5 L ZebTEC aquaria at 27°C ± 1°C with a photoperiod of 14 h light: 10 h dark. Males and females were paired in spawning boxes the day before spawning in a ratio of 2:2. Spawning was triggered once the light was turned on and was usually completed within 30 min.

Fertilized batches of eggs with a fertilization rate of at least 90% were collected within 30–60 min after spawning and rinsed several times in Dutch Standard Water (DSW; demineralized water supplemented with 100 mg/L $NaHCO_3$, 20mg/L $KHCO_3$, 200 mg/L $CaCl_2 \cdot 2H_2O$, and 180 mg/L $MgSO_4 \cdot 7H_2O$ and then aerated for 24 h at 27°C). The eggs were evenly distributed among the test compounds and concentrations after removal of any debris or coagulated eggs. Embryos within the 4- to 32-cell stage were selected and transferred to a 24-well plate with similar test compound and concentration. One embryo was transferred to one well containing 2 mL test medium and 10 embryos per test concentration were used. Embryos were kept in an incubator at 26.5 ± 1°C with a photoperiod of 14 h light:10 h dark.

The triazoles hexaconazole, cyproconazole, triadimefon, myclobutanil, and triticonazole were tested in the range of 0.1 to 316 µM. All triazoles were dissolved in DMSO and further diluted in DWS with a final DMSO concentration of 0.2% (v/v). The control group was exposed to 0.2% DMSO.

Zebrafish – Morphological assessment:

Morphological evaluation of the embryos was performed at 72 h post fertilization (hpf). GMS was recorded using the GMS system (Fig. 6.6.2.3-2). This scoring system is developed similar to the one used for rat WEC and comprises the normal development of a zebrafish embryo up to 72 hpf. The semi-quantitative assessment of specific developmental endpoints supports standardization of the evaluation. An experimental embryo is compared to the reference embryo in the scoring matrix and receives points for each developmental hallmark dependent on its stage of development. All deviations, for instance incomplete detachment of the tail, will result in a lower point score, which corresponds to a certain extent of developmental retardation. Malformations and other teratogenic effects are separately recorded as present or absent. The test was considered valid if <10% of the control embryos showed coagulation or effects.

General Morphology Score

Hpf	12	24	48	72
Detachment of tail	 0  *18hpf* 1	 2	 3	 3
Somite formation	No = 0	Yes = 1	Yes = 1	Yes = 1
Eye development	 1	 2	 2 + 1 for pigment	 2 + 1 for pigment
Movement	No = 0	Yes = 1	Yes = 1	Yes = 1
Heartbeat	No = 0	Yes = 1	Yes = 1	Yes = 1
Blood circulation	No = 0	No = 0	Yes = 1	Yes = 1
Pigmentation head-body	0	 0	 1	 1
Pigmentation tail	0	 0	 1	 1
Pectoral fin	0	0	 0	 1
Protruding mouth	0	0	 0	 1
Hatching	No = 0	No = 0	No = 0	Yes = 1
GMS	***1***	***7***	***12***	***15***

Figure 6.6.2.3-2: General morphology scores

Benchmark dose determination:

The results of the ZET data were analyzed using the benchmark dose (BMD) approach, in which the benchmark concentration (BMC) at a predefined benchmark response (BMR) was calculated using a fitted dose-response curve. For the tested compounds a decrease of 5% in GMS was defined as the BMR for calculating the corresponding BMC (BMC_{GMS}). This BMR level was arbitrarily selected to obtain the concentration related to the threshold of effect outside the normal variation. In this procedure a nested family of concentration–response curves with an increasing number of parameters is fitted and the log likelihood of each model is calculated to determine its goodness of fit. The model with the lowest number of parameters which gave the best fit was selected to calculate the BMC_{GMS}.

The BMC for teratogenicity (BMC_T), with teratogenicity defined as the fraction of embryos with one or more teratogenic effects, was calculated with a BMR defined as a 5% increase in the fraction of affected embryos. This level was also arbitrarily selected in the same manner as for the BMC_{GMS}. For these quantal data, four models with statistically similar goodness of fit were fitted, namely log–logistic, Weibull, log-probit and gamma. The model with the lowest BMC outcome was chosen. However, compounds within the same class are expected to have similar mechanisms of action. Therefore, based on the analysis of individual compounds the most conservative model per class of compounds was selected for final BMC calculation. For the group of the triazole anti-fungals the Weibull model was selected to fit the concentration–response curves.

Results

Effects on morphology and teratogenicity

Triticonazole showed minor effects only in the highest concentration tested and was indicated as the least potent triazole with a BMC_{GMS} of 80.5 µM. Furthermore, it should be noted that the confidence interval of the triticonazole BMC_{GMS} exceeded the highest tested concentration. Comparable patterns of teratogenic effects were observed for all triazoles, however, at different concentrations, indicative of differences in potency. Triticonazole showed a BMC_T of 40.0 µM, however, even at the highest tested concentration it did not cause 100% teratogenicity in contrast to the other compounds. The study authors concluded that « in the ZET all tested triazoles, except trticonazole, showed teratogenic effects of a comparable nature, although at different doses, indicative of differences in potency ».

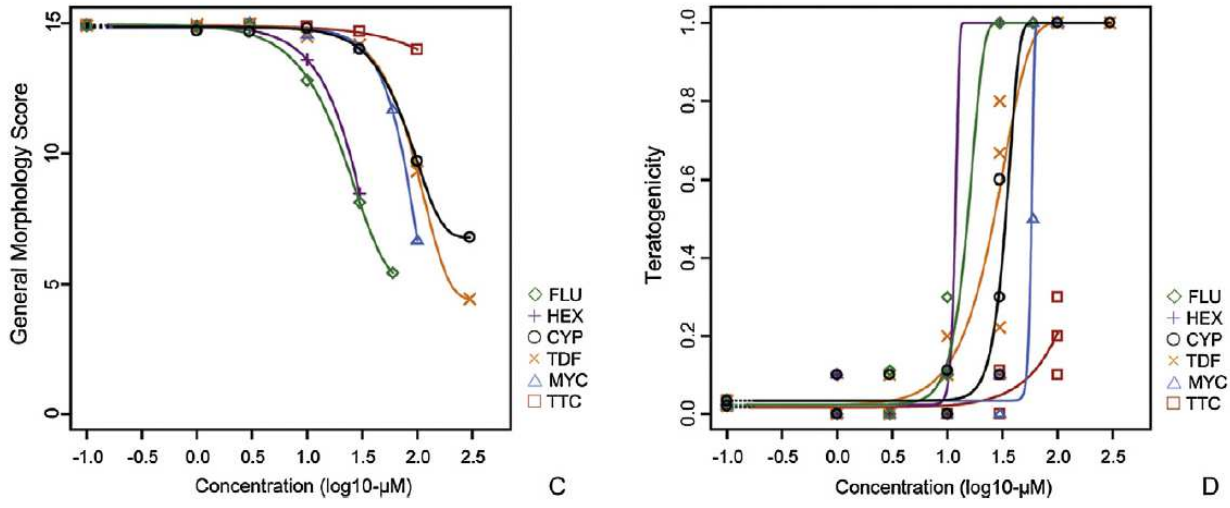


Figure 6.6.2.3-3: Concentration response curves of general morphology score and teratogenicity (fraction of embryos with at least one teratogenic effect) scored at 72 h post fertilization

Despite the different concentrations at which the various triazoles exerted their effects, the patterns of teratogenic effects appeared very similar (please see figure below), mostly comprising head and heart malformations, scoliosis, yolk deformation and edema in exposed embryos.

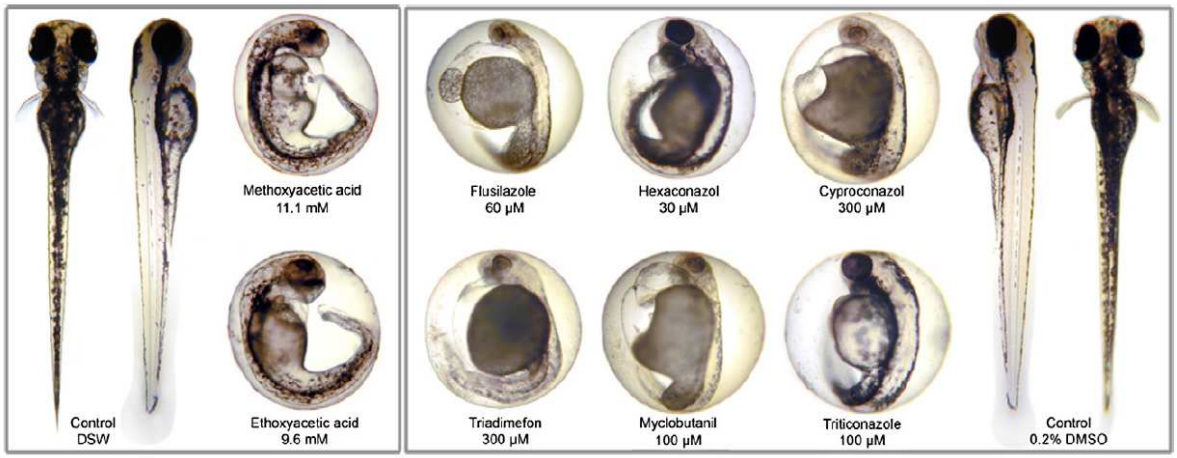


Figure 6.6.2.3-4: representative pictures of zebrafish embryos at 72 h post fertilization exposed to DSW (control) and glycol ether metabolites (left panel) and DMS (control and 1,2,4-triazoles (right panel)

Triticonazole showed to be the least potent compound. In general, the ranking of these compounds with the ZET is comparable to the ranking in vivo.

Table 6.6.2.3-2: Benchmark concentrations for the endpoints GMS and teratogenicity for different traizoles in the ZET

Triazoles	BMC_{GMS}[a] (µM)	BMC_T[b] (µM)	dLEL[c] (µmol/kg bw/day)
FLU	4.8 (4.3–5.4)	8.1 (5.4–11.3)	1.3
HEX	7.0 (6.1–7.9)	10.1 (7.1–19.0)	8.0
CYP	27.7 (22.3–34.7)	19.8 (8.4–29.7)	41.1
TDF	29.2 (23.1–37.5)	6.6 (3.5–12.3)	170.2
MYC	30.2 (28.0–32.5)	51.4 (25.8–53.8)	1083.9
TTC	80.5 (66.7–101.5[d])	40.0 (16.2–96.2)	3146.5

[a] Benchmark concentration for general morphology score at a 5% benchmark response.
[b] Benchmark concentration for teratogenicity at a 5% benchmark response.
[c] dLEL: lowest effect level for any developmental effect derived from the ToxRefDB.
[d] Exceeding highest concentration tested.

Conclusion

For triticonazole benchmark concentrations of 80.5 µM and 40 µM were derived for general morphology and teratogenicity. Triticonazole was found to be the least potent triazole with regard to general developmental and to specific teratogenic endpoints in a zebrafish embryotoxicity test, where zebrafish embryos have been evaluated 72 h post fertilization. The study gives an overview on ranking of tested substances.

Previous evaluation:	No

DRAR (2016)	Literature data

Reference:	Triazole-induced gene expression changes in the zebrafish embryo
Author(s), year:	Hermsen S.A.B. et al., 2012
Report/Doc. number::	2012/1369002/ -
Guideline(s):	No
GLP:	No
Deviations from OECD Guideline	No Guideline study
Acceptability:	Yes, additional information

Executive Summary

The zebrafish embryo is considered to provide a promising alternative test model for developmental toxicity testing. Most systems use morphological assessment of the embryos, however, microarray analyses may increase sensitivity and predictability of the test by detecting more subtle and detailed responses.

The purpose of the study was to investigate the possibility of relating gene expression profiles of structurally similar chemicals tested in a single concentration, to the complete transcriptomic concentration-response of flusilazole (FLU) as positive control. Besides triticonazole also hexaconazole, cyproconazole, triadimefon and myclobutanil at equipotent concentrations (based on morphological alterations) have been tested. The gene expression profiles of triticonazole (TTC) was assessed at the highest possible concentration of 100 µM. Compared to the other azoles, this was the highest concentration tested. Zebrafish embryos within the 4- to 64 –cell stage were incubated with triticonazole solution. Cultures were checked for morphology and parallel cultures were used for gene expression analysis.

Triticonazole induced very little morphological effects and showed a low absolute average fold change. It was stated, that the nonembryotoxic triticonazole induces a 4.7% decrease in GMS (general morphology score) at 100 µM, the highest concentration testable. Also the absolute average fold change (aaFC) and the expression of all upregulated genes were very low and similar to the low flusilazole concentration.

Slight induction (1.2-1.4 fold) was observed for genes of transcriptional, or retinoid metabolism pathways, however to a much lesser degree for triticoanzole compared to the other triazoles. The genese indicative for fatty acid metabolism were also slightly upregulated (roughly 1.2-fold). A higher fold-increase of 1.5 was observed for the steroid biosynthesis pathways, triticonazole showing the highest effect compared to the other triazoles in this test system. It was discussed that for triticoanzole, the cyp51 expression and steroid biosynthesis regulation is high compared to other other azoles and together with the low retinol metabolism regulation it is suggested that this compound has the least unwanted effects with respect to developmental toxicity. Based on 205 concentrations-response genes correlated to GMS, triticonazole also appeared as the least potent compound.

Materials and methods

Test Material:	Triticonazole (CAS No. 131983-72-7, TTC), Hexaconazole (CAS No. 79983-71-4, HEX), Cyproconazole (CAS No. 94361-06-5, CYP), Triadimefon (CAS No. 43121-43-3, TDF), Myclobutanil (CAS No. 88671-89-0, MYC)
Vehicle and/or positive control:	DMSO (vehicle control), Flusilazole (CAS No. 85509-19-9, positive control)

Test animals:

Species:	Zebrafish (Danio rerio) embryos
Strain:	Singapore wild type stock
Sex:	Male and female
Diet:	Feeding twice daily with dry flakes (Special Diet Services, Tecnilab-BMI BV, The Netherlands) and once daily with defrosted *Artemia* (Landman BV, The Netherlands) in a quantity that was consumed within 5 min. Three days before spawning, females were separately housed and fed only thawed *Artemia*, both to optimize egg production.
Source:	Ruinemans Aquarium BV, Montfoort, The Netherlands; maintained and bred in the laboratory for several generations
Environmental conditions:	
Temperature:	27 ± 1°C
Photo period:	14:10 h light:dark cycle

Zebrafish husbandry and exposure:

Adult zebrafish were kept in 7.5 L ZebTEC aquaria at 27°C ± 1°C with a photoperiod of 14 h light: 10 h dark. Males and females were paired in spawning boxes the day before spawning in a ratio of 2:2. Spawning was triggered once the light was turned on and was usually completed within 30 min.

Fertilized batches of eggs with a fertilization rate of at least 90% were collected within 30–60 min after spawning and rinsed several times in Dutch Standard Water (DSW; demineralized water supplemented with 100 mg/L $NaHCO_3$, 20mg/L $KHCO_3$, 200 mg/L $CaCl_2 \cdot 2H_2O$, and 180 mg/L $MgSO_4 \cdot 7H_2O$ and then aerated for 24 h at 27°C). The eggs were evenly distributed among the test compounds and concentrations after removal of any debris or coagulated eggs. Embryos within the 4- to 64-cell stage were selected and transferred to a 24-well plate with similar test compound and concentration. One embryo was transferred to one well containing 2 mL test medium. Embryos were kept in an incubator at 26.5 ± 1°C with a photoperiod of 14 h light:10 h dark.

The triazoles hexaconazole, cyproconazole, triadimefon, and myclobutanil were tested at 17, 64.7, 64.9, and 57.4 µM, respectively. Triticonazole was tested at the highest soluble concentration of 100 µM. All triazoles were dissolved in DMSO and further diluted in DWS with a final DMSO concentration of 0.2% (v/v). The control group was exposed to 0.2% DMSO.

Zebrafish – Morphological assessment:

Morphological assessment of the embryos was done microscopically at 24 and 72 hpf using the general morphology score (GMS ; please see Figure 6.6.2.3-2) system to quantify developmental retardation and teratogenicity, whereas parallel cultured embryos at 24 hpf were used for microarray analyses. The experiment was conducted in three independent series. In each independent series, 24 embryos per compound per concentration were used for evaluation of the GMS. The results of the scoring at 72 hpf were used for analyses of the morphological effect size.

Zebrafish – RNA isolation and processing:

For microarray analysis, 6 independent biological replicates, each including 20 pooled zebrafish embryos, for each of the experimental FLU concentrations, and 8 biological replicates for the controls and other triazoles tested in one

concentration, were collected at 24 hpf. This was done in three independent series, which included all the concentrations of FLU and the other triazoles per series. Embryos were stored in RNAlater to stabilize RNA and kept in RNAlater for at least 1 day at 4°C. Before RNA isolation, RNAlater was removed and embryos were homogenized using a pestle in an Eppendorf tube. RNA was isolated using the RNeasy mini kit including an additional DNase treatment. RNA concentration was measured on the NanoDrop spectrophotometer and RNA integrity was assessed on the Bioanalyzer 2100 using the RNA 6000 Nano Chip kit by automated gel electrophoresis. RNA samples were hybridized onto 4×180k D. rerio microarrays. ACy3/Cy5 dual colour array with a common reference design was used. Quality control was performed on raw microarray data using a scatterplot and MA-plot as well as a normal probability plot to assess signal distribution. The positive and negative controls on the slides were only used for quality control and excluded from further analysis.

Data analyses:

Microarray data was normalized and further analyzed using R statistical software (version 2.11.0). For the concentration–response of FLU the same data was used as previously published. However, the data for the additional triazoles was generated in the same experiment as the concentration–response FLU data, but have not been published before. The FLU data were used for comparison with the additional triazoles. Normalization of the Cy3/Cy5 extracted signals was done in a three-step approach as described by Janssen et al. (2007)[9]. Principal Component Analyses (PCA) [44] were done to detect nuisance effects of experimental variables "series", "labeling", "slide", "array", "RNA isolation batch". A clustering of samples was observed according to "series". For this reason a linear model was fitted with experiment group and "series" as fixed factors, as "series" did not have enough levels to effectively model as random. Therefore a microarray analysis of variance (MAANOVA) package in R was used. The nuisance effect caused by "series" was removed from the data. Probes that were significantly differentially expressed between control and treatment group were identified using one-way ANOVA with a Benjamini and Hochberg False Discovery Rate (FDR) adjusted p-value of <0.05. After selection, probes were pooled on their Entrez gene ID to obtain a list of unique genes.

In a previous study, genes showing monotonous and biphasic concentration-dependent changes were selected and four significantly enriched terms, which were transcription, retinol metabolism, steroid biosynthesis and fatty acid metabolism were identified with these genes. Gene expression patterns based on these 205 concentration–response genes and terms, were visualized using maximal fold change (FC) per gene determined between the experimental and control group using the average normalized signal values per group and created into a heatmap using GeneMaths XT. Furthermore, the absolute average fold change (aaFC) of the 205 genes and the genes present in the terms assigning the same value, e.g. a 2-fold down and a 2-fold upregulated gene.

The average of these values was defined as the aaFC. In addition, the overlap between genes significant for each treatment group was determined for which aaFC was determined as well. Additional pathway analyses for all compound groups was done using PathVisio 2 for KEGG and Wiki pathways (http://www.pathvisio.org/) and DAVID (http://david.abcc.ncifcrf.gov/) for GO terms with the genes that were significantly differentially expressed compared to the controls (FDR < 0.05). Pathways were considered significantly enriched with a z-score of >1.64 (PathVisio) or Benjamini <0.1 (DAVID).

[9] Janssen R, Pennings J, Hodemaekers H, Buisman A, van Oosten M, de Rond L, et al. Host transcription profiles upon primary respiratory syncytial virus infection. Journal of Virology 2007;81:5958–67.

Results

Effects on morphology

In order to confirm the $BMC_{GMS}20$ of the compounds, control and exposed zebrafish embryos were evaluated at 72 hpf using the classical read-out GMS, in parallel to incubations for transcriptomics analysis at 24 hpf. In previous experiments, FLU caused a concentration-dependent decrease in GMS at 72 hpf. All other triazoles also showed a decrease in GMS at 72 hpf. Triticonazole induced a 4.7% decrease in GMS at 100 µM, the highest concentration testable, which was in concordance with previous results.

The FLU $BMC_{GMS}20$-concentration group (13.5µM) showed a decrease of 19.6% in GMS compared to the controls. In addition, an increase in teratogenic effects such as pericardial edema and malformations of head and heart was observed (Figure 6.6.2.3-5). At 24 hpf no decrease in GMS was observed for all the tested triazoles, including the FLU concentration groups from 13.5 µM downward. A delay in development at 24 hpf was only observed in the 28 µM and 60 µM FLU treatment groups.

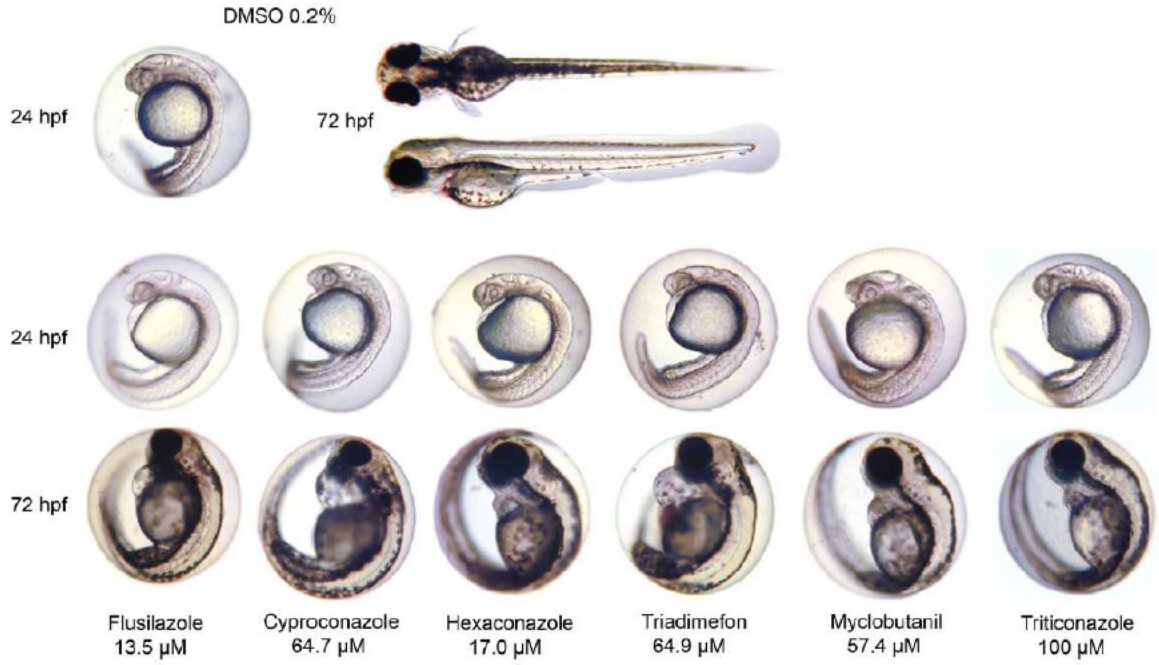


Figure 6.6.2.3-5: representative pictures of zebrafish embryos exposed to different traizoles and DMS= (vehicle) at 24 and 72 hpf

Gene expression changes and GMS

In a former study 205 genes were identified that were responding with different concentrations of FLU. To compare the different potencies of the compounds tested at a single concentration to a complete concentration–response these genes were used to calculate absolute average fold change (aaFC). When comparing the GMS to the aaFC, triticonazole induced very little morphological effects and also showed a lower aaFC in gene expression at 100 µM, similar to the low (0.28 – 2.8 µM) FLU concentrations. The overall gene expression induction by triticonazole and MYC deviates from the other triazoles. The aaFC for the genes that were significantly differentially expressed in all triazoles (8 genes) was calculated and it was found that triticonazole showed a good overlap with the FLU 6.0 µM

concentration group, with a similar aaFC and GMS. The expression of all upregulated genes after exposure to triticonazole was not as high as for the other triazoles and was more comparable to lower concentrations of FLU.

Pathway analysis

Triticonazole did not show a high aaFC for transcription, but an aaFC comparable to the lowest FLU concentrations. For retinol metabolism similar patterns were observed, however, aaFC is higher overall than for transcription. Triticonazole showed a low aaFC and individual gene expression compared to the other compounds tested. Steroid biosynthesis was highly regulated in triticonazole, even higher than for FLU, with triticonazole giving the highest upregulation.

The fatty acid metabolism gene set is almost equally regulated in all the compounds at equipotent concentrations regarding morphological effects, except for triticonazole, which showed less morphological effect, but equal disruption of fatty acid metabolism.

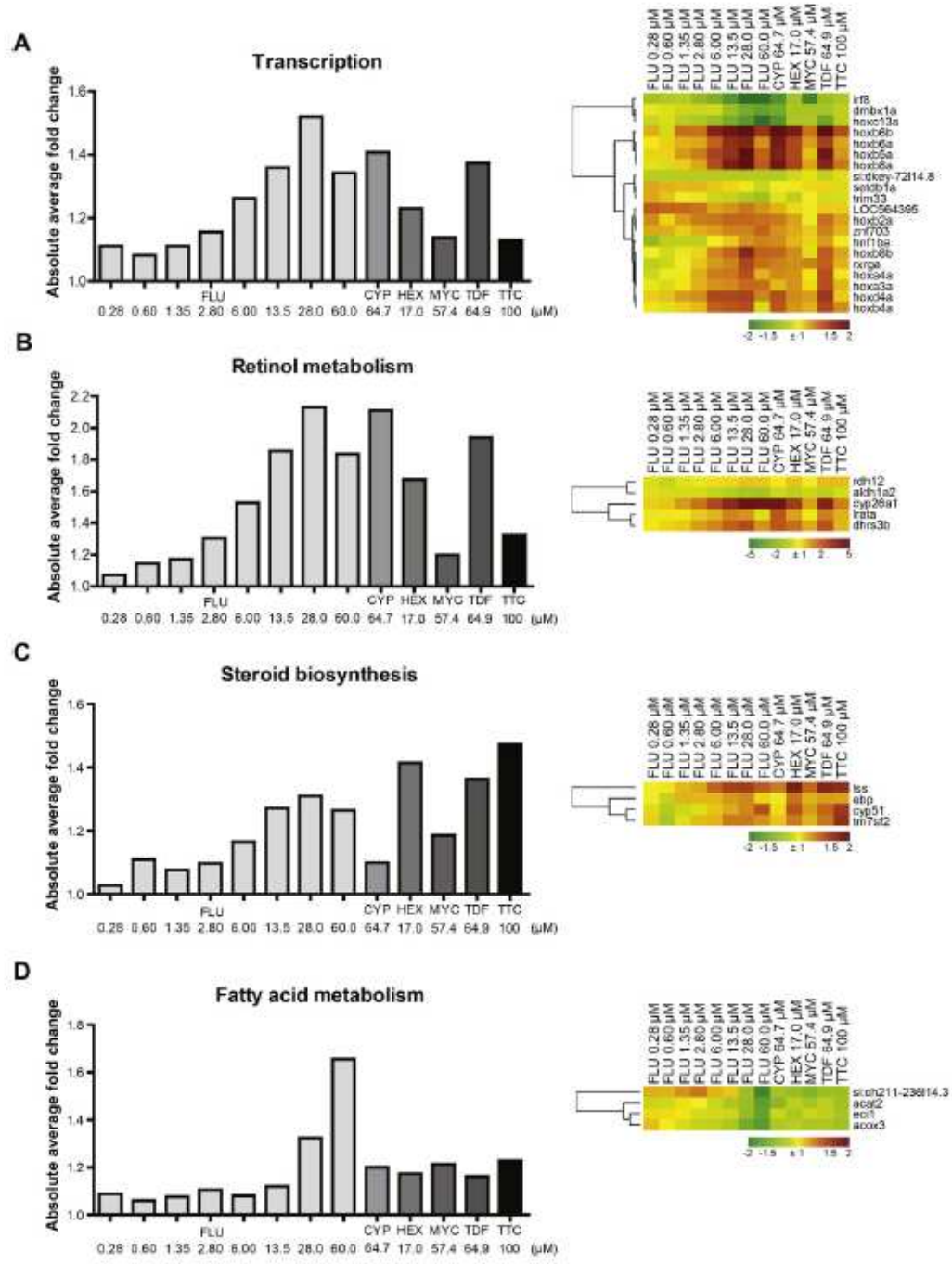


Figure 6.6.2.3-6: Absolute average fold change for the genes present in transcription (A), retinol metabolism (B), steroid bioysynthesis (C) and fatty acid metabolism (D) calculated for each exposure group, with heatmaps showing individual gene expression

Discussion

For the triazoles it has been proposed that their mechanism of developmental toxicity is related to retinol metabolism disruption, possibly via Cyp26a1 expression in this pathway. In this study, all triazoles regulated retinol metabolism including Cyp26a1 expression. Triticonazole regulated the retinol metabolism pathway to a lesser degree compared to other tested traizoles.

The anti-fungal mechanism of action for the triazoles is related to steroid biosynthesis mediated by Cyp51. This process was regulated by all compounds in the same direction. For triticonazole a high aaFC was observed, indicating that the mechanism of action is highly regulated at the $BMC_{GMS}20$.

For triticonazole Cyp51 expression and steroid biosynthesis regulation is high compared to other triazoles. Together with the low retinol metabolism regulation, it suggests that this compound has the least unwanted effects with respect to developmental toxicity. These findings correspond well with the morphological results. Morphological effects showed that all compounds at the concentrations tested, including FLU at 13.5 µM, decreased the GMS with the expected 20%, except for the negative compound triticonazole. Triticonazole showed only minor effects at 72 hpf as expected, which is in line with the limited regulation of retinol metabolism. Based on the 205 concentration–response genes correlated to GMS, triticonazole appeared as the least potent tested traizole in terms of aaFC (absolute average fold change) in transcription and GMS (general morphology score).

Conclusion

Triticonazole is considered to be non-embryotoxic, based on very little morphological changes induced in zebra fish embryos. Triticonazole regulated the retinol metabolism pathway to a lesser degree compared to other tested triazoles but Cyp51 expression and steroid biosynthesis regulation was higher than for other tested triazoles. Cyp51 catalyzes the first step following cyclization in sterol biosynthesis such as removal of the 14 alpha-methyl group from lanosterol in the cholesterol biosynthetic pathway, leading to formation of the initial substrate in steroid hormone biosynthesis.In the study of Karmaus et al., 2016 (B.6.8.3.5) triticonazole induced slight decrease of progesterone, deoxycorticosterone and cortisol levels (for last two only at cytotoxic levels) but it is recognised that other enzymes (e.g. Cyp17, Cyp11B1, Cyp21, HSD3B2) are involved in their synthesis.

The results of the gene expression data and the concentration-response genes correlated to GMS (general morphology score) suggest that triticonazole has the least unwanted effects – compared to other tested traizoles – with respect to developmental toxicity.

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Comparison of the mouse embryonic stem cell test, the rat whole embryo culture and the zebrafish embryotoxicity test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles
Author(s), year:	Jong E. de et al., 2011
Report/Doc. number::	2011/1297792/ -
Guideline(s):	No
GLP:	No
Deviations from OECD Guideline	No Guideline study
Acceptability:	Yes; additional information

Executive Summary

Triticonazole was investigated in three widely studied alternative assays for developmental toxicity, the mouse Embryonic Stem cell Test (EST), the Zebrafish Embryotoxicity Test (ZET) and the rat post-implantation Whole Embryo Culture (WEC). Again in this comparative study 5 further 1,2,4-triazoles were tested in all test systems

(flusilazole, hexaconazole, cyproconazole, triadimefon, and myclobutanil). For EST an established cell line was used, while the WEC and the ZET used mammalian and non-mammalian embryos, respectively. The results were analyzed using the Benchmark Dose (BMD) approach and compared with known BMD for skeletal variations and developmental lowest effect levels (dLEL) derived from an in vivo study from the ToxREF database (EPA). The rat developmental toxicity study conducted with triticonazole [redacted], 1991; DocID C018955) has been evaluated.

For Triticonazole benchmark concentrations of 35.8, 272.1 and 80.5 µM were derived for the differentiation of embryonic stem cells into beating cardiomyocytes (EST) and on embryonic development using total and general morphological score (WEC and ZET, respectively), respectively. Compared to the other test triazoles, triticonazole was least potent in the embryonic stem cell test and in the zebrafish assay (see above: Hermsen et al., 2011 and 2012) and together with cyproconazole also least potent in the Whole Embryo Culture test.

Also the in vivo results from rat developmental toxicity studies of the six triazoles have been compared. For triticonazole, the BMD_{10} for skeletal variations was calculated to be 3720 µmol/kg, which is above the highest dose tested in the in vivo rat [Crl:CD(SD)] teratology study [redacted] 1991; DocID C018955). It was stated, that triticonazole is not causing malformations, such as cleft palate, renal malformations and hydrocephaly.

Material and methods

Test Material:	Triticonazole (CAS No. 131983-72-7, TTC), Hexaconazole (CAS No. 79983-71-4, HEX), Cyproconazole (CAS No. 94361-06-5, CYP), Triadimefon (CAS No. 43121-43-3, TDF), Myclobutanil (CAS No. 88671-89-0, MYC), Flusilazole (CAS No. 85509-19-9)
Vehicle and/or positive control:	DMSO (vehicle control in EST, WEC), 5-Fluorouracil (dissolved in PBS; positive control in EST)

Cell culture (for EST):

Cell line:	Mouse D3 embryonic stem (ES) cells
Maintainance:	Sub-culturing was performed every 2-3 days
Source:	[redacted]
Medium:	DMEM supplemented with 20% fetal calf serum, glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 µg/mL), non-essential amino acids (1%) and β-mercaptoethanol (0.1 mM). 1000 U/mL murine leukemia inhibiting factor (mLIF) was added to maintain pluripotency.

Test animals (for WEC):

Species:	Rat
Strain:	not stated
Sex:	Female
Age:	7-10 weeks
Source:	not stated
Diet:	Feed and water ad libitum (not further specified)
Housing:	Single housing after mating
Environmental conditions:	
Temperature:	20-24°C
Humidity:	50-70%

Cell viability assay:

Mouse embryonic ES-D3 cells were seeded in 96-well plates (500 cells/well) in routine culture medium and incubated for 2 h to allow cell adherence. Afterwards the cells were exposed to the chemicals at equal concentrations as used in the differentiation assay. After 3 days the exposure medium was removed and fresh medium was added containing the corresponding compound for exposure. After 2 more days of incubation and approximately 80% cell confluence, CellTiterblue was added to each well and incubated for 2 h. The fluorescence was read using a spectrofluorometer at 544 nm (excitation) and 590 nm (emission). Three independent experiments were done for each compound.

Cardiomyocyte differentiation assay:

At the start of the assay 20 µl of an ES cell suspension (3.75×10^4 cells/mL) was placed onto the inner side of the lid of a 10-cm Petri-dish containing 5 mL of PBS resulting in drops containing 750 cells. After an incubation period of 3 days in a humidified atmosphere at 37 °C and 5% CO_2 the formed embryoid bodies (EBs) were transferred to bacteriological Petri dishes. After 2 days the EBs were transferred to 24-well plates with one EB per well and 24 EBs in total per concentration. On day 10 of culture cardiac differentiation was determined by light microscopic evaluation of the number of beating EBs. Cells were exposed to the test compound in concentrations up to 100–300 µM from day 0 onward with a medium renewal containing the corresponding compound for exposure on day 3 and day 5. Solvent controls were included in each experiment. Tests were accepted for further analysis if the solvent control consisted of at least 21 beating EBs out of 24 EBs incubated. In each experiment, 5-fluorouracil was used as a positive control at a concentration leading to approximately 50% decline in the number of beating EBs (0.3 µM). Three independent experiments were done for each of the compounds.

Immunohistochemistry:

On day 10 of cardiomyocyte differentiation, EBs exposed to cyproconazole, hexaconazole and myclobutanil (triticonazole not investigated) were stained using immunofluorescence histochemistry for the presence of βIII-tubulin, an early marker for neurons and MAP2, a marker for mature neurons. The cells were exposed to the primary antibody, βIII-tubulin, diluted in HEPES buffered saline solution (HBSS) supplemented with 0.1% saponin (Sigma-Aldrich), for 1 h at room temperature and to MAP2 overnight at 4°C. Samples were examined under a BX51 fluorescence microscope with CellF software for analysis.

Rat postimplantation embryo culture (WEC):

At day 10 of gestation, rat embryos with 2–5 somites were explanted from the uterus and cultured in 2 mL serum mixture. Embryos from each dam were distributed as evenly as possible over the solvent control and the various concentrations tested. Culture flasks were gassed with increasing oxygen concentrations twice daily during culture. After 48 h of culture at 37°C, the embryos were morphologically examined without prior fixation. Solvent controls (DMSO) were included in each experiment. For this study the total morphological score (TMS) was selected for further analysis. Three independent experiments were done for each compound.

Zebrafish embryotoxicity test (ZET):

The developmental toxicity data of the triazoles in the ZET were taken from Hermsen et al., 2011 (described above). Briefly, embryos within the 4- to 32-cell stage were selected for exposure to the triazoles. The embryos were

morphologically evaluated at 72 h post fertilization using the general morphology score system. The resulting BMC05 values for the general morphological score ($BMC05_{GMS}$) as stated in the study were used for correlation with the in vivo developmental toxicity of the compounds.

Results

Embryonic stem cell test (EST)

All tested triazoles inhibited the differentiation of embryonic stem cells into cardiomyocytes in a concentration–response manner. Myclobutanil and triticonazole among other triazole compounds showed little difference in their effects on cardiomyocyte differentiation with BMCd50 values between 30.5 and 35.8 µM. All compounds reduced the cell viability of the ES-D3 cells. Triticonazole had the strongest effect on cell viability at the BMCd50 with a cell viability of 76%. Morphologic evaluation on day 10 of culture showed differences in the cell types occurring after exposure to the different triazole compounds.

Whole embryo culture (WEC)

The concentration–response curves obtained testing the various triazoles in the WEC were used to calculate BMC values associated with a 5% decline in TMS ($BMC05_{TMS}$). Triticonazole and cyproconazole had the lowest effect on embryonic development with $BMC05_{TMS}$ values of 272.1 and 335.9 µM, respectively. At the end of the 48 h of exposure, all triazoles showed a common developmental effect pattern albeit with different potencies. The effects on morphology occurred at the level of the pharyngeal apparatus with reduction and fusion of the first and second branchial arches. Abnormalities of the mandibular process that develop from the first branchial arch were also detected. Furthermore, triazole exposure resulted in short tail and failure to close the posterior neuropore and otic vesicle.

Table 6.6.2.3-3: Effect of the six tested triazoles on the differentiation of ES cells into beating cardiomyocytes (EST) and on embryonic development in the WEC and the ZET

Compound	EST BMC_d50 (µM)	WEC $BMC05_{TMS}$ (µM)	ZET $BMC05_{GMS}$ (µM)[a]
Hexaconazole	16.6 (13.7–19.9)	149.9 (102.6–202.5)	7.0 (6.1–7.9)
Flusilazole	5.7 (4.8–6.7)	19.0 (18.0–20.1)	4.8 (4.3–5.4)
Cyproconazole	31.8 (30.1–40)	335.9 (270.0–471.4)	27.7 (22.34–34.7)
Triadimefon	32.2 (30.4–41.1)	178.6 (149.8–209.6)	29.2 (23.1–37.5)
Myclobutanil	30.5 (29.2–34.1)	138.6 (88.2–195.7)	30.2 (28.0–32.5)
Triticonazole	35.8 (33.4–38.8)	272.1 (145.4–441.7)	80.5 (66.7–101.5)

Note. BMC_d50 = concentration at which the fraction of beating EBs is reduced by 50% in the EST. $BMC05_{TMS}$ = concentration at which the total morphological score is reduced by 5% in the WEC. $BMC05_{GMS}$ = concentration at which the general morphological score is reduced by 5% in the ZET. Values are expressed as the averages of three independent experiments with the 90% confidence intervals given between brackets.

[a] Estimated in a previous study (Hermsen et al., 2011). Values are expressed as the averages of three independent experiments with the 90% confidence intervals given between brackets.

In vivo developmental toxicity

In vivo studies on the developmental toxicity of the triazoles tested were obtained from industry. The type of abnormalities described in the in vivo studies after triazole exposure mainly consisted of skeletal defects. Furthermore, triticonazole did not cause treatment-related malformations, such as cleft palate, renal malformations and hydrocephaly. Triticonazole only affected skeletal development at high doses. It should be noted that the calculated BMD_{10} value for triticonazole was slightly above the highest dose tested.

Table 6.6.2.3-4: Developmental toxicity after exposure to the triazole compounds via gavage

Compounds	Rat strain	Dose (µmol/kg bw/day)	Days	BMD10 skeletal variation (µmol/kg)[a]	dLEL (µmol/kg)[b]	Reference
Hexaconazole	Wistar	0, 8.0,79.6, 795.6	GD7-16	7.9 (1.8–27.2)	8.0	(Killick et al., 1984)
Flusilazole	Crl:CD(SD)	0, 1.3, 6.3, 31.7, 158.5, 792.6	GD7-16	9.1 (6.0–13.2)	1.3[c]	(Lamontia et al., 1984)
Cyproconazole	Wistar	0, 20.6, 41.1, 82.2, 164.5	GD6-15	53.4 (38.4–66.7)	41.1	(Becker, 1985)
Triadimefon	Crl:CD(SD)	0, 34.0, 85.1, 170.2, 306.4	GD6-15	91.5 (74.7–115.0)	170.2	(Unger et al., 1982)
Myclobutanil	Sprague–Dawley	0, 108, 325, 1082, 1624	GD6-15	1090 (1020–1170)	1082	(Costlow and Kane, 1984)
Triticonazole	Crl:CD(SD)	0, 126, 629, 3147	GD6-15	3720 (2540–9000)[d]	3147	(Burns, 1991)

[a] BMD10 values for skeletal variations include supernumerary ribs, and absent sternebrae. 90% confidence intervals are given between brackets.
[b] dLELs were taken from the ToxREF database.
[c] Unlike the other dLELs and BMDs, this dLEL was based on urogenital malformations at 1.3 µmol/kg.
[d] BMD10 value lies above highest dose tested.

Conclusion

In the EST and the ZET assay triticonazole was the lowest potent compared to the other azoles. In the WEC triticonazole was the second-least potent compound (here cyproconazole was least potent followed by triticonazole). WEC, EST and ZET assays correctly identified the potency of triticonazole for developmental effects, based on in vivo data, as triticonazole did not induce malformations such as cleft palate, renal malformations and hydrocephaly.

B.6.7. NEUROTOXICITY

B.6.7.1. Neurotoxicity studies in rodents

Triticonazole does not belong to a chemical family for which testing for delayed neurotoxicity is required. However, there was indication of neurotoxicity seen at the top dose in the 52-week dog study (tremors, ataxia, convulsions), but no microscopic findings in brain, spinal cord or isciatic nerves were seen. For further clarification, studies on neurotoxicity after acute and repeated oral exposure to rats according to relevant guidelines have been performed and evaluated already in DAR (2003).

B.6.7.1.1. Benchmark and time-to-peak effect neurotoxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Triticonazole – Benchmark and time-to-peak effect neurotoxicity study in rats
Author(s), year:	[redacted] 1997
Report/Doc. number::	R012965 / -
Guideline(s):	US EPA guideline 82-7
GLP:	Yes
Deviations from OECD Guideline	No OECD Guideline
Acceptability:	Yes; additional information (range finding study)

Material and methods

4 Male and 4 female rats (strain: Crl:CD®[SD]BR VAF/plus; source: [redacted]) received single doses of 0 (vehicle control), 50, 1000 or 2000 mg/kg bw triticonazole (batch no. 9550347; purity 97.2 %, dissolved in 0.5 % w/v methylcellulose in water) by oral gavage at a dose volume of 10 ml/kg (groups 1, 2, 3, 4) to establish a dose range for subsequent studies. An additional group of 8 male rats (group 5) were dosed at 2000 mg/kg bw on the following day to determine the time-to-peak effect.

Animals were observed twice daily for mortalities and clinical signs; individual body weights were recorded on the day of dosing. Neurobehavioural tests, consisting of an abbreviated functional observational battery (during handling, in an open arena, and during manipulations to assess reflexes and physiological parameters) and locomotor activity assessment in a circular open field enclosure for 60 minutes (cumulative counts of photobeam breaks in 10-minute intervals) were done for all animals predose and approx. 2 hours after dosing (groups 1, 2, 3, 4), and approx. 1 and 4 hours postdose for the males in the additional group (four males at each interval).

On day 4, the animals were sacrificed and the carcasses were discarded.

Results

There were no clear test material-related observations noted during the FOB at any dose level. The FOB results were similar across all groups, both sexes and across the three intervals the animals were assessed.

Motor activity appeared higher for males given 2000 mg/kg bw than of the controls especially during the 20 to 40-minute interval of the motor activity session, and appeared to peak between 2 and 4 hours after dosing (highest counts for most of the intervals whilst motor activity session occurring for animals tested 2 hours post-dose).

Conclusion

Based on the results, dose levels of 80, 400 and 2000 mg/kg bw were recommended for further testing. In addition, a time-to-peak effect for motor activity testing of approx. 2 - 3 hours after dosing was recommended for the acute study. No time-to-peak effect for FOB testing was established.

B.6.7.1.2. Acute neurotoxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Triticonazole – Acute neurotoxicity study in rats
Author(s), year:	[redacted] 1997
Report/Doc. number::	R012968 / -
Guideline(s):	US EPA guideline 81-8, OECD 424
GLP:	Yes
Deviations from OECD Guideline 424 (1997)	No
Acceptability:	Yes

Material and methods

Groups of 10 male and 10 female rats (strain: Crl:CD®[SD]BR VAF/plus; source: [redacted]) received single doses of 0 (vehicle control), 80, 400 and 2000 mg/kg bw triticonazole (batch no. 9550347; purity 97.2 %, dissolved in 0.5 % w/v methylcellulose in water) by oral gavage.

Animals were observed twice daily for mortalities and clinical signs; individual body weights were recorded on days 1, 8 and 15. All animals were subjected to a functional observational battery (FOB) and an assessment of locomotor activity in a circular open field enclosure for 40 minutes (cumulative counts of photobeam breaks in 10-minute intervals) prior and also at 2 hours, 7 and 14 days after treatment. The FOB included testing of various sensorimotor functions, reflexes, behavioural functions and also body temperature and comprised: (i) set of observations while the animals were in their home cages; (ii) set of observations when initially handling the animals; (iii) set of observations in an open test arena and (iv) set of observations during manipulation/specific testing. After 15 or 16 days post-treatment, macroscopic neuropathological examination (including measurement of length, width and height of each cerebrum and cerebellum) was performed on all animals. Histopathology of designated nervous tissues (7 sections of the brain; pituitary gland; 3 sections of the spinal cord; cervical, lumbar and trigeminal ganglion; optic, sciatic, tibial and sural nerv), eyes and skeletal muscle was performed, taken from 6 animals/sex in the control and high-dose groups, resp.

Results

Clinical signs and mortality: There were no significant toxicological findings noted at any dose level. All animals survived the scheduled sacrifice. In addition, body weights and weight gains were unaffected by treatment.

FOB/locomotor activity: The results obtained were similar across all groups, both sexes and across the testing intervals. Numerous variations were noted but considered of normal biological differences because they occurred in control animals as well as triticonazole-treated animals. Of the data evaluated statistically, significant decreases in the number of fecal boli were observed on day 1 for males receiving 2000 mg/kg and in the number of rears and body temperature, resp. on day 8 for males given 2000 mg/kg when compared with controls. These significant differences, however, were not considered to be toxicologically relevant because the statistical differences were either consequences of the variation noted for the control animals or the mean values of the males in these groups were similar to the mean values observed for the same animals predose.

Pathology: There were no macroscopic or microscopic findings related to the administration of triticonazole observed at any dose level. The only statistical significant finding was a smaller mean brain size for females given 400 and 2000 mg/kg, but the differences from control females were negligible (<1.5 %), were not found in males, and were not associated with any other pathological abnormalities or behavioural abnormalities. Therefore, this finding was not considered biologically relevant or related to triticonazole.

Conclusion

Based on the results of the study, the NOAEL for acute oral neurotoxicity of triticonazole to rats is greater than 2000 mg/kg bw.

B.6.7.1.3. Repeated neurotoxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Triticonazole – 13 week dietary neurotoxicity study in rats
Author(s), year:	[redacted] 1997
Report/Doc. number::	R012967 / -

Guideline(s):	US EPA guideline 82-7, OECD 424
GLP:	Yes
Deviations from OECD Guideline 424 (1997)	-functional observational battery (FOB) and assessment of locomotor activity in a circular open field were not measured in the first or second week of treatment
Acceptability:	Yes

Material and methods

Four groups of 10 rats/sex/dose (strain: Crl:CD®[SD]BR VAF/plus; source: [redacted]) received triticonazole (batch no. 9550347; purity 97.2 %) with their diet at dose levels of 0, 500, 2500 and 10000 ppm for 13 weeks (equivalent to mean values of 32.5, 169.9 and 695.1 mg/kg bw per day for males and 38.5, 199.4 and 820.3 mg/kg bw per day for females).

Throughout the study, clinical signs, body weight and food consumption were recorded. Neurotoxicological examinations included a functional observational battery (FOB) and an assessment of locomotor activity in a circular open field enclosure prior and during the 4th, 8th and 13th week of treatment. The functional observational battery comprised of the same 4 sets of observations as described in the acute neurotoxicity study. At terminal necropsy after 13 weeks of treatment, a macroscopic examination was performed on all animals. Histopathology of designated nervous tissues (7 sections of the brain; pituitary gland; 3 sections of the spinal cord; cervical, lumbar and trigeminal ganglion; optic, sciatic, tibial and sural nerv), eyes and skeletal muscle was performed; the tissue was taken from 6 animals/sex in the control and high-dose groups, respectively.

Results

General observations: All animals survived to the scheduled sacrifice. There were no treatment-related clinical signs noted at any dose level. During the first 4 weeks of treatment, statistically significant lower mean body weight gains (79.4% of control) were observed among females receiving 10000 ppm. Considering the whole period (week 1 to 13), body weight gain was not statistically significantly lower either in males or in females of 10000 ppm group, and was about 90% of control values for both males and females. Body weight at the end of the study was not impaired at 10000 ppm (95% of control in males and 96% in females). No effects on body weight or body weight gain were observed at lower doses.

FOB/locomotor activity: The FOB results were similar across all groups, both sexes and at all testing intervals. Variations were observed during all test intervals in the resistance of animals when being removed from the cage, the activity in the open field, and the response of the animals to being touched with a penlight. However, these findings were attributed to normal biological variation because they occurred in control animals as well as in triticonazole-treated animals and the occurrence was unrelated to dose. Of the FOB parameter evaluated statistically, significant differences in the number of rears were noted at week 8 for males given 10000 ppm and at week 13 for females given 500 or 10000 ppm and in the hindlimb grip strength of females given 2500 ppm at week 8. These statistical differences were not considered treatment-related because they were not dose-related and they occurred at week 8 but not at the week 13 interval.

There were no significant differences in the locomotor activity of animals during the treatment with triticonazole.

Pathology: There were no significant differences in brain size measurements between control and treated animals. In addition, no macroscopic or microscopic findings related to the administration of triticonazole were observed at any dose level.

Conclusion

Based on the results of the study, the NOAEL for systemic toxicity is set at 2500 ppm (169.9 mg/kg bw per day in males and 199.4 mg/kg bw per day in females) based on reduction in body weight gain (90% of control animals) in both males and females at 10000 ppm. The NOAEL for neurotoxicity in rats given diets containing triticonazole is > 10000 ppm (695.1 mg/kg bw per day for males and 820.3 mg/kg bw per day for females).

B.6.7.2. Delayed polyneuropathy studies

No delayed polyneuropathy studies were performed and are not considered necessary.

B.6.8. OTHER TOXICOLOGICAL STUDIES

B.6.8.1. Metabolites

Triticonazole belongs to the class of triazole fungicides and is used almost exclusively as seed treatment for cereals. Treatment of these crops might consequently result in low residues in plant items destined for human food or animal feed. Additionally to seed treatment, triticonazole is sometimes used as fungicide in ornamentals. The spectrum of uses is very limited and there is not any indication this this will be changed in the future. Therefore, it is considered that current evaluation is very robust towards the variety of potential exposure situations and presents a reasonable worst case scenario that takes into account the complexity of the temporal and spatial changes that can occur with the residues. The residue definition for triticonazole is considered robust against future regulatory changes (e.g. extension of authorisations) covering all available residue data in terms of the full array of uses intended.

The estimated human exposure to triticonazole and metabolites in residues is very low. None of the metabolites identified in plant or animal items is expected in groundwater > 0.1 µg/l.

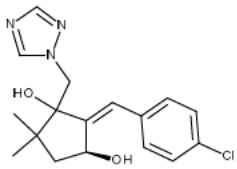
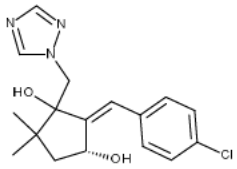
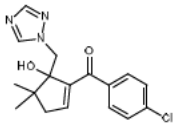
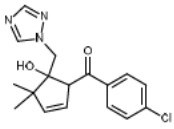
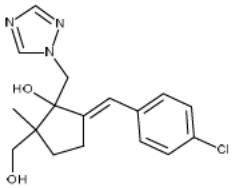
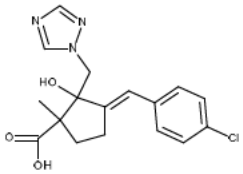
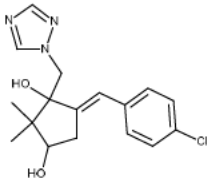
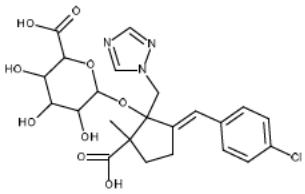
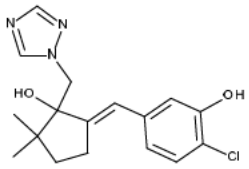
In 2016, EFSA published a guidance document for the establishment of the residue definition for dietary risk assessment (see EFSA Scientific Opinion Guidance on the establishment of the residue definition for dietary risk assessment (EFSA Journal 2016, 14(12): 4549)). Although the Guidance Document is not adopted or formally implemented at the time the DRAR on triticonazole is written, a metabolite assessment had been conducted following the guidance document.

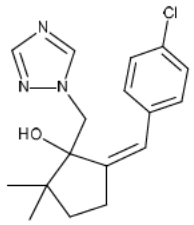
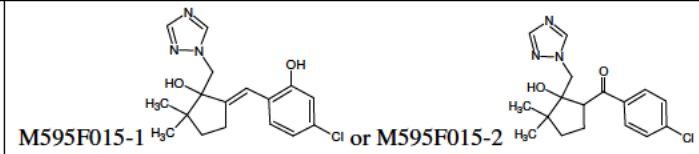
According to the results of the metabolism studies, triticonazole is metabolized in primary and rotational crops as well as in livestock. The main key transformation steps were similar for both plant and animal:

- Hydroxylation of the chlorobenzene ring
- Hydroxylation of the cyclopentane ring in various positions
- Glucuronidation
- Oxidation of hydroxyl groups
- Oxidation on the benzyl/styrene group

Table 6.8.1 -1: Metabolites of triticonazole found in metabolism studies

Metabolite code	Metabolite structure	Occurrence

Metabolite code	Metabolite structure	Occurrence
M595F001 (Reg. 5079285 RPA 404766)		Plants: Wheat (straw, hay), Barley (straw, grain) Rotational Crops: Wheat (straw)
M595F002 (Reg. 5059144 RPA 406341)		Plants: Wheat (straw), Barley (straw, grain) Rotational Crops: Wheat (straw)
M595F004*	 M595F004-1 or M595F004-2 	Plants: Wheat (straw, hay)
M595F005 (Reg. 5079247 RPA 404886)		Plants: Wheat (straw), Barley (straw, grain) Rotational Crops: Wheat (straw) Rat (feces; RLD): M: 15.14%dose F: 24.12 %dose
M595F006 (Reg. 5079450 RPA 406972)		Livestock: Goat (liver, kidney, muscle) Rat (urine, RLD): M: 2.18 % administered dose = 17.9 %absorbed dose (urine) F: 11.23 % administered dose = 55.9%absorbed dose (urine) Rat (feces): M: 34.16 %dose F: 21.59 %dose
M595F007 (Reg. 5079286 RPA 406780)		Plants: Wheat (straw), Barley (straw, grain)
M595F010		Livestock: Goat (liver)
M595F013 (Reg. 5079288 RPA 407922)		Plants: Wheat (straw, hay), Barley (straw)

Metabolite code	Metabolite structure	Occurrence
M595F014 (Z-isomer; Reg. 5079359 RPA 406203)		Plants: Barley (straw)
M595F015*	 M595F015-1 or M595F015-2	Plants: Wheat (straw)
[redacted] (technical impurity of TGAI)		Plants: Wheat (straw, hay) (considered to be an artefact)

*The structures of these residues have not been identified with certainty (MS structure elucidation only); thus separate QSAR assessments of all possible structures have been conducted, while each one exposure assessment had been done

Explanation for QSAR models used

For some of the metabolites studies are available, however, for most metabolites QSAR models have been applied to address genotoxicity.

In an initial assessment, a number of publicly available QSAR models were used. These included VEGA, OECD Toolbox and the commercial application OASIS Times, also implemented into the OECD Toolbox. A detailed description of the respective models is given below.

QSAR predictions are generally only considered predictive, if the training database is representative for the chemistry of interest. It was found that all predictions with the software tools mentioned above for triticonazole and its metabolites were outside of the applicability domain. The training databases of VEGA (or to be more precise, the implemented algorithms CAESAR, Sarpy for Ames mutagenicity) and OASIS Times are largely composed of chemicals and only contain limited data on pharmaceuticals and plant protection active ingredients. Plant protection metabolites are generally not included.

The training databases of each of the mentioned tools adequately cover the so called parametric domain. This means that they include functional groups or so called atom centered fragments that are also common in plant protection chemistry. However, functional group reactivity is always a function of the molecular environment, thus even functional groups that are highly reactive in a short chain alkene will not be reactive in an unsaturated long chain fatty acid or in a molecule stabilized via an extensive π-electron system. As a consequence, the parametric domain alone, or individual structural elements alone are not sufficient to indicate a mutagenic alert.

To overcome this lack of predictivity, BASF has acquired the commercial software package CASE Ultra. The model is described in more detail below. The QMRFs for each module used are provided as separate documents. CASE Ultra features one expert/rule based model for Ames mutagenicity and a number of statistical modules for Ames mutagenicity and in vivo MNT. BASF has extracted genotoxicity information from EFSA conclusions (period ~2006-2016) as well as BASF internal genotoxicity information of plant protection chemistry and used this information to train the respective statistical models.

This includes Ames mutagenicity data for 33 and in vitro/in vivo MNT data for 22 azoles and their metabolites. In addition, data on the classical triazole metabolites were included. Generally, azoles lack a genotoxic potential. From all azoles investigated in the EFSA conclusions only bromuconazole has been reported as mutagenic in some assays. However, this is likely due to the presence of bromide and not typical for azole structures.

A number of azole/imidazole fungicides are used as pharmaceuticals. Pharmaceutical testing usually includes extensive studies on mutagenicity, including Ames and in vivo micronucleus or adequate surrogate assays. Mutagenicity is generally not acceptable for pharmaceuticals, unless this is part of the intended treatment regimen, e.g. in chemotherapy. For fungicides, mutagenicity is definitely an exclusion parameter. Marketed azole/imidazole fungicides include voriconazole, econazole, fluconazole, genaconazole, itraconazole, ketoconazole, omoconazole, oxiconazole, posaconazole, saperconazole, sulconazole and terconazole. Each is negative for Ames mutagenicity and several negative in vivo MNT assay reports have been published. For the predictions for triticonazole and its metabolites this data has however not yet been implemented into the used Case Ultra models. An in-depth investigation, including review of the FDA or EMEA documentation would be useful to extend the respective databases. It should be noted however, that Ames mutagenicity data for pharmaceuticals, including a number of azole fungicides are part of the review database of the CASE Ultra Konsolidator for Ames and have been used to derive the expert rules in the CASE Ultra Expert system.

The lack of genotoxicity as seen for plant protection and pharmaceutical azole chemistry is in so far highly important, as predictions using OASIS Times and the rule based model Toxtree often report positive predictions based on one or the other structural alert: One commonly seen structural alert is that of hydroxylated phenols or of halogenated phenols. Virtually all azoles contain phenolic groups, usually halogenated/chlorinated in one or two positions. As outlined above for triticonazole, the most common metabolic reaction encountered is that of hydroxylation via one of the Cytochrome P450 isoenzymes which is followed by phase II conjugation. This common reaction is seen for multiple other azoles and often results in metabolites at urine/plasma/bile concentrations above 10%. Thus, it can be assumed that this is also a common reaction encountered after addition of S-9 mix in the classical in vitro genotoxicity tests of azoles or has been part of the in vivo micronucleus test. Therefore, it is apparent, that hydroxylation of the phenyl ring or the halogenated phenol ring does not increase the frequency of mutations or chromosome aberrations for azole fungicides. Consequently, predictions as encountered after simulated metabolic activation using OASIS Times or the respective algorithm in the OECD toolbox can be dismissed as false positive.

The following paragraphs give a brief introduction to the QSAR methods used in the evaluation of Triticonazole metabolites.

OASIS TIMES (v12.13)

OASIS TIMES is a hybrid statistical and knowledge-based model for toxicity prediction. The Tissue Metabolism Simulator (TIMES), developed by LMC (Bourgas University, Bulgaria; http://oasis-lmc.org/) integrates on the same platform a metabolic simulator and QSAR models for predicting toxicity of selected metabolites. The metabolic simulator generates plausible metabolic maps from a comprehensive library of biotransformations and abiotic reactions. It allows prioritization of chemicals according to toxicity of their metabolites. The reports for the evaluations have been provided in the dossier.

The reactivity model describing interactions of chemicals with DNA is based on an alerting group approach. Only those toxicophores extracted from the training set having clear interpretation for the molecular mechanism causing the ultimate effect included in the model. The mechanistic interrelation between alerts and related parametric ranges generalizing the effect of the rest of the molecules on the alert is also considered. The structural component of the model is based on the structural similarity between chemicals in the training set which were correctly predicted by the model. The structural neighborhood of atom-centered fragments is used to determine this similarity. The training set consists of 1514 chemicals for Ames and 808 chemicals for chromosomal aberration.

The derived model is combined with metabolic simulator TIMES used for predicting metabolic activation of chemicals with the S9 mix. The metabolic simulator is trained to reproduce documented maps for mammalian liver metabolism for 261 chemicals. Parent chemicals and each of the generated metabolites are submitted to a battery of models to screen for a general effect and mutagenicity mechanisms. Thus, chemicals are predicted to be mutagenic as parents only, parents and metabolites, and metabolites only. Mutagenicity could be due to the parent chemical only or as a result of its metabolic activation (i.e., the parent is inactive but it is transformed to a mutagenic metabolite), or both parent structure and metabolites could be mutagenic.

This OASIS QSAR system is also included in the OECD Toolbox (but not in combination with TIMES), in order to make use of (Q)SAR approaches also in the assessment of chemicals under REACH (OECD, 2008).

For the final evaluation of triticonazole metabolites, predictions using OASIS Times and its metabolism predictor were dismissed, as the predictions were out of domain. It should be noted however, that the models for Ames and in vitro CA without metabolic activation were always negative. The in vitro CA model with simulated S9 gave several positive alerts based on the structure of substituted phenols. This alert is clearly not valid for azole fungicides, as outlined above. Other alerts identified are also dismissed as they are not predictive for plant protection chemistry, as can be seen using the trained CASE Ultra models.

VEGA

Using the VEGA platform, access to a series of QSAR (quantitative structure-activity relationship) models for regulatory purposes was obtained. Of the models offered by VEGA [http://www.vega-qsar.eu/] only the two independent statistical prediction models for mutagenicity (Ames) were selected. The data obtained for triticonazole and its metabolite are not reported separately but are included in dossier. The first model is an implementation of CAESAR, which makes predictions based on the comparison of the structure of interest to the CAESAR database of mutagenicity data of substances in the structure database. A score is provided for the match of the structures, and the mutagenicity data of the closest related substances compared to the structure of interest. Consequently, if a structure is not adequately presented in the database, the prediction is only of very limited validity. It is important to note, that although the chemical space of any moiety of azole fungicides is covered no significant match to triticonazole was identified.

The second algorithm SarPy searches for isolated structural alerts of substructures in the molecule. Again, this is based on the mutagenicity data provided in the structure database.

It should be noted, that the QPRF reports generated by VEGA are not transparent enough, to derive the real rationale for predictions. In many cases a structural alert is shared by multiple compounds in the database, but only a limited number are provided in the report. Those are most often not of similar structure.

Predictions using VEGA were generally out of domain (low to moderate reliability) and therefore disregarded for the final evaluation. Nonetheless it should be noted, that all predictions using VEGA were negative.

CASE Ultra

CASE Ultra (Version 1.6.0.0) is a combination of a number of statistical models to predict Ames mutagenicity or the potential to form micronuclei (in vivo MNT).

QSAR models used were:

- GT1_A7B for Bacterial mutagenicity in diverse Salmonella substrains (TA97, TA 98, TA100, TA1535, TA1536, TA1537, TA1538)
- GT1_AT_ECOLI (featuring data from Salmonella TA102 and E.coli)
- GT3_MNT_Mouse
-

The QMRFs for the models included in the commercial package are included in the dossier.

Each statistical model works as multilinear regression QSAR. A set of multilinear regressions are derived with a stepwise increment method, one set per structural alert. Structural alerts are derived by fragmentation of the training dataset with an initial pool of ~1000 descriptors. Fragments are counted and compared to the number of substances in the training database containing this fragment and their respective activity in the test organism. Additional structural alerts as well as positive and negative modulators are taken into consideration.

In addition, CASE Ultra also features a konsolidator which combines test results and compares the test chemical and alerts with the test results of 11461 chemicals with experimental data. The consolidator also allows adding experimental data to the evaluation. Thus, experimental data available for parent and tested metabolites/impurities are taken into consideration. The konsolidator is only available for Ames mutagenicity.

BASF has further trained the above-mentioned models using genotoxicity information for plant protection chemistry as derived from EFSA conclusions over a time-period spanning 2006 to 2016. Thereby, the respective training dataset for the statistical models have been expanded.

Prediction model, number of substances underlying standard version and number of substances in trained version:

- GT1_A7B for Bacterial mutagenicity 3979 → 4624
- GT1_AT_ECOLI 1199 → 1861
- GT3_MNT_Mouse 610 → 1106

Statistical features of the original models were retained. Models were validated using three different methods, as available under default settings for CASE Ultra:

- Leave N%Out Cross Validation
- Bootstrap
- Y Scrambling

For Ames mutagenicity, CASE Ultra also features an expert, rule based model. This module is not trainable. The model is described in:

Alexander Sedykh, Suman Chakravarti, and Roustem Saiakhov. MultiCASE rule-based expert system for mutagenicity prediction: creating and validating Genetic Toxicity Association Annual Meeting, 2015, Delaware. Poster presentation.

The model is based on structural fragments probability for each structural alert. The probability for each alert is built on the mutagenicity outcomes for an Ames mutagenicity test for 10 777 compounds. The model consists of 175 structural alerts, of which 40 represent general mechanisms and the remainder accompanies them as refining factors. Expert alerts were collected from the public literature, refined and benchmarked on a reference set of over 11.000 chemicals with known mutagenicity outcomes.

It should be noted that this reference dataset consists of chemicals, pharmaceuticals and partially plant protection active ingredients. However, it does generally not contain data on metabolites or conjugates. Glutathione conjugates or glucuronides are generally missing. Sulfuric acid is present as a moiety or functional group, but not as part of phase II conjugates. Sugar conjugates, typically seen in plant metabolism are also clearly under represented.

Based on the rationale provided in the paragraphs above, only results form CASE Ultra for Ames and CASE Ultra and Toxtree for the in vivo MNT are considered relevant for the assessment of triticonazole metabolites.

Table 6.8.1 -2: Assessment of genotoxicity following EFSA guidance on residue definition Module 1: (steps 1-9)

Parent/ Metabolite		Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
	Assignment to Groups	creation of an inventory	compounds of no toxicological concern	classified as genotoxic	genotoxicity characterised	QSAR assessments revised[1]	Read-across analysis[3]	Exposure calculation (against 0.0000025 mg/kg bw)
BAS 595 F			Parent	No	Yes	Ames: Negative CA: Negative		
M595F001	1	yes	No	No	No	Ames: Negative	Not genotoxic (same structural alert as parent)	n.c.
M595F002	1	yes	No	No	Partly (Ames negative)	CA: Negative		n.c.
M595F004-1	single	yes	No	No	No	Ames: Negative CA: Negative	n.c.	No exposure estimation against genotox TTC done since the metabolite(s) detected only in feed items (straw) and not in animal tissues.
M595F004-2			No	No	No	Ames: Negative CA: Positive SA 10^2 in Toxtree, CASE Ultra negative	n.c.	
M595F005	2	yes	No	No	No	Ames: Negative CA: Negative	Not genotoxic (same alert as for parent; structural similarity to M595F006	n.c.
M595F006	2	yes	No	No	Yes (Covered by parent)	Ames: Negative CA: Negative	n.n.	n.c.
M595F007	single	yes	No	No	No	Ames: Negative CA: Negative	Not genotoxic (same alert as for parent)	n.c.
M595F010	2	yes	No	No	Yes (glucuronide of M595F006)	Ames: Negative CA: Negative	n.n	n.c.
M595F013	single	yes	No	No	No	Ames: Negative CA: Negative	Not genotoxic (same structural alert as parent)	n.c.
M595F014	single	yes	No	No	Yes (negative In vitro MNT and Ames)	Ames: Negative CA: Negative	n.n.	n.c.
M595F015-1	single	yes	No	No	No	Ames: Negative CA: Negative	Not genotoxic (same alert as for parent)	n.c.
M595F015-2			No	No	No	Ames: Negative CA: Negative	Not genotoxic (same structural alert as parent)	
[redacted]	1	yes	No	No	Partly (Ames negative)	Ames: Negative CA: Negative	Not genotoxic (same structural alert as parent)	n.c.
							Sum:	n.c, not necessary

1 QSAR analysis was revised, because the initial QSAR calculation performed with VEGA and OASIS Time were all out of the applicability domain. Revised calculations were performed with CASE Ultra and for in vivo chromosome aberration with Toxtree. For Toxtree the structural alert SA 34 H-acceptor path; 3 H acceptor is disregarded based on the low predictivity
2 Toxtree prediction SA 10: alpha, beta unsaturated carbonyls. This alert is largely valid for small molecules only. It is common in plant protection active ingredients and their metabolites and consequently not associated in the statistical prediction model for in vivo micronucleus prediction of CASE Ultra.
3 Profiling using the OECD Toolbox profilers were initially done to comply with the guidance document. Due to in domain negative QSAR predictions this step can be omitted and is only listed for completeness.
n.n = not necessary; n.c = not conducted

Since no testing for genotoxicity on group representatives is considered necessary, steps 8-9 of the EFSA guidance can be omitted.

Table 6.8.1 -3: Assessment of general toxicity and derivation of residue definition following EFSA guidance Module 2: (steps 10-19)

Metabolite	Step 10	Step 11	Step 12-19
	General tox characterised	Exposure [%] (against TTC CCIII = 1.5 µg/kg bw/day (chronic) or 5.1 µg/kg bw/d (acute)) for ALL cereals, not only wheat	Decision on residue definition
M595F001	No	n.c	-
M595F002	No	n.c	-
M595F004	No	n.c	-
M595F005	No	n.c	-
M595F006	Yes	n.c	-
M595F007	No	n.c	-
M595F010	Yes	n.c	-
M595F013	No	n.c	-
M595F014	No	n.c	-
M595F015	No	n.c	-
[redacted]	No	n.c	-
SUM of all metabolites in Plant and Livestock		**Chronic:** Max 0.4% (WHO Cluster diet B. wheat) **Acute:** *Children*: 0.5% Bovine: Liver 0.1% Wheat 0.1% Bovine: Kidney 0.1% Maize 0.1% Rye *Adults:* 0.2% Bovine: Liver	n.n

		0.1% Sheep: Liver 0.1% Wheat 0.1% Barley 0.1% Bovine: Kidney	

n.c = no calculation for single metabolites conducted since the sum of all exposure estimates (plant and livestock) is far below the TTC values for acute and general toxicity

n.n = not necessary; neither chronic nor acute TTC for general toxicity exceeded, therefore stopped at Step 11 and no further need for steps 12-19 (according to Guidance document.)

B.6.8.1.1. M595F001 (Reg. 5079285, RPA 404766)

M595F001 is enantiomer of M595F002. M595F001was detected and identified by LC/MS analysis in rat urine at levels below the sensitivity of the radioactivity detector used for quantification of metabolites. No studies are available with M595F001 itself (but with the enantiomer).

For metabolite M595F001, the structure is close to the structure of triticonazole parent, only hydroxylation occurs.

For M595F002 a negative Ames assay is available. In addition, QSAR analysis was conducted. There was no alert identified for Ames mutagenity with any of the applied models. For Chromosome aberration in vitro using OASIS Times a positive out of domain prediction was identified based on two structural alerts: 1) substituted phenols – interaction with topoisomerase and 2) alpha-beta-unsaturated carbonyls and related compounds. The prediction is out of domain and identified alerts do not fit to the experimental database available for Triticonazole and other azoles.

Evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

For consumer risk assessment, it can be assumed that M595F001 has no genotoxic concern.

Table 6.8.1.1-1: Summary table for prediction of Ames mutagenicity for M595F001 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F001	Enantiomer of M595F002, which is negative	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.1-2: Summary table for prediction of DNA damage for M595F001

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F001	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.2. M595F002 (Reg. 5059144, RPA 406341)

M595F002 is enantiomer of M595F001.

- RPA 406341 was detected and identified by LC/MS analysis in rat urine at levels below the sensitivity of the radioactivity
- Acute oral LD50 value > 2000 mg/kg bw (evaluated already in DAR (2003)
- negative AMES test (evaluated already in DAR (2003)

For metabolite M595F002, the structure is close to the structure of triticonazole parent, only hydroxylation occurs.

For M595F002 a negative Ames assay is available. In addition, QSAR analysis was conducted. There was no alert identified for Ames mutagenity with any of the applied models. For Chromosome aberration in vitro using OASIS Times a positive out of domain prediction was identified based on two structural alerts: 1) substituted phenols – interaction with topoisomerase and 2) alpha-beta-unsaturated carbonyls and related compounds. The prediction is out of domain and identified alerts do not fit to the experimental database available for Triticonazole and other azoles.

Evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

For consumer risk assessment, it can be assumed that M595F002 has no genotoxic concern.

Table 6.8.1.2-1: Summary table for prediction of Ames mutagenicity for M595F002 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F002	negative	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.2-2: Summary table for prediction of DNA damage for M595F002

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F002	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.2.1. Acute oral toxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	An acute oral toxicity study in rats with RPA 406341
Author(s), year:	[redacted] 1999
Report/Doc. number::	R000206 / -
Guideline(s):	OECD 401 (1987) and US EPA OPPTS guideline 870.1100
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Materials and methods

Groups of 5 rats/sex (strain: Sprague Dawley® SD®; source: [redacted].) weighing between 238 and 309 g received per gavage a single oral dose of 2000 mg/kg bw RPA 406341 (batch no BESS0599B, purity not given, suspended in cornoil 50% w/v). After administration all animals were kept under observation for 14 days. Clinical observations were made 3 times during the first day and at least daily thereafter. Body weights were recorded prior to dosing and on days 7 and 14 of the study. At termination all surviving rats were examined at necropsy for abnormalities. Necropsies were also performed on those animals that had died during the study.

Results

Clinical signs and mortality: The clinical abnormalities observed during the study included decreased activity, eyelids partially closed, reduced defecation, soft/mucoid faeces, rough haircoat, piloerection, dehydration, hunched posture and nasal discharge. These symptoms cleared by study day 4 in males and by study day 9 in females.
One female was found dead on day 8.
Body weight gain was normal for all surviving animals except in 4 of 5 females, which showed body weight loss during the first 7 days after treatment. From day 7 to 14 females again gained weight.
Pathology: In the animal that died, the only notable finding at necropsy was depletion of body fat. No significant gross internal findings were observed at necropsy on study day 14 for the remaining animals.

Conclusion:

Under the conditions of the study and based on the information given in the study report, oral LD_{50} of RPA 406341 in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute oral toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.1.2.2. Genotoxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	Mutagenicity test with RPA 406341 in the Salmonella – Escherichia coli/mammalian-microsome reverse mutation assay with a confirmatory assay
Author(s), year:	Mecchi M., 1999
Report/Doc. number::	R000208 / -
Guideline(s):	OECD 471 and 472 (1983)
GLP:	Yes
Deviations from OECD 471 (1997):	No
Acceptability:	Yes

Material and methods

The test article RPA 406341 (batch no. BESS0599B; purity stated to be 100 %) was tested in the Ames test using histidine dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA 1535, TA 1537, TA 98 and TA 100) and the tryptophan deficient strain WP2uvrA of *Eschericia coli*. The substance (dissolved in DMSO) was tested at concentrations of 0 (solvent control), 33.3, 100, 333, 1000, 3300 and 5000 µg/plate via the plate incorporation methode (without pre-incubation) both in the presence and absence of S-9 mix (liver preparations from Aroclor 1254-induced male Sprague-Dawley rats). The dose levels were established on the basis of a dose

range-finding study using tester strains TA 100 and WP2uvrA. In this pre-test, concentrations of up to 5000 µg/plate produced no cytotoxicity in either the presence or in absence of S-9 mix as evidenced by no decrease in the number of revertants per plate. In two independent experiments, three replicates per concentration were incubated at 37°C for 52 hours.

As positive controls benzopyrene (2.5 µg/plate, 2-nitrofluorene (1 µg/plate), 2-amino-anthracene (2.5 µg/plate), sodium azide (2 µg/plate), 4-nitroquinoline-N-oxide (1 µg/plate) and "ICR-191" (2 µg/plate) were used.

Evaluation criteria: The test was considered positive if an at least a 2-fold increase in the mean revertants per plate of at least one of the tester strains over the mean revertants per plate of the appropriate vehicle control was produced. This increase had to be accompanied by a dose response to increasing concentrations of the test substance.

Results

In both experiments, no statistically significant positive increases in the mean number of revertants per plate were observed following treatment with RPA 406341 with any of the tester strains at any concentration, neither in the presence nor in the absence of S-9 mix. Also the mean number of revertant colonies on negative control plates were within acceptable ranges while the the positive control materials elicited the expected positive responses.

Conclusion

It can be concluded that RPA 406341 did not induce reverse gene mutations in any *Salmonella typhimurium* and *Escherichia coli* strains when tested at dose levels up to 5000 µg/plate in this test system.

B.6.8.1.3. M595F004-1 and M595F004-2

The structures of these residues have not been identified with certainty (MS structure elucidation only).

The metabolite M595F004 has not been tested for genotoxicity.

No specific QSAR assessments had been conducted for the two possible structures underlying M595F004, however a positive alert with regard to α, ß-unsaturated carbonyl group can be assumed.

Re-evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) for in vivo MNT gives a clear in domain negative prediction (see table below). Toxtree identified a positive alert SA10: α, ß-unsaturated carbonyl group. As outlined above, the alert is frequently observed for pesticides and larger molecules like unsaturated fatty acids, which are not mutagenic and do not have chromosome damaging properties. This alert is largely valid for short molecules where the double bond is not stabilized by mesomeric interactions. The in vivo MNT model in CASE Ultra trained with plant protection chemistry does not indicate that this alert is valid for plant protection active ingredients in general and not relevant for triticonazole in particular. Furthermore, the molecule is mesomeric stabilized, albeit the molecular enthalpy indicates the M595F004-2 form is energetically preferred. Therefore, some doubts remain, and it cannot be excluded with certainty, that M595F004-2 is mutagenic.

Table 6.8.1.3-1: Summary table for prediction of Ames mutagenicity for M595F004 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F004-1	-	Negative	Negative	Negative	Negative	Negative	Negative
M595F004-2	-	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.3-2: Summary table for prediction of DNA damage for M595F004

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F004-1	**Negative**[3]	**Negative**[3]
M595F004-2	**Negative**[3]	Positive[4,5]

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

[3] Negative in domain predictions are depicted in **bold**

[4] Positive in domain predictions are depicted underscored

[5] Positive alert for Toxtree stems from the structural alert SA10, alpha, beta unsaturated carbonyls; this structural alert is not supported by the information extracted from the trained Case Ultra model; This structural alert is frequently observed in pesticides and larger molecules like unsaturated fatty acids which are not mutagenic and do not have chromosome damaging properties. This alert is largely valid for short molecules where the double bound is not stabilized by mesomeric effects. This is indirectly confirmed by the negative prediction in the Ames Expert module.

Genotoxicity of either M595F004-1 or M595F004-2 cannot be excluded, thus an assessment was done with the 0.0000025 mg/kg bw per day value in the Module 1. As Toxtree did not find this alert in other metabolites risk assessment was restricted to this metabolite only and an individual risk assessment against the genotoxicity TTC threshold was conducted. Even if M595F004 is considered genotoxic, only minor % of genotoxicity TTC (0.0000025 mg/kg bw per day) is utilised, for both acute and chronic exposure (please see table 6.8.1-2).

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.4. M595F005 (Reg. 5079247, RPA 404886)

The metabolite M595F005 is precursor of metabolite M595F006 which is considered non-genotoxic based on occurrence in rat metabolism study. M595F005 is a hydroxylation of triticonazole. It was measured only in rat faeces (15% in males and 24% in females after repeated low dose), but not in urine. Assuming high bioavailability via bile, there is nevertheless some indication, that M595F005 is probably also covered via triticonazole testing.

No studies with M595F005 were conducted.

QSAR results of M595F005 gave the same structural alerts as identified for parent. M595F005 can be grouped with M595F006 and M595F010, thus the absence of genotoxicity can be assumed via coverage of M595F006 and

M595F010 in rat metabolism study with triticonazole. A separate QSAR evaluation gave no indication of a mutagenic alert. Thus, metabolite M595F005 is considered to be not genotoxic.

Table 6.8.1.4-1: Summary table for prediction of Ames mutagenicity for M595F005 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F005	-	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.4-2: Summary table for prediction of DNA damage for M595F005

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F005	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.5. M595F006 (Reg. 5079450, RPA 406972)

For metabolite M595F006, the structure is close to the structure of triticonazole parent.

M595F006 has been identified in the rat metabolism study. M595F005 is a hydroxylation of triticonazole, while M595F006 is the further oxidation step. M595F006 metabolite has been determined in males up to 2.18% of the dose in urine after repeated low dose administration (RLD), which corresponds to an amount of 17.9% absorbed dose and in females up to 11.23% dose, corresponding to a level of 55.9% absorbed dose. The differences between %dose and %absorbed dose levels can be explained by an overall low radioactive recovery in urine in the repeated low dose group (12.18% in males and 20.08% in females). Radioactive recovery in bile is much higher, as shown in the bile study; overall triticonazole is considered to be entirely bioavailable. Relative high %dose values of metabolite M595M006 in fecal extracts of the repeated low dose group (34.16 and 21.59% in males and females) indicate further, that the toxicity of this metabolite is covered by toxicological testing of triticonazole.

No QSAR alert was found for M595F006. However, the structure was out of domain using VEGA and OASIS TIMES.

Evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

Table 6.8.1.5-1: Summary table for prediction of Ames mutagenicity for M595F006 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F006	Covered by parent	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.5-2: Summary table for prediction of DNA damage for M595F006

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F006	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

M595F006 is considered to be sufficiently present in rat metabolism (> 10% absorbed dose) to conclude that its toxicity is covered by studies conducted with parent. In case that reference values are needed, those from parent triticonazole apply.

B.6.8.1.6. M595F007 (Reg. 5079286, RPA 406780)

For metabolite M595F007, the structure is close to the structure of triticonazole parent, only hydroxylation occurs. M595F007 was detected and identified by LC/MS analysis in rat urine at levels below the sensitivity of the radioactivity detector used for quantification of metabolites For M595F007 there was no alert identified for Ames mutagenity with any of the applied models. For Chromosome aberration in vitro using OASIS Times a positive out of domain prediction was identified based on a structural alert: 1. substituted phenols – interaction with topoisomerase. The prediction is out of domain and identified alerts do not fit to the experimental database available for Triticonazole and other azoles.

Evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

Table 6.8.1.6-1: Summary table for prediction of Ames mutagenicity for M595F007 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F007	-	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.6-2: Summary table for prediction of DNA damage for M595F007

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F007	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.7. M595F010

M595F010 is conjugated M595F006. As stated in the EFSA guidance for residue definition, "*Conjugated metabolites (glycosides, glucuronides) are assumed being covered in their toxicological properties by their respective aglycons*". Therefore, M595F010 is considered covered by the assessment of M595F006.

Evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

Table 6.8.1.7-1: Summary table for prediction of Ames mutagenicity for M595F010 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F010	Glucuronide of M595F006	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.7-2: Summary table for prediction of DNA damage for M595F010

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F010	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

M595F010 has not been measured in rat metabolism but is a conjugate of M595F006. M595F006 is considered to be sufficiently present in rat metabolism to conclude that its toxicity (and the toxicity of its conjugates) is covered by studies conducted with parent. In case that reference values are needed, those from parent triticonazole apply.

B.6.8.1.8. M595F013 (Reg. 5079288, RPA 407922)

For metabolite M595F013, the structure is close to the structure of triticonazole parent, only hydroxylation occurs on the chlorobenzene ring.

According to the QSAR analysis, no Ames mutagenicity was predicted in none of the analysis. Predictions using VEGA or OASIS Times were out of domain. In an initial assessment for chromosome aberration using OASIS Times, an alert for chromosome aberration with S9 had been predicted. The structural alerts were: 1. substituted phenols – interaction with topoisomerase; 2. hydroxylated phenols and 3. quinones and trihydroxybenzenes, hydroxylated phenols. Predictions were out of domain. As outlined in the introduction to the QSAR models, OASIS Times is not adequately trained on the chemistry of plant protection or in particular on the chemistry of triazole fungicides. Therefore, the predictions of OASIS Times are rejected.

Re-evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

Table 6.8.1.8-1: Summary table for prediction of Ames mutagenicity for M595F013 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F013	-	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.8-2: Summary table for prediction of DNA damage for M595F013

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F013	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.9. M595F014 (Z-isomer, Reg. 5079359, RPA 406203)

M595F014 is Z-isomer of triticonazole.

- Not identified in rat (photolysis metabolite)
- Acute oral LD50 value > 2000 mg/kg bw (evaluated already in DAR (2003)
- negative AMES test (evaluated already in DAR (2003)
- negative *in vitro* micronucleus test (submitted for the purpose of renewal)

A normal practice for cereal seed treatment as well as storage is the exclusion of light in order to avoid seed germination. Further, treated seeds are also stored in brown paper bags. However, to complete the genotoxicity package of Z-isomer of triticonazole, which can be formed under UV-light via treatment/handling of triticonazole-

treated seeds (exposed to sunlight), the notifier submitted a new *in vitro* micronucleus test for the purpose of renewal of triticonazole. Additionally, a non-GLP study has been conducted (details please see section Ecotoxicology, CP, Spangler et al., 2015) on conversion from triticonazole to Z-isomer after fresh treatment of seeds with triticonazole and UV light exposure in a period of 10 days. In the study was shown that ratio of triticonazole / Z-isomer is in the range of 99.0 / 1.0% (day 1) to 97.6 / 2.4 % (day 10) and therefore Z-isomer is considered as very minor.

Additionally, QSAR assessment has been submitted for purpose of renewal. Evaluation of the mutagenic potential using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

Table 6.8.1.9-1: Summary table for prediction of Ames mutagenicity for M595F014 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F014	negative	Negative	Negative	Known negative	Negative	Known negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.9-2: Summary table for prediction of DNA damage for M595F014

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F014	Known Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.9.1. Acute oral toxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	An acute oral toxicity study in rats with RPA 4062031
Author(s), year:	[redacted] 1998
Report/Doc. number::	R000127 / -
Guideline(s):	OECD 401 (1987) and USEPA (=EPA) 81-1
GLP:	Yes
Deviations:	No
Acceptability:	Yes

Material and Methods:

Groups of 5 fasted male and female Sprague Dawley CD rats (9 and 12-week old male and female, respectively) received a single oral administration of RPA 406203 by gavage at dose levels of 2000 mg/kg body weight. RPA 406203 was suspended in corn oil.

Animals were observed twice during the first day and at least daily thereafter for clinical signs and mortality until death or sacrifice on Day 14. Body weights were recorded prior to dosing, on the day of dosing and weekly thereafter.

At termination of the study, all surviving animals were autopsied and subjected to a macroscopic examination.

Results

No treatment-related deaths were observed throughout the study. Clinical signs included dark material around the facial area and faecal stain. All animals had recovered on Day 4 except one female which showed hair loss on Day 12. The body weight evolution was normal for all animals.

Gross examination revealed the presence of foci on the lungs of 4 out of 5 male rats. However, the toxicological significance of this finding is unclear since these foci have been observed in untreated animals in the same strain of rats in the performing laboratory.

Conclusion

Under the conditions of the study and based on the information given in the study report, oral LD_{50} of RPA 406203 in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute oral toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.1.9.2. Genotoxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment; tables with results added

Reference:	Salmonella - Escherichia coli / Mammalian-microsome reverse mutation assay with a confirmatory assay with RPA406203 - Amended final report
Author(s), year:	Lawlor T.E., 1999
Report/Doc. number::	R000129 / -
Guideline(s):	OECD 471 (1997) and USEPA (=EPA) 84.2
GLP:	Yes
Deviations from OECD 471 (1997):	No
Acceptability:	Yes

Material and Methods

RPA 406203 (batch N° OB0012, purity 99.8 %) was tested for its ability to induce mutation in 4 histidine dependent auxotrophic mutant strains TA98, TA100, TA1535 and TA1537 of *Salmonella typhimurium* and the tryptophan deficient strain WP2 uvrA of *Escherichia coli*. The study consisted of a cytotoxicity range-finding experiment conducted in TA100 and WP2 uvrA followed by two independent experiments, each conducted in the absence and presence of metabolic activation by an Aroclor 1254 induced rat liver post-mitochondrial fraction (S-9). Negative and appropriate positive controls were included in each experiment.

Results

Cytotoxicity range-finder experiment

RPA 406203 was dissolved in DMSO and tested from 6.67 to 5000 µg/plate in TA100 and WP2 uvrA.

Cytotoxicity evidenced by a thinning of the background lawn of non-revertant cells and a reduction in revertant colony numbers was observed in TA 100 tester strain at 1000 and 3330 µg/plate in the presence and absence of S9, respectively. Indications of cytotoxicity were noted in WP2 tester strain at 3330 µg/plate in the absence of S9 whereas no cytotoxicity was observed in the presence of S9.

Experiment 1 (standard plate incorporation)

Accordingly, RPA 406203 was tested at 10.0, 33.3, 100, 333, 1000 and 5000 µg/plate.

The mean numbers of revertant colonies on negative control plates were within acceptable ranges while the mean number of revertant colonies in positive control plates were significantly increased.

RPA 406203 treatments produced no statistically significant increases in the mean numbers of revertant colonies in any tested strains both in the absence and presence of S-9.

Table 6.8.1.9.2-1: Number of revertant colonies in Salmonella typhimurium strains following treatment with RPA 406203 - Experiment 1

Without metabolic activation					
Treatment and concentration (µg/plate)	**Mean revertants per plate (± SD)**				
	TA 98	**TA 100**	**TA 1535**	**TA 1537**	**WP2uvrA**
Solvent control	13 ± 1	76 ± 6	8 ± 4	3 ± 1	8 ± 1
RPA 406203 10	15 ± 5	76 ± 8	9 ± 4	3 ± 1	13 ± 3
RPA 406203 33.3	17 ± 3	80 ± 6	10 ± 2	5 ± 4	13 ± 4
RPA 406203 100	16 ± 4	81 ± 10	11 ± 3	6 ± 2	8 ± 2
RPA 406203 330	17 ± 3	83 ± 6	6 ± 2	4 ± 1	11 ± 3
RPA 406203 1000 sp	14 ± 2	79 ± 11	5 ± 2	3 ± 2	10 ± 2
RPA 406203 5000 mp - *	4 ± 1	63 ± 10	6 ± 4	4 ± 2	9 ± 1
2-nitrofluorene 1	147 ± 25	-	-		
Sodium azide 2	-	735 ± 145	659 ± 1	-	-
ICR-191 2	-	-	-	902 ± 84	-
4-nitroquinoline-N-oxide 1	-	-	-	-	328 ± 47

With metabolic activation					
Treatment and concentration (µg/plate)	**Mean revertants per plate (± SD)**				
	TA 98	**TA 100**	**TA 1535**	**TA 1537**	**WP2uvrA**
Solvent control	19 ± 5	95 ± 2	13 ± 4	10 ± 1	14 ± 3
RPA 406203 10	25 ± 3	97 ± 10	10 ± 2	9 ± 2	11 ± 1
RPA 406203 33.3	25 ± 3	105 ± 10	12 ± 1	7 ± 1	14 ± 3
RPA 406203 100	17 ± 2	103 ± 6	12 ± 3	12 ± 4	10 ± 3
RPA 406203 330	22 ± 4	98 ± 7	10 ± 1	9 ± 3	15 ± 2

RPA 406203 1000	sp	20 ± 6	89 ± 7	18 ± 2	6 ± 2	11 ± 3
RPA 406203 5000	mp - *	21 ± 3	108 ± 2	9 ± 2	7 ± 4	18 ± 3
Benzo[a]pyrene 2.5		388 ± 20	-	-	-	-
2-aminoanthracene 2.5		-	622 ± 29	111 ± 8	181 ± 7	-
2-aminoanthracene 25		-	-	-	-	421 ± 27

SD: standard deviation; sp: slight precipitate; mp: moderate precipitate; * : presence of cytotoxicity

Experiment 2 (standard plate incorporation)

RPA 406203 was tested at the same final concentrations.

Similarly to Experiment 1, RPA 406203 treatments produced no statistically significant increases in the mean numbers of revertant colonies in any tested strains both in the absence and presence of S-9.

Table 6.8.1.9.2-2: Number of revertant colonies in Salmonella typhimurium strains following treatment with RPA 406203 - Experiment 2

Without metabolic activation						
Treatment and concentration (µg/plate)		**Mean revertants per plate (± SD)**				
		TA 98 *	**TA 100 ***	**TA 1535**	**TA 1537 ***	**WP2uvrA**
Solvent control		13 ± 5	69 ± 5	9 ± 6	3 ± 1	10 ± 5
RPA 406203 10		15 ± 4	73 ± 6	9 ± 2	6 ± 2	14 ± 1
RPA 406203 33.3		12 ± 3	67 ± 9	11 ± 2	5 ± 2	17 ± 1
RPA 406203 100		17 ± 4	71 ± 17	9 ± 2	5 ± 1	9 ± 4
RPA 406203 330		11 ± 2	83 ± 12	12 ± 4	4 ± 3	10 ± 4
RPA 406203 1000	sp	13 ± 3	63 ± 14	10 ± 0	6 ± 1	9 ± 2
RPA 406203 5000	mp - *	6 ± 4	53 ± 3	7 ± 4	1 ± 1	9 ± 4
2-nitrofluorene 1		196 ± 28	-	-		
Sodium azide 2		-	648 ± 24	594 ± 32	-	-
ICR-191 2		-	-	-	612 ± 70	-
4-nitroquinoline-N-oxide 1		-	-	-	-	374 ± 76

With metabolic activation						
Treatment and concentration (µg/plate)		**Mean revertants per plate (± SD)**				
		TA 98	**TA 100**	**TA 1535**	**TA 1537**	**WP2uvrA**
Solvent control		27 ± 4	97 ± 4	13 ± 3	7 ± 4	14 ± 8
RPA 406203 10		26 ± 7	80 ± 13	13 ± 3	9 ± 3	15 ± 7
RPA 406203 33.3		30 ± 5	85 ± 4	11 ± 2	6 ± 1	17 ± 2
RPA 406203 100		23 ± 4	105 ± 10	14 ± 1	11 ± 5	14 ± 3
RPA 406203 330		24 ± 9	86 ± 7	14 ± 6	7 ± 4	14 ± 7
RPA 406203 1000	sp	22 ± 6	81 ± 11	12 ± 1	6 ± 3	14 ± 5
RPA 406203 5000	mp - *	13 ± 2	75 ± 11	11± 5	5 ± 3	10 ± 4
Benzo[a]pyrene 2.5		447 ± 31	-	-	-	-
2-aminoanthracene 2.5		-	913 ± 66	122 ± 11	157 ± 6	-

2-aminoanthracene 25	-	-	-	-	282 ± 24

SD: standard deviation; sp: slight precipitate; mp: moderate precipitate; * : presence of cytotoxicity

Conclusion

Following 2 independent experiments that were performed in the absence and presence of a rat liver metabolic activation system, RPA 406203 did not induce reverse gene mutation in any *Salmonella typhimurium* and *Escherichia coli* strains when tested at dose levels up to 5000 µg/plate.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	Reg.No. 5079359 (Metabolite of BAS 595 F, Triticonazole) Z-isomer: Micronucleus Test In Human Lymphocytes In Vitro
Author(s), year:	Chang, S., 2016
Report/Doc. number::	-/2016/1039622
Guideline(s):	OECD 487 (2014)
GLP:	Yes
Deviations from OECD 487 (2014):	A series of in-house non-GLP validation experiments was performed to get distinct responses of statistical significance when using the specified positive controls. To achieve such response the test design, specifically for the treatment, the recovery phase and harvest time, was slightly modified comparing the current proposal given in the OECD Guideline 487 (harvest time point was approximately 2 – 2.5 x AGT (average generation time))..
Acceptability:	Yes

Material and methods

Test Material:	Reg.No. 5079359 (Metabolite of BAS 595 F, Triticonazole - Z-isomer)
Description:	solid / white
Lot/Batch #:	BESS0578
Purity/content:	99.9% (Tolerance +/- 1.0%)
Stability of test compound:	The stability of the test item under storage conditions (at +2°C to +8 °C) over the study period was guaranteed by the sponsor (expiry date April 01, 2024). The stability of the test item in DMSO over a period of 4 hours was verified.
Vehicle used:	DMSO
Control Materials:	
Negative:	No negative control was employed in this study.
Vehicle control:	DMSO (final concentration 1%)
Positive control:	Without metabolic activation: Mitomycin C (MMC, 1.5 µg/mL; pulse treatment) dissolved in deionized water; Demecolcin (100 ng/mL; continuous treatment) dissolved in deionized water With metabolic activation: Cyclophosphamide (CPA, 15 µg/mL for Experiment I.A and 12.5 µg/mL for Experiment II) dissolved in saline (0.9% NaCl)
Activation:	S9 was produced from the livers of rats pre-treated with β-naphthoflavone/phenobarbital. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature mixed with an appropriate

volume of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.
The S9-mix was prepared immediately before use and had the following composition:

Component	**Concentration**
Phosphate buffer (pH 7.4)	100 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM

The protein concentration of the S9 preparation used for this study was 27.2 mg/mL that resulted in final protein concentration of 0.75 mg/mL in the cultures.

Test system: Human peripheral blood lymphocytes from healthy non-smoking donors not receiving medication. Experiment I.A: 35 years old female donor
Experiment I.B: 29 years old female donor
Experiment II: 22 years old male donor

Culture media: Dulbecco´s Modified Eagles medium/Ham´s F12 (1:1) with GlutaMAX™ (200 mM) supplemented with 10% (v/v) fœtal bovine serum (FBS), Pen / Strep (100 U/mL / 100 µg/mL), HEPES (10 mM), heparin (125 U.S.P.-U/mL), phytohemeagglutinine (PHA, 3 µg/mL).

Test concentrations:

Micronucleus assay

Experiment I.A
(4-h exposure, + S9): 12.99, 22.74, **39.79, 69.63, 121.9**, 213.2, 373.2, 653.1, 1142.9, 2000 µg/mL (evaluated concentrations are indicated in bold)

Experiment I.B
(4-h exposure, - S9): 12.99, 22.74, **39.79, 69.63, 121.9**, 213.2, 373.2, 653.1, 1142.9, 2000 µg/mL (evaluated concentrations are indicated in bold)

Experiment II
(4-h exposure, +S9): 12.99, 22.74, **39.79, 69.63, 121.9**, 213.2, 373.2, 653.1, 1142.9, 2000 µg/mL (evaluated concentrations are indicated in bold)

(20-h exposure, -S9): 12.99, 22.74, 39.79, 69.63, 121.9, **213.2, 373.2, 653.1**, 1142.9, 2000 µg/mL (evaluated concentrations are indicated in bold)

Culture conditions:

Blood cultures were established by preparing an 11 % mixture of whole blood in culture medium within 30 hrs after blood collection. Human lymphocytes were stimulated for proliferation by the addition of the mitogen PHA to the culture medium for a period of 48 hours. All incubations were done at 37 °C with 5.5 % CO_2 in humidified air.

Dose selection:

Dose selection was performed according to the current OECD Guideline for the *in vitro* micronucleus test. The highest test item concentration should be 2000 µg/mL, 2 µL/mL or 10 mM, whichever is the lowest. At least three test item concentrations should be evaluated for cytogenetic damage.

2000 µg/mL was applied as top concentration for treatment of the cultures. Test item concentrations ranging from 12.99 to 2000.0 µg/mL (with and without S9 mix) were chosen for the evaluation of cytotoxicity. Cytotoxicity was characterized by the percentages of reduction in the CBPI in comparison with the controls (% cytostasis) by counting 500 cells per culture in duplicate.
Precipitation of the test item was observed at the end of treatment at 121.9 µg/mL and above in the absence and presence of S9 mix. No cytotoxicity was observed in Experiment IA and IB up to the highest applied concentration. Therefore, 2000 µg/mL (with and without S9 mix) were chosen as top concentration in Experiment II.

Micronucleus test:

Pulse exposure:

About 48 hrs after seeding 2 blood cultures (10 mL each) were set up in parallel in 25 cm² cell culture flasks for each test item concentration. The culture medium was replaced with serum-free medium containing the test item. For the treatment with metabolic activation the culture medium was supplemented with approx. 2.5 % S9 fraction (50 µL S9 mix/mL culture medium). After 4 hrs the cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were resuspended in and washed with "saline G" (pH 7.2, containing 8000 mg/L NaCl, 400 mg/L KCl, 1100 mg/L glucose • H_2O, 192 mg/L Na_2HPO_4 • 2 H_2O and 150 mg/L KH_2PO_4). The washing procedure was repeated once as described. The cells were resuspended in complete culture medium with 10 % FBS (v/v) and cultured for a 16-hour recovery period. After this period Cytochalasin B (4 µg/mL) was added and the cells were cultured another approximately 20 hours until preparation.

Continuous exposure

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

Preparation of cells

The cultures were harvested by centrifugation 40 hrs after beginning of treatment. The cells were spun down by gentle centrifugation for 5 minutes. The supernatant was discarded and the cells were re-suspended in approximately 5 mL "saline G" and spun down once again by centrifugation for 5 minutes. Then the cells were resuspended in 5 mL KCl solution (0.0375 M) and incubated at 37 °C for 20 minutes. 1 mL of ice-cold fixative mixture of methanol and glacial acetic acid (19 parts plus 1 part, respectively) was added to the hypotonic solution and the cells were resuspended carefully. After removal of the solution by centrifugation the cells were resuspended for 2 x 20 minutes in fixative and kept cold. The slides were prepared by dropping the cell suspension in fresh fixative onto a clean microscope slide. The cells were stained with Giemsa.

Statistics:

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

Cytotoxicity evaluation:

Evaluation of cytotoxicity and cytogenetic damage

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

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

CBPI Cytokinesis-block proliferation index

n Total number of cells

MONC Mononucleate cells

BINC Binucleate cells

MUNC Multinucleate cells

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

T Test item

C Solvent control

Evaluation criteria:

Acceptability criteria:

- The rate of micronuclei in the solvent controls falls within the historical laboratory control data range.
- The rate of micronuclei in the positive controls is statistically significant increased.
- The quality of the slides must allow the evaluation of a sufficient number of analysable cells.

Evaluation criteria:

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

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

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

- the number of micronucleated cells is not in the range of the historical laboratory control data and

- either a concentration-related increase in three test groups or a statistically significant increase in the number of micronucleated cells is observed.

If the above mentioned criteria for the test item are not clearly met, the test item will be classified as equivocal or a confirmatory experiment may be performed. However, results may remain questionable regardless of the number of times the experiment is repeated.

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

Results

The stability of test item preparations in DMSO over a period of 4 hours at room temperature was demonstrated analytically.

Precipitation of the test item in the culture medium was observed at 121.9 µg/mL and above in Experiment IA and II in the presence of S9 mix and in Experiment IB in the absence of S9 mix. In Experiment II in the absence of S9 mix precipitation was observed at 653.1 µg/mL and above at the end of treatment.

No relevant influence on osmolarity or pH was observed. The osmolarity is generally high compared to the physiological level of approximately 300 mOsm. This effect however, is based on a final concentration of 1% DMSO in medium. As the osmolarity is measured by freezing point reduction, 1% of DMSO has a substantial impact on the determination of osmolarity.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest evaluated concentration, which showed test item precipitation.

Micronucleus assay

In each experimental group two parallel cultures were analysed. 1000 binucleate cells per culture were evaluated for cytogenetic damage on coded slides.

In the absence and presence of S9 mix, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed after treatment with the test item.

In both experiments, either Demecolcin (100 ng/mL), MMC (1.5 µg/mL) or CPA (12.5 or 15.0 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei that were within the range of the respective historical control data. The solvent control data were within the range of the historical negative control data.

Table 6.8.1.9.2-3: Summary of results of the in vitro micronucleus test in human lymphocytes with Reg.No. 5079359 (Metabolite of BAS 595 F, Triticonazole - Z-isomer)

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %[a]	Micronucleated cells in %[b]
Exposure period 4 hrs without S9 mix					
I.B	40 hrs	Solvent control[1]	2.11		0.40
		Positive control[2]	1.59	46.5	**11.35***

Table 6.8.1.9.2-3: Summary of results of the in vitro micronucleus test in human lymphocytes with Reg.No. 5079359 (Metabolite of BAS 595 F, Triticonazole - Z-isomer)

Exp.	Preparation interval	Test item concentration in µg/mL	Proliferation index CBPI	Cytostasis in %[a]	Micronucleated cells in %[b]
		39.79	2.02	7.6	0.35
		69.63	1.99	10.5	0.50
		121.9[P]	1.89	19.5	0.30
Exposure period 20 hrs without S9 mix					
II	40 hrs	Solvent control[1]	1.83		0.15
		Positive control[3]	1.57	31.7	**2.75**[*]
		213.2	1.54	35.5	0.10
		373.2	1.73	12.1	0.05
		653.1[P]	1.75	10.1	0.25
Exposure period 4 hrs with S9 mix					
I.A	40 hrs	Solvent control[1#]	1.96		1.03
		Positive control[4]	1.38	60.7	**4.70**[*]
		39.79[#]	1.94	2.9	0.88
		69.63	1.98	n.c.	0.80
		121.9[P]	1.76	21.2	0.75
II	40 hrs	Solvent control[1#]	2.08		0.10
		Positive control[5]	1.61	43.8	**2.25**[*]
		39.79	2.01	6.3	0.20
		69.63	1.97	9.6	0.15
		121.9[P]	1.96	10.8	0.10

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

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

* $p<0.05$ (Chi-Square test)

[#] The number of micronucleated cells was determined in a sample of 4000 binucleated cells

[P] Precipitation occurred microscopically at the end of treatment

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

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

[1] DMSO 1.0 % (v/v)

[2] MMC 1.5 µg/mL

[3] Demecolcin 100.0 ng/mL

[4] CPA 15.0 µg/mL

[5] CPA 12.5 µg/mL

Conclusion

Under the experimental conditions reported, RPA 406203 did not induce micronuclei *in vitro* micronucleus test in human lymphocytes and is considered to be non-mutagenic when tested up to the precipitating concentration.

B.6.8.1.10. M595F015-1 and M595F015-2

The structures of these residues have not been identified with certainty (MS structure elucidation only).

M595F015-01 (one of the possible structures underlying the peak of M595F015), the same structural alerts had been identified than for parent, it can thus be concluded that M595F015-1 is not genotoxic. For M595F015-2 there was no alert identified for Ames mutagenicity with any of the applied models. For chromosome aberration in vitro using OASIS Times a positive out of domain prediction was identified based on three structural alerts. The structural alerts were: 1. substituted phenols – interaction with topoisomerase; 2. hydroxylated phenols and 3. quinones and trihydroxybenzenes, hydroxylated phenols. All predictions were out of domain and were disregarded for the forther evaluation.

Re-evaluation of the mutagenic potential of M595F015-01 and M595F015-02 using CASE Ultra for Ames (statistical and rule based models) and CASE Ultra (statistical) and Toxtree (rule based) for in vivo MNT gives a clear in domain negative prediction.

Table 6.8.1.10-1: Summary table for prediction of Ames mutagenicity for M595F015 using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
M595F015-1	-	Negative	Negative	Negative	Negative	Negative	Negative
M595F015-2	-	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.10-2: Summary table for prediction of DNA damage for M595F015

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
M595F015-1	Negative	Negative
M595F015-2	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.11. [redacted]

For [redacted] an Ames assay is available giving no indication for genotoxicity. Further, in a QSAR evaluation there were no structural alerts for genotoxicity obtained.

Table 6.8.1.11-1: Summary table for prediction of Ames mutagenicity for [redacted] using CASE Ultra models

Substance name	Experimental	Konsolidator Outcome [1]	GT1_A7B Salmonella[2]	GT1_ A7B Salmonella Trained [3]	GT1_Ecoli[2]	GT1_Ecoli Trained[3]	GT Expert[2]
[redacted]	negative	Negative	Negative	Negative	Negative	Negative	Negative

[1] Konsolidator model provided with Case Ultra; combines evaluation from the two basic statistical models GT1_A7B and GT1_E.coli with the results from the rule based model GT_Expert; this is than supplemented with a comparison to experimental data from a structure database of >11.000 chemicals

[2] Models as provided with CASE Ultra version 1.6.0.

[3] Models were trained with data extracted from EFSA conclusions (period 2006 to 2016) and further expanded by BASF proprietary data to enter the chemical space of plant protection products into the models

Table 6.8.1.11-2: Summary table for prediction of DNA damage for M595F015

Substance name	Case Ultra in vivo MNT prediction[1]	Toxtree[2]
[redacted]	Negative	Negative

[1] CASE Ultra predictions depicted are those performed with the model GTS_MNT_Trained_PPPs plus BASF, as this model is most representative for the chemical space of DMPT and its metabolites; the basic in vivo MNT model was not predictive for triticonazole and/or other azoles

[2] Toxtree Version 2.6; Structural alert SA 34: H-acceptor-path3-H-acceptor is disregarded as mentioned in the guidance document for plant residues; chemicals only displaying SA 34 are depicted as negative

No information on general toxicity is considered necessary. If general toxicity reference values of 1.5 µg/kg bw/d (chronic) and 5 µg/kg bw/d (acute) for Cramer Class III substances are taken into account, % exposure is near zero.

B.6.8.1.12 Triazole alanine and 1,2,4 triazole

Triazole-alanin was identified in grain and and 1,2,4 triazole in goat muscle and milk.

With regard to the triazole-derived metabolites, no toxicological assessment has been conducted as that would be beyond the scope of the triticonazole assessment and will be reconsidered based on the outcome of the on-going TDM assessment.

B.6.8.1.12. Metabolite fraction MET 6 (MWT 333)

RMS (fate and behaviour section) identified a metabolite fraction called “MET 6 (MWT 333)”, which might occur in groundwater < 0.2 µg/l. The potential metabolite(s) belonging to this fraction is/are not yet identified, but based on the molecular weight it is strongly assumed that it is a mono-hydroxylated parent triticonazole.

All currently identified mono-hydroxylated metabolites of triticonazole (M595F001, M595F002, M595F004, M595F007, M595F013), hydroxylated on different part of parent molecule and investigated based on their occurrence in residues of plant and animal origin, were devoid of any genotoxic concern (proven in an extensive QSAR evaluation; for details please see B.6.8.1). Although it is not *a priori* expected that unidentified fraction

"MET 6 (MWT 333)", assumed to be a mono-hydroxylated parent, is a fraction of genotoxic concern, this assumption cannot be currently substantiated by data (since no identification has been done yet).

B.6.8.2. Impurities

Toxicological information on starting material, reaction intermediates, theoretical impurities or any other molecules where the information on their names or structure is confidential are evaluated in Volume 4. In Volume B6 only toxicological studies are summerised.

B.6.8.2.1. [redacted] *(Reg.No.* [redacted]

- Acute oral LD50 value > 2000 mg/kg bw (evaluated already in DAR (2003)
- Acute dermal LD50 value > 2000 mg/kg bw (evaluated already in DAR (2003)
- 14 days study in rats: NOAEL = 100 mg/kg bw per day (evaluated already in DAR (2003)
- negative AMES test (evaluated already in DAR (2003)
- positive gene mutation MLA (new for renewal) with S9; based on proportion and absolute amount of small colonies indication of a clastogenic rather than a gene mutation effect
- negative TK6 gene mutation assay (new for renewal)
- negative micronucleus assay *in vitro* (new for renewal)
- QSAR analysis (new for renewal, included in Volume 4)

Based on the content of [redacted] in triticonazole and its toxicological profile, [redacted] is considered to be a non-relevant impurity. Detailed evaluation and conclusion according to Guidance document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) 1107/2009) (SANCO/10597/2003-rev.10.1, July 2012) is included in Volume 4.

B.6.8.2.1.1. Acute oral toxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	[redacted]: Acute oral limit test in rats
Author(s), year:	[redacted] 1993
Report/Doc. number::	R013083 / -
Guideline(s):	OECD 401 (1987)
GLP:	Yes
Deviations from OECD 401 (1987):	No
Acceptability	Yes

Material and methods

Five male and five female Sprague Dawley rats (source: [redacted]) weighing between 306 to 314 g for the males and 203 to 223 g for the females received a single oral dose of 2000 mg/kg bw [redacted] (batch no. TV3198C; purity 99.9 %; suspended in 0.5 % w/w methylcellulose in distilled water) by gavage. Observations were

made for 15 days following dosing, after which survivors were sacrificed and examined at necropsy for abnormalities. Body weights were recorded prior to dosing and weekly thereafter.

Results

There were no mortalities, and no clinical signs were observed during the study. The body weight gains were normal for all animals, and there were no abnormal necropsy findings at termination of the study.

Table 6.8.2.1.1-1: Mortality induced by [redacted] in Sprague Dawley rats after a single oral administration

	Male		Female	
Dose (mg/kg)	**Mortality**	**Time of death in days (number of rats)**	**Mortality**	**Time of death in days (number of rats)**
2000	0/5	-	0/5	-

Conclusion

Under the conditions of the study and based on the information given in the study report, oral LD_{50} of [redacted] in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute oral toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.2.1.2. Acute dermal toxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	[redacted] Acute dermal limit test in rats
Author(s), year:	[redacted] 1992
Report/Doc. number::	R013084 / -
Guideline(s):	OECD 402 (1987)
GLP:	Yes
Deviations from OECD 402 (1987):	No
Acceptability	Yes

Material and methods

Groups of five male and five female rats (strain: Sprague Dawley; source: [redacted]) weighing between 200 to 300 g received a single topical application of [redacted] (batch no. TV3198C; purity 99.9 %; moistened with 0.3 ml of 0.9% saline) at a dose level of 2000 mg/kg bw, which was placed on a gauze patch and applied on the shaven dorsal skin. The treated area (approx. 10 % of the body surface) was then secured with a bandage. After the 24-hour exposure period, the bandage and patch were removed and any residual test substance was eliminated by washing with water.

Animals were observed frequently during the first day and at least twice daily thereafter for clinical signs (systemic and local toxicity) and mortality until sacrifice on day 14. Body weights of the animals were recorded on the day before treatment and on days 1, 8 and 15. At termination, all animals were subjected to a macroscopic post mortem examination.

Results

There were no deaths related to treatment (one animal died due to incorrect application of the securing bandage). There were neither clinical signs of toxicity nor signs of local skin irritation considered to be related to treatment throughout the study period.

Table 6.8.2.1.2-1: Mortality induced by [redacted] in Sprague Dawley rats after a single dermal administration

	Male		Female	
Dose (mg/kg)	**Mortality**	**Time of death in days (number of rats)**	**Mortality**	**Time of death in days (number of rats)**
2000	0/5	-	0/5	-

At necropsy, the only finding noted was focally dilated sinusoids in one male rat; this finding was not confirmed histologically. Therefore, this change was considered to be incidental.

Conclusion

Under the conditions of the study and based on the information given in the study report, dermal LD_{50} of [redacted] in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute dermal toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.2.1.3. 14 days repeated dose rat study

Previous evaluation: DRAR (2016)	DAR (2003) NOAEL for [redacted] (impurity) was originally set in DAR 2003 at 10 mg/kg bw per day. However, since only finding at 100 mg/kg bw per day was the increase in relative liver weight (107% of control animals) this dose was considered to be NOAEL during th re-assessment of the study for DRAR (2016)

Reference:	RPA 400727/[redacted]: Fourteen day comparative oral toxicity study in the rat
Author(s), year:	[redacted] 1992
Report/Doc. number::	R013086/ -
Guideline(s):	OECD 407 (1981)
GLP:	Yes
Deviations from OECD 407 (2008):	Lower number of animals per group and sex (5), not all organs according to OECD 407 (2008) weighed and investigated
Acceptability	Yes

Material and methods

Triticonazole (batch no DA 646; purity 96.8 %) as well as [redacted] (batch no. HUT 689C; purity 99.3 %) was administered by gavage to groups, each of five male and five female Spargue Dawley CD rats (source: [redacted]) for 14 consecutive days at dose levels of 10, 100 and 1000 mg/kg bw per day. A control group of five males and five females were dosed with the vehicle alone (arachis oil, 4 ml/kg bw/d).

Animals were observed daily (i.e. prior to dosing and 1 and 5 hours after dosing) for clinical signs and mortality. Body weights were recorded twice weekly whereas food consumption was noted weekly. Haematology (haematocrit, haemoglobin concentration, RBC, MCHC, MCV, MCH, WBC total, WBC differential, platelet count, clotting time) and clinical chemistry analysis (AP, ALT, AST, urea, creatinine, glucose, bilirubin, total protein, albumin, globulin, calcium, phosphate, sodium, potassium, chloride) were performed for all animals at the end of the

study. At necropsy, each animal was subjected to a gross pathology examination and selected organs (adrenals, brain, heart, kidneys, liver, ovaries, pituitary, spleen, testes) were weighed. Microscopic examinations were performed on the following tissues from all high dose and control group animals: adrenals, heart, kidneys, liver, spleen, stomach and testes. Due to indications of treatment-related findings, microscopic examination of liver and stomach was extended to all animals.

Results:

General observations: There were no deaths during the study. Animals treated with 1000 mg/kg triticonazole showed sporadic signs of increased salivation immediately after dosing. No signs were detected in the remaining animals treated with triticonazole or in any of the dose groups treated with [redacted]

There was some evidence for a slight reduction in body weight gain among males in both high dose groups compared with controls. However it was stated in the report that these values were within the normal range for rats of this strain and age. At the end of the study, males treated with triticonazole at 1000 mg/kg bw per day had 93% body weight compared to control while males treated with [redacted] had 92% body weight compared to control males. Females treated with 1000 mg/kg bw per day had 104% body weight of control animals; those treated with [redacted] had 102% body weight of controls.

Haematology: In males treated with 1000 mg/kg triticonazole, a statistically significant reduction in mean corpuscular haemoglobin in comparison with controls (92.8%) was observed, but also these values were noted to be within the historical control range. Statistically significant reductions in haemoglobin and haematocrit values were observed in low and high dose females treated with [redacted], but there was no convincing dose-relationship. It was considered that neither test material caused adverse effects on haematological parameters.

Blood chemistry investigations indicated a significant reduction in plasma urea in males treated with 1000 mg/kg triticonazole, and in females treated with 100 and 1000 mg/kg [redacted] (with no dose-relation), but all of these values were noted to be within the historical control range for rats of this strain. It was concluded that neither test material caused adverse effects on clinical chemistry parameters.

At necropsy, high dose males treated with triticonazole showed thickening of the glandular gastric epithelium whilst females appeared to have thickened forestomachs. In the [redacted] treated groups, only one high dose female showed thickening of the forestomach. However, none of the affetced animals showed any associated histopathological changes in the stomach.

Organ weight analysis revealed increased relative liver weights in males treated with 1000 mg/kg triticonazole (112% of control), and also in males (120% of controls) and females (116% of controls) treated with 1000 mg/kg [redacted] There was also a dose-related significant increase in relative kidney weights in females treated with 100 (107 %) and 1000 mg/kg [redacted] (113 %); absolute organ weights were also elevated in the high dose females only. Although a dose relationship was apparent, there was no histopathological or biochemical evidence to support any adverse effect of treatment on kidneys. Other minor diferences in organ weights did not show any consistent dose-related trend and are considered to be coincidential.

Table 6.8.2.1.3-1: Liver weight changes, group mean values

	Dose group level (mg/kg/day) Male				Female			
	0	**10**	**100**	**1000**	**0**	**10**	**100**	**1000**
Triticonazole								
Absolute weight (g)	11.908	10.882	11.320	11.389	8.831	7.700	8.146	10.176
Relative weight (%)	4.559	4.213*	4.516	4.621	4.806	4.178**	4.386*	5.389**
(% control)								(112%)
[redacted]								
Absolute weight (g)	11.908	11.035	10.733	13.182	8.831	7.505	7.914	10.200
Relative weight (%)	4.559	4.410	4.273	5.467***	4.806	4.195*	4.344	5.574**
(% control)				(120%)				(116%)

Significantly different from control; * p<0.05; ** p< 0.01; *** p<0.001; F-max test for homogeneity of variance and Kruskal Wallis or Mann Whitney test thereafter

Microscopic examination revealed treatment-related hepatic and gastric changes: Minimal to slight hepatocyte vacuolation was observed in 3/5 females receiving 1000 mg/kg triticonazole and in 2/5 males and 3/5 females receiving 1000 mg/kg [redacted]. It was stated that the type of cytoplasmic vacuolation in hepatocytes observed is commonly seen in association with lipid accumulation. In addition, minimal acanthosis and hyperkeratosis were noted in the forestomach of 2/5 males treated with 1000 mg/kg [redacted].

Table 6.8.2.1.3-2: Group incidence of histopathological changes in the liver and the stomach

Organ	Dose group level (mg/kg/day) Male				Female			
Finding description	**0**	**10**	**100**	**1000**	**0**	**10**	**100**	**1000**
Triticonazole								
Liver (number examined)	5	5	5	5	5	5	5	5
Hepatocyte vacuolation	0	0	0	0	0	0	0	3
[redacted]								
Liver (number examined)	5	5	5	5	5	5	5	5
Hepatocyte vacuolation	0	0	0	2	0	0	0	3
Stomach (number examined)	5	5	5	5	0	0	0	0
Acanthosis	0	0	0	1	-	-	-	-
Hyperkerathosis	0	0	0	2	-	-	-	-

Conclusion:

Treatment with 1000 mg/kg bw per day [redacted] was associated with similar findings to those seen with 1000 mg/kg bw per day triticonazole (slightly reduced body weight gain, increased liver weight and hepatocytic vacuolation). The only effects associated with treatment with [redacted] but not triticonazole were increased kidney weight (> 10% of controls) in females receiving 1000 mg/kg bw per day [redacted], and acanthosis and hyperkeratosis in the stomach of males treated with 1000 mg/kg bw per day [redacted]

The NOAEL for both triticonazole and [redacted] in this 14-day study can be considered at 100 mg/kg bw per day.

B.6.8.2.1.4. Genotoxicity

Previous evaluation:	DAR (2003)
DRAR (2016)	No remarks on the original assessment

Reference:	[redacted]: Salmonella typhimurium Reverse mutation assay (Ames Test)
Author(s), year:	Percy A., 1993
Report/Doc. number::	C019500/ -
Guideline(s):	OECD 471 (1983)
GLP:	Yes
Deviations from OECD 471 (1997):	No
Acceptability	Yes

Material and methods

[redacted] (batch no. HUT 689C; purity 99.3 %) was tested in the Ames test using histidine dependent auxotrophic mutants of *Salmonella typhimurium* (strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100). The substance (dissolved in DMSO) was tested at concentrations of 0 (solvent control), 100, 250, 5000, 1000 and 2500 µg/plate via the plate incorporation method both in the presence and absence of S-9 mix (liver preparations from Aroclor 1254-induced male Sprague-Dawley rats). The dose levels were established on the basis of a dose range-finding study using the strain TA 100. (In this pre-test, a precipitate which interfered with the reading of the plates was observed at 5000 µg/plate.) In two independent experiments, three replicates per concentration were incubated at 37°C for 52 hours.

As positive controls 2-nitrofluorene (1 µg/plate), 2-amino-anthracene (2 µg/plate), sodium azide (1 µg/plate) and 9-aminoacridine (50 µg/plate) were used.

Evaluation criteria: The test substance was considered mutagenic under the conditions of this assay if in the two mutagenicity tests the value of the ratio of the mean number of revertant colonies induced on plates of a given substance concentration to the mean number of revertant colonies spontaneously induced on plates of the negative solvent control is concentration dependent and reaches at least two or is greater or equal to two at the highest concentrattion employed or at the highest concentration that does not exhibit clear cytotoxicity.

Results

In both experiments a precipitate and cytotoxicity evidenced by a thinning of the background lawn was noted at 2500 µg/plate. However, [redacted] produced no significant positive increases in the mean number of revertants per plate in any of the strains at any concentration tested either in the presence or absence of S-9 mix. Also the mean number of revertant colonies on negative control plates were within acceptable ranges while the the positive control materials elicited the expected positive responses.

Table 6.8.2.1.4-1: Number of revertant colonies in Salmonella typhimurium strains following treatment with [redacted] - Experiment 1

Without metabolic activation					
Treatment and concentration (µg/plate)	**Mean revertants per plate (± SD)** **TA 98**	**TA 100**	**TA 1535**	**TA 1537**	**TA 1538**
Solvent control	25 ± 5.6	125 ± 12.0	21 ± 3.5	14 ± 3.4	12 ± 5.4
[redacted] 100	28 ± 8.5	112 ± 21.9	25 ± 1.5	15 ± 1.0	10 ± 0.0
[redacted] 250	30 ± 7.5	123 ± 13.2	22 ± 3.2	13 ± 3.6	14 ± 2.1
[redacted] 500	29 ± 4.0	109 ± 7.2	20 ± 1.5	10 ± 1.7	12 ± 3.0
[redacted] 1000	25 ± 5.9	98 ± 8.7	20 ± 4.0	13 ± 3.8	13 ± 5.3
[redacted] 2500	20 ± 6.1 PC	110 ± 8.6 PC	16 ± 4.5 PC	4 ± 1.2 PC	9 ± 4.2 PC
2-nitrofluorene 1	285 ± 18.8	-	-	-	361 ± 16.9
Sodium azide 1	-	723 ± 75.8	546 ± 25.0	-	-

9-aminoacridine 50	-	-	-	359 ± 38.4	-
With metabolic activation					
Treatment and concentration (µg/plate)	**Mean revertants per plate (± SD)** **TA 98**	**TA 100**	**TA 1535**	**TA 1537**	**TA 1538**
Solvent control	33 ± 2.2	105 ± 10.2	13 ± 2.2	13 ± 4.9	24 ± 6.3
[redacted] 100	34 ± 5.0	114 ± 13.1	15 ± 3.2	13 ± 4.4	25 ± 9.5
[redacted] 250	39 ± 0.6	120 ± 8.1	15 ± 3.1	17 ± 7.4	21 ± 6.1
[redacted] 500	38 ± 2.5	116 ± 3.5	13 ± 4.4	18 ± 2.5	26 ± 3.8
[redacted] 1000	36 ± 1.2	97 ± 4.6	14 ± 5.1	13 ± 1.2	16 ± 2.5
[redacted] 2500	25 ± 2.1 PC	116 ± 7.6 PC	10 ± 3.2 PC	11 ± 3.8 PC	18 ± 8.1 PC
2-aminoanthracene 2	2365 ± 167	2137 ± 114	247 ± 19	284 ±15	1930 ±106

SD: standard deviation; C: presence of cytotoxicity: P: presence of precipitate

Table 6.8.2.1.4-2: Number of revertant colonies in Salmonella typhimurium strains following treatment with [redacted] – Experiment 2

Without metabolic activation					
Treatment and concentration (µg/plate)	**Mean revertants per plate (± SD)** **TA 98**	**TA 100**	**TA 1535**	**TA 1537**	**TA 1538**
Solvent control	27 ± 8.4	131 ± 9.9	21 ± 2.9	13 ± 3.9	16 ± 1.6
[redacted] 100	31 ± 4.0	120 ± 5.5	22 ± 0.0	22 ± 0.0	16 ± 1.6
[redacted] 250	29 ± 9.5	111 ± 0.0	24 ± 4.0	14 ± 3.5	16 ± 3.5
[redacted] 500	21 ± 2.1	128 ± 15.0	24 ± 4.7 ±	13 ± 3.2	17 ± 3.2
[redacted] 1000	31 ± 6.6	134 ± 19.0	24 ± 7.1	13 ± 3.2	17 ± 3.2
[redacted] 2500	27 ± 6.1P	112 ± 9.1 P	15 ± 5.0 P	10 ± 4.4 P	11 ± 3.5 P
2-nitrofluorene 1	1974 ± 202	-	-	-	1927 ± 177
Sodium azide 1	-	1850 ± 144	296 ± 20.4	-	-
9-aminoacridine 50	-	-	-	289 ±36.9	-
With metabolic activation					
Treatment and concentration (µg/plate)	**Mean revertants per plate (± SD)** **TA 98**	**TA 100**	**TA 1535**	**TA 1537**	**TA 1538**
Solvent control	36 ±8.5	114 ± 12.3	19 ± 7.6	17 ± 3.0	23 ± 3.7
[redacted] 100	30 ± 7.5	127 ± 7.0	16 ± 5.5	18 ± 0.6	25 ± 1.5
[redacted] 250	39 ± 4.6	127 ± 6.9	19 ± 3.1	21 ± 6.4	21 ± 2.9
[redacted] 500	40 ± 10.4	129 ± 10.6	16 ± 2.5	15 ± 4.6	23 ± 4.0
[redacted] 1000	31 ± 1.7	120 ± 15.0	16 ± 2.9	10 ± 1.2	25 ± 2.0
[redacted] 2500	26 ± 4.0 PC	109 ± 2.1 PC	13 ± 0.6 PC	13 ± 3.8 PC	27 ± 2.9 PC
2-aminoanthracene 2	1974 ± 202	1850 ± 144	296 ± 20	289 ± 36	1927 ± 177

SD: standard deviation; C: presence of cytotoxicity: P: presence of precipitate

Conclusion:

It can be concluded that [redacted] did not induce reverse gene mutations in any *Salmonella typhimurium* strains when tested at dose levels up to 2500 µg/plate either in the presence or absence of metabolic activation.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted](impurity of BAS 595 F, Triticonazole) - Salmonella typhimurium / Escherichia coli reverse mutation assay
Author(s), year:	Woitkowiak C., 2016
Report/Doc. number::	2016/1319709/ -
Guideline(s):	OECD 471 (1997)

GLP:	Yes
Deviations from OECD 471 (1997):	No
Acceptability	Yes

Materials and methods

S. typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and a strain of E. coli WP2 uvrA were exposed with Reg.No. 505519 (batch: L76-158, purity: 97.2%) in the presence and absence of metabolic activation for 48 - 72 hours. Vehicle (DMSO) and appropriate positive controls were included in each experiment. In the Ames standard plate test (SPT) and the pre-incubation test (PIT), the test item was tested in triplicates of six concentrations in a range from 33 to 5200 µg/plate with and without S9 mix (phenobarbital/ß-naphthoflavone-induced rat liver S9 fraction). As positive controls 2-amino-anthracene (with S9 mix) and N-methyl-N-nitro-N-nitrosoguanidine (MNNG), 4-nitro-o-phenylenediamine (NOPD), 9-aminoacridine (AAC) and 4-nitroquinoline-N-oxide (4-NQO) (all without S9 mix) were used.

Results

Cytotoxicity

A weak bacteriotoxic effect (slight decrease in the number of his^+ or trp^+ revertants) was occasionally observed in the SPT and PIT depending on the strain and test conditions from about 2600 µg/plate onward.

Solubility

Test substance precipitation was found from about 1000 µg/plate onward in PIT with metabolic activation and from about 2600 µg/plate onward for any other condition.

Mutagenicity

In the SPT and PIT experiments (Table 6.8.1.3.4-3) with and without metabolic activation no biologically relevant increase in number of his^+ or trp^+ revertants was observed in any strain tested.

The positive controls yielded revertant numbers in a range expected for the respective strains (within the range of the historical positive control data of the performing laboratory) and thus demonstrated the sensitivity of the test system.

The vehicle control induced number of revertans that was within the range of the historical negative control data of each strain.

Table 6.8.2.1.4-3: Standard plate Ames tests with [redacted] (impurity of BAS 595 F, Triticonazole) - Mean number of revertants

Experiment 1: Standard plate test										
Strain	**TA 100**		**TA 1535**		**TA 1537**		**TA 98**		**WP2 uvrA**	
Metabol. activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Vehicle control										
DMSO	104.0	111.3	14.3	11.0	9.7	8.0	18.3	28.0	24.7	26.3
SD	*4.6*	*5.5*	*2.9*	*3.5*	*0.6*	*2.0*	*4.0*	*2.6*	*5.8*	*3.5*
Test item [µg/plate]										
33	81.7	92.7	13.3	14.7	6.7	6.3	16.3	33.7	24.7	27.7
SD	*7.6*	*8.3*	*5.0*	*5.5*	*0.6*	*3.2*	*4.0*	*4.0*	*4.0*	*1.5*
100	81.3	102.7	12.3	10.0	7.3	10.0	21.0	23.7	24.0	25.7
SD	*18.0*	*10.7*	*1.5*	*2.6*	*2.1*	*2.6*	*7.5*	*6.0*	*8.7*	*2.5*
333	88.7	102.0	11.3	10.0	10.3	8.7	15.3	26.0	22.7	31.7
SD	*8.4*	*8.7*	*4.2*	*2.6*	*2.1*	*2.5*	*6.0*	*9.8*	*5.0*	*10.0*
1000	91.3	97.3	12.3	10.3	10.3	11.0	24.7	26.3	17.3	34.3
SD	*12.2*	*2.1*	*6.1*	*2.5*	*5.5*	*4.4*	*4.5*	*10.0*	*1.2*	*8.6*
2600	104.7 P	100.0 P	10.7 P	7.3 P	3.7 P	5.3 P	18.7 P	16.0 P	17.3 P	17.3 P
SD	*11.0*	*34.6*	*4.0*	*1.2*	*2.1*	*1.5*	*3.1*	*3.5*	*4.5*	*4.6*
5200	89.0 P	94.3 P	9.3 P	7.7 P	5.0 P	5.3 P	19.3 P	20.7 P	16.0 P	15.3 P
SD	*6.0*	*5.5*	*0.6*	*3.5*	*1.0*	*2.5*	*7.0*	*4.2*	*6.1*	*2.3*
Pos. control										
substance	MNNG	2-AA	MNNG	2-AA	AAC	2-AA	NOPD	2-AA	4-NQO	2-AA
[µg/plate]	5	2.5	5	2.5	100	2.5	10	2.5	5	60
mean revert.	3769.7	1276.7	4902.7	263.7	937.0	97.3	991.0	492.7	869.0	111.7
SD	*277.7*	*45.7*	*98.3*	*31.6*	*110.8*	*20.4*	*73.0*	*29.0*	*49.3*	*12.7*
Experiment 2: Pre-incubation test										
Strain	**TA 100**		**TA 1535**		**TA 1537**		**TA 98**		**WP2 uvrA**	
Metabol. activation	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**
Vehicle control										
DMSO	83.7	99.7	7.0	7.3	8.3	7.0	19.3	19.3	26.7	23.0
SD	*5.9*	*4.0*	*3.6*	*2.1*	*0.6*	*2.6*	*4.7*	*0.6*	*5.0*	*5.2*
Test item [µg/plate]										
33	101.7	89.7	11.7	10.7	5.7	6.3	18.3	28.3	23.0	27.0
SD	*11.1*	*10.6*	*1.2*	*3.1*	*1.2*	*2.5*	*4.2*	*7.5*	*5.0*	*7.5*
100	97.7	98.0	11.3	7.7	9.7	9.3	24.3	22.3	24.7	23.0
SD	*2.9*	*10.0*	*0.6*	*1.5*	*3.5*	*0.6*	*4.2*	*4.0*	*5.7*	*3.5*
333	83.7	101.3	8.3	8.7	6.7	8.3	21.0	22.3	22.7	22.7
SD	*8.3*	*6.4*	*2.1*	*2.1*	*3.1*	*1.2*	*1.7*	*2.3*	*4.7*	*3.1*
1000	104.3	98.7 P	12.0	9.0 P	6.7	8.3 P	24.3	21.7 P	28.0	18.0 P
SD	*16.9*	*19.9*	*2.6*	*0.0*	*2.3*	*2.1*	*4.7*	*5.0*	*2.0*	*1.0*
2600	100.3 P	108.3 P	8.3 P	8.3 P	5.3 P	6.7 P	16.7 P	19.0 P	17.0 P	16.3 P
SD	*14.0*	*13.6*	*1.5*	*2.3*	*0.6*	*1.5*	*3.1*	*5.6*	*3.5*	*0.6*
5200	90.7 P	86.0 P	10.0 P	9.3 P	4.0 P	4.0 P	14.0 P	17.3 P	19.3 P	17.7 P
SD	*10.7*	*10.8*	*1.0*	*2.1*	*1.0*	*1.7*	*2.6*	*2.1*	*4.7*	*2.5*
Pos. control										
substance	MNNG	2-AA	MNNG	2-AA	AAC	2-AA	NOPD	2-AA	4-NQO	2-AA
[µg/plate]	5	2.5	5	2.5	100	2.5	10	2.5	5	60
mean revert.	2282.7	1163.7	2947.3	137.3	854.3	141.0	1034.0	2145.3	703.3	151.0
SD	*233.1*	*54.5*	*230.4*	*21.7*	*283.8*	*19.7*	*36.8*	*85.9*	*99.7*	*3.5*

2-AA = 2-Aminoanthracene; AAC = 9-Aminoacridine; MNNG = N-methyl-N'-nitroso-guanidine; NOPD = 4-nitro-o-phenylenediamine; 4-NQO = 4-Nitroguinoline-N-oxide
P = precipitation

Conclusion

Based on the results of the present study [redacted] is not mutagenic in the Ames standard plate test and pre-incubation test with and without metabolic activation under the chosen experimental conditions.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] (Impurity of BAS 595 F, Triticonazole): In vitro gene mutation test in L5178Y Mouse Lymphoma cells (TK+/- Locus Assay, microwell version)
Author(s), year:	Schulz M. and Landsiedel R, 2016
Report/Doc. number::	BASF DocID 2016/1226269
Guideline(s):	OECD 490 (2015), EC 440/2008 B.17, EPA OPPTS 870.5300
GLP:	Yes
Deviations from OECD 490 (2016):	No
Acceptability	Yes

Material and Methods

[redacted] ([redacted] / Impurity of BAS 595 F (Triticonazole); batch: L76-158, purity 97.2%) was tested in vitro for its ability to induce forward mutations in mammalian cells by assessing the mutation of the TK locus in Mouse Lymphoma L5178Y cells with the microwell method. Three valid and independent experiments were conducted in the presence or absence of metabolic activation with two parallel cultures each. Based on the results of a preliminary cytotoxicity assay, concentrations ranging from 6.3 to 600 µg/mL and 4.7 to 400 µg/mL without and with addition of the S9 mix, respectively, were used in the main experiments. The treatment intervals for both experiments in the presence and absence of metabolic activation were generally 4 hours, except in experiment II in the absence of metabolic activation, where a treatment interval of 24 h was applied. Methyl methane sulfonate (MMS) served as positive controls in the experiments without metabolic activation and Cyclophosphamide (CPA) as well as 7,12-Dimethylbenz[a]anthracene (DMBA) served as positive controls in the experiments with metabolic activation. After the incubation period, treatment media were replaced by culture medium and the cells were incubated for 48 h for expression of mutant cells. This was followed by incubation of cells in selection medium containing TFT for about 10 days. Finally, the number of large and small colonies was determined.

Results

Osmolality and pH values were not relevantly influenced by test substance treatment.

In this study, in the absence and the presence of S9 mix, test substance precipitation in culture medium at the end of treatment was observed from about 180 µg/mL onward in all experiments.

Cytotoxicity indicated by reduced relative total growth was observed in all experiments.

The negative controls gave mutant frequencies within the range expected for the cell line used. All positive controls led to the expected increase in the frequencies of forward mutations. Thus, the validity of the present study as well as the sensitivity of the test system used is demonstrated.

In the absence of the metabolic activation, no biological relevant increase of the mutant frequency was observed neither after a short nor a long term exposure with the test item. The corrected mutant frequencies obtained for both experiments were always below the calculated threshold taking in consideration the Global Evaluation Factor (GEF; 126 plus the mutant frequency of the respective negative control).

In the presence of metabolic activation, the test substance caused a partly concentration-dependent increase in the mutant frequencies in two out of three experiments after 4 hours exposure. In the study report it is stated that a

clastogenic mode of action has to be considered. A reproducible and dose-related shift in the ratio of small versus large colonies can be considered indicated (especially in the 3rd experiment) as obvious when mean absolute counts are compared. Thus, in the presence of metabolic activation, a clastogenic rather than a gene mutation effect is concluded in this assay.

Table 6.8.2.1.4-4: MLA gene mutation in mammalian cells - 1st experiment

Test group	Corrected Mutant frequency [per 10^6 cells]	Mean colony counts [%] (Mean absolute counts)		Toxicity data			Cloning efficiency (CE_2 -viability)	
		Small colonies	Large colonies	TSG	RSG	RTG	absolute	relative
Without metabolic activation; 4-hour exposure period								
Vehicle (DMSO)	67.7	89 (16)	11 (2)	22.9	100.0	100.0	130.0	100.0
MF threshold[§]	194							
Test item [µg/mL]								
9.4	81.4	89 (16)	11 (2)	20.9	91.2	78.6	112.0	86.2
18.8	93.7	84 (16)	16 (3)	20.8	90.9	68.0	97.3	74.8
37.5	75.9	89 (16)	11 (2)	17.7	77.5	69.2	116.0	89.3
75.0	71.7	93 (14)	7 (1)	17.7	77.3	66.6	112.0	86.2
150.0	77.4	89 (17)	11 (2)	14.4	63.1	63.1	130.0	100.0
300.0 [P]	91.7	81 (17)	19 (4)	10.0	43.8	38.8	115.0	88.5
600.0 [P]	n. c.							
MMS								
15 µg/mL	**1077.2**	90 (128)	10 (15)	13.0	57.0	31.4	71.7	55.1
With metabolic activation; 4-hour exposure period								
Vehicle (DMSO)	116.9	82 (18)	18 (4)	15.5	100.0	100.0	95.7	100.0
MF threshold§	243							
Test item [µg/mL]								
4.7	69.2	93 (13)	7 (1)	13.3	85.9	92.3	102.9	107.5
9.4	82.4	81 (13)	19 (3)	12.7	81.9	86.7	101.2	105.8
18.8	97.7	84 (16)	16 (3)	12.0	77.2	77.8	96.5	100.8
37.5	84.2	81 (13)	19 (3)	12.4	80.0	81.3	97.3	101.6
75.0	76.3	80 (16)	20 (4)	10.1	65.2	91.2	134.0	140.0
150.0	**266.2**	94 (48)	6 (3)	4.4	28.2	30.1	102.1	106.6
300.0 [P]	164.7	90 (27)	10 (3)	4.3	28.0	28.6	98.0	102.4
CPA								
2.5 µg/mL	**652.3**	93 (98)	7 (7)	9.6	61.9	56.1	86.6	90.5
DMBA								
1 µg/mL	**1361.4**	84 (92)	16 (17)	5.2	33.7	**15.4**	43.6	45.6

[§] = $MF_{\text{vehicle control corr}}$ + GEF (126 x 10^{-6}), rounded
n. c. = not continued due to strong cytotoxicity
[P] = precipitation occurred at the end of the exposure period

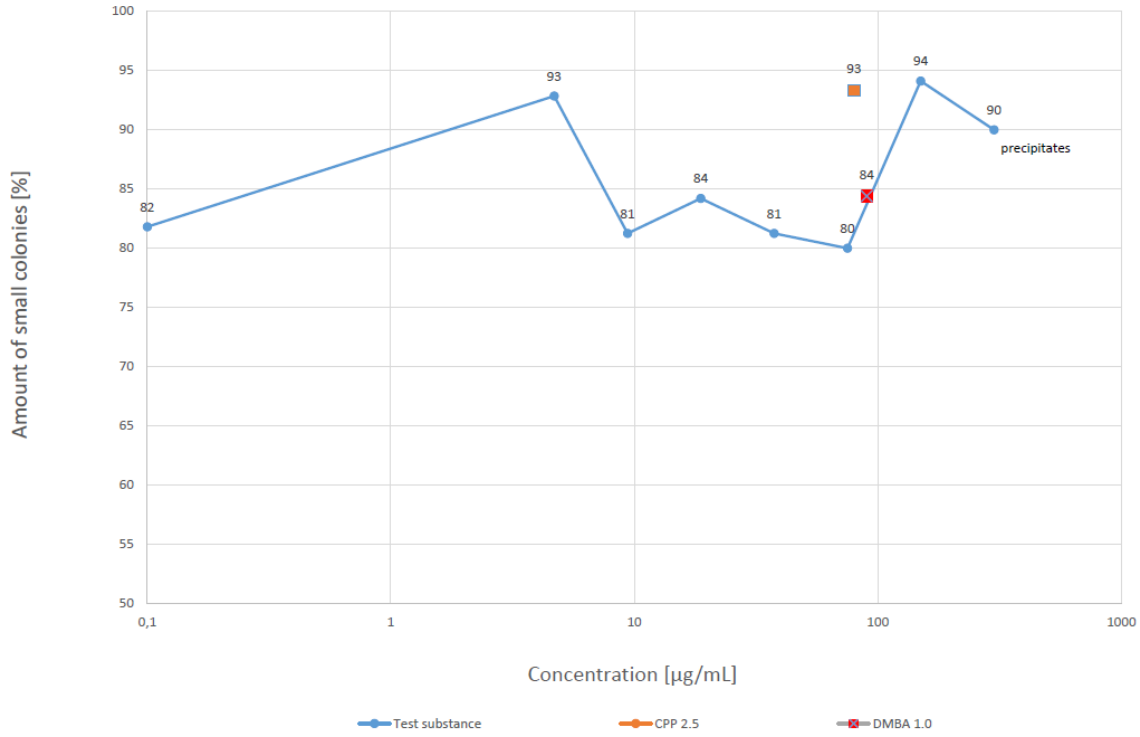


Figure 6.8.2.1.4-1: Proportion of small colonies in 1st experiment with metabolic activation

Table 6.8.2.1.4-5: MLA gene mutation in mammalian cells – 2nd experiment

Test group	Corrected Mutant frequency [per 10^6 cells]	Mean colony counts [%] (Mean absolute counts)		Toxicity data			Cloning efficiency (CE_2 -viability)	
		Small colonies	Large colonies	TSG	RSG	RTG	absolute	relative
Without metabolic activation; 24-hour exposure period								
Vehicle (DMSO)	64.6	83 (10)	17 (2)	86.9	100.0	100.0	96.5	100.0
MF threshold§	191							
Test item [µg/mL]								
6.3	49.9	80 (8)	20 (2)	84.4	97.1	96.3	95.7	99.2
12.5	42.9	80 (8)	20 (2)	70.3	80.9	99.0	118.1	122.4
25.0	39.6	78 (7)	22 (2)	48.9	56.2	68.2	117.1	121.3
50.0	47.2	70 (7)	30 (3)	27.3	31.4	34.9	107.3	111.2
100.0	51.9	88 (7)	13 (1)	4.5	5.2	**4.7**	86.6	89.8
200.0 P	46.4	78 (7)	22 (2)	9.1	10.5	**11.2**	102.9	106.6
400.0 P	n. c.							
MMS								
5 µg/mL	**418.2**	76 (52)	24 (16)	58.0	66.7	60.8	88.0	91.2
With metabolic activation; 4-hour exposure period								
Vehicle (DMSO)	54.9	73 (8)	27 (3)	18.7	100.0	100.0	108.2	100.0
MF threshold§	181							
Test item [µg/mL]								
6.3	53.2	85 (11)	15 (2)	19.2	102.9	111.4	117.1	108.2
12.5	68.0	69 (11)	31 (5)	18.2	97.6	110.5	122.6	113.3
25.0	50.3	75 (9)	25 (3)	18.1	96.7	105.6	118.1	109.2
50.0	56.0	83 (10)	17 (2)	17.9	95.6	100.7	114.0	105.3

Test group	Corrected Mutant frequency [per 10^6 cells]	Mean colony counts [%] (Mean absolute counts)		Toxicity data			Cloning efficiency (CE_2 -viability)	
		Small colonies	Large colonies	TSG	RSG	RTG	absolute	relative
100.0	57.7	83 (10)	17 (2)	14.6	78.0	72.4	100.4	92.8
200.0 [P]	n. c.							
400.0 [P]	n. c.							
CPA								
2.5 µg/mL	**460.3**	85 (62)	15 (11)	13.1	70.2	55.4	85.4	78.9
DMBA								
1 µg/mL	**794.3**	80 (79)	20 (20)	11.9	63.8	41.4	70.1	64.8

[§] = $MF_{vehicle\ control\ corr}$ + GEF (126 x 10^{-6}), rounded
n. c. = not continued due to strong cytotoxicity
[P] = precipitation occurred at the end of the exposure period

Table 6.8.2.1.4-6: MLA gene mutation in mammalian cells – 3rd experiment

Test group	Corrected Mutant frequency [per 10^6 cells]	Mean colony counts [%] (Mean absolute counts)		Toxicity data			Cloning efficiency (CE_2 -viability)	
		Small colonies	Large colonies	TSG	RSG	RTG	absolute	relative
With metabolic activation; 4-hour exposure period								
Vehicle (DMSO)	74.5	80 (12)	20 (3)	14.8	100.0	100.0	103.8	100.0
MF threshold[§]	200							
Test item [µg/mL]								
40.0	76.2	87 (13)	13 (2)	14.8	99.8	101.5	105.5	101.7
60.0	84.2	80 (16)	20 (4)	11.3	76.2	89.2	121.5	117.1
80.0	112.2	88 (21)	13 (3)	8.6	58.0	63.2	113.0	108.9
100.0	141.7	88 (28)	13 (4)	5.9	39.9	43.8	114.0	109.8
120.0	137.5	89 (25)	11 (3)	4.5	30.4	32.5	111.0	107.0
140.0	**213.6**	91 (39)	9 (4)	3.4	22.9	24.5	111.0	107.0
160.0	**231.8**	86 (32)	14 (5)	3.6	24.2	**19.0**	81.6	78.7
180.0	**302.9**	94 (51)	6 (3)	3.4	22.9	22.0	99.6	96.0
200.0	144.8	93 (26)	7 (2)	2.3	15.7	**14.5**	95.7	92.3
CPA								
2.5 µg/mL	**673.7**	92 (87)	8 (8)	9.0	60.6	45.7	78.1	75.3
DMBA								
1 µg/mL	**805.7**	83 (86)	17 (17)	8.0	54.0	35.5	68.2	65.7

[§] = $MF_{vehicle\ control\ corr}$ + GEF (126 x 10^{-6}), rounded
n. c. = not continued due to cytotoxicity
[P] = precipitation occurred at the end of the exposure period

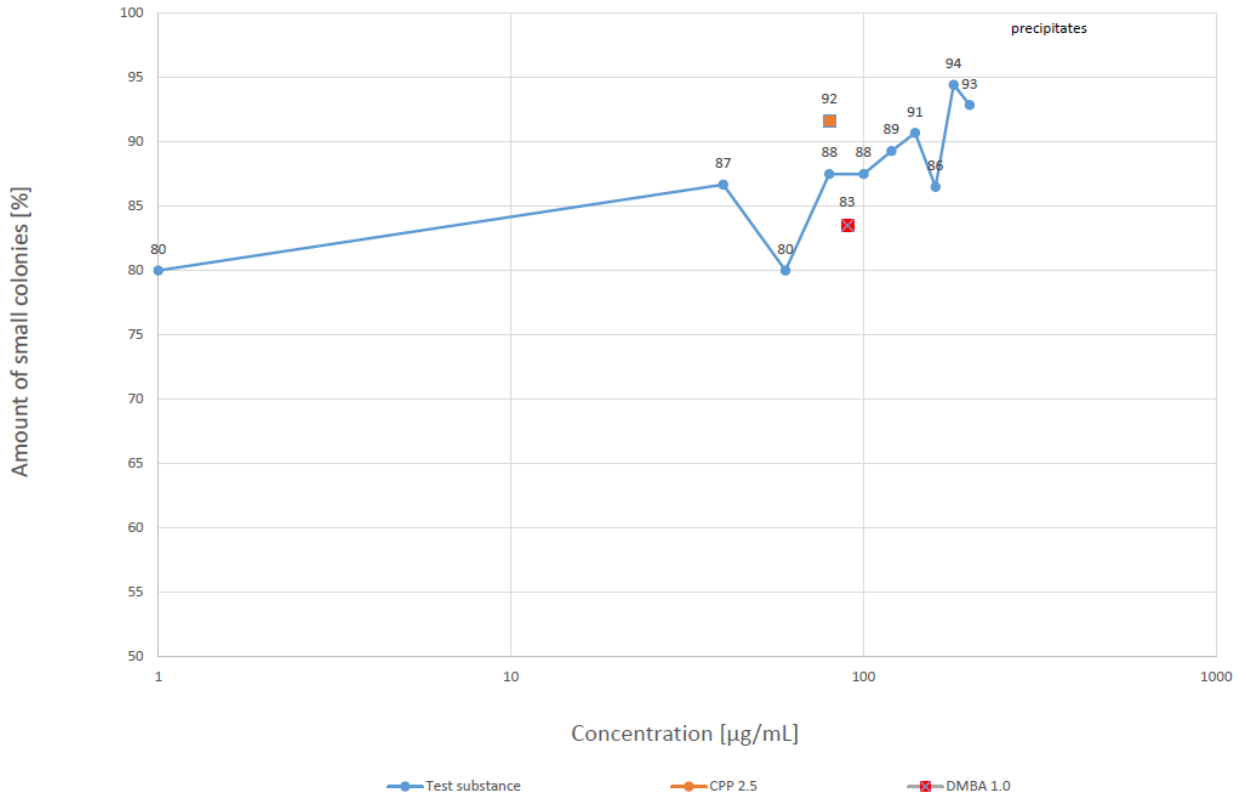


Figure 6.8.2.1.4-2: Proportion of small colonies in 3rdt experiment with metabolic activation

Conclusion

Under the experimental conditions reported, the test item [redacted] (Impurity of BAS 595 F, Triticonazole) induces gene mutations in the *in vitro* Mouse Lymphoma assay in the presence of a metabolic system. A reproducible and dose-related shift in the ratio (and absolute counts) of small versus large colonies was observed, indicating a clastogenic effect.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	In vitro mammalian cell gene mutation tests in TK6 cells treated with [redacted] (impurity of BAS 595 F, Triticonazole)
Author(s), year:	Takayuki Fukuda T., 2017
Report/Doc. number::	2017/1129485
Guideline(s):	OECD 490 (2016)
GLP:	No

- No inspection by the Quality Assurance Unit was performed during the performance of the in vitro study. However, all information and raw data are well-documented in a GLP-conform manner and the study procedure was according to the OECD test guideline. The laboratory is a GLP laboratory; for TK6 assay the validation of SOPs and analytical methods is outstanding.

- BoZo participated in a collaborative study of the TK6 gene mutation assay assessing intra- and inter-reproducibility by testing different chemicals.
From the proficiency chemicals listed in the OECD 490 (2016), BoZo tested

	Methyl methane sulphonate as a mutagen active without metabolic activation and Cyclophosphamide as mutagens requiring metabolic activation. - In summary, 27 TK6 assays were conducted according to the test guideline TG 490 in the time period from 2015 to 2017. - Thereby, TK6 tests were conducted with the following substances: Cisplatin Cyclophosphamide Methyl methane sulfonate Tamoxifen Arsenic (III) trioxide Hydrogen peroxide Auramine
Deviations from OECD 490 (2016):	No
Acceptability	Yes

Material and Methods

[redacted] ([redacted] Lot Number: L76-158, purity 97.2%) was tested *in vitro* for its ability to induce forward mutations in mammalian cells by assessing the mutation of the TK locus in DNA-repair proficient, p53-competent human lymphoblastoid TK6 cells. Two independent experiments were conducted in the presence or absence of metabolic activation with two parallel cultures each. Based on the results of a preliminary cytotoxicity assay, four concentrations ranging from 31.3 to 250 µg/mL without and with addition of the S9 mix, respectively, were used in the main experiment. The treatment period for both experiments in the presence and absence of metabolic activation was 4 hours. Additionally, a treatment interval of 24 hours was applied, in one experiment in the absence of metabolic activation, using five concentrations ranging from 15.6 to 250 µg/mL. Furthermore, a confirmatory test with metabolic activation was conducted using five concentrations in a range of 120 to 200 µg/mL. Methyl methane sulfonate (MMS) served as positive controls in the experiments without metabolic activation and Cyclophosphamide (CPA) served as positive controls in the experiments with metabolic activation. After the respective incubation period, treatment media were replaced by culture medium and the cells were incubated 3 days for expression of mutant cells. This was followed by incubation of cells in selection medium containing TFT for 12 to 14 days. Based on early appearing and late appearing mutants, small and large colonies were recorded and mutant frequencies calculated.

Judgements of results:

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

a) at least one of the test concentrations exhibits a statistically significant increase compared with the concurrent negative control

b) the increase is concentration-related when evaluated with an appropriate trend test

c) any of the results are outside the distribution of the historical negative control data

When all of these criteria are met, the test chemical is then considered able to induce mutation in this test system.

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

a) none of the test concentrations exhibits a statistically significant increase compared with the

concurrent negative control,

b) there is no concentration-related increase when evaluated with an appropriate trend test

c) all results are inside the distribution of the historical negative control data

The test chemical is then considered unable to induce mutations in this test system.

Results

In the preliminary cytotoxicity assay using concentrations ranging from 24.7 to 2000 µg/mL (24.7, 74.1, 222, 667, 2000 µg/mL) without and with addition of the S9 mix, severe precipitation in the medium was observed at dose levels of 667 to 2000 µg/mL and a few precipitates at dose levels of 222 µg/mL. Cytotoxicity was noticed in the presence of strong precipitation, at the dose levels of 667 to 2000 µg/mL, revealing 28% of relative survival (RS) at 222 µg/mL of the continuous experiment conducted without metabolic activation. In accordance to OECD TG 490, based on cytotoxicity with approximately 20% RS at continuous treatment the highest concentration was selected for the main experiments, which is also the only one concentration producing a visible precipitate.

In the gene mutation assay, precipitation of the test substance in culture medium at the end of treatment was observed at the highest dose level of 250 µg/mL in the absence and in the presence of S9 mix and at 200 µg/mL in the confirmation test.

No cytotoxicity was observed with short-term treatment in the presence or absence of metabolic activation as well as with long-term treatment in the absence of metabolic activation.

No biological relevant and reproducible as well as dose dependent increase in mutant colony numbers was observed in main experiments with and without addition of the metabolizing system.

The negative control values were within the range of the historical control data of the performing laboratory. All positive controls led to distinct increase in the frequencies of forward mutations. Thus, the validity of the present study as well as the sensitivity of the test system used is demonstrated.

Table 6.8.2.1.4-7: TK gene mutation in mammalian cells - experiment with and without metabolic activation - 4-hour exposure period – Experiment I

Test group	Total mutant frequency (TMF) [per 10^{-6} cells]	Small mutant frequency[1] [%]	Relative survival (RS) [%]
Without metabolic activation; 4-hour exposure period			
Vehicle (DMSO)	2.78	40	100
Test item [µg/mL]			
31.3	4.04	43	88
62.5	2.29	47	97
125	1.86	42	90
250 [p]	2.62	42	100
MMS			
µg/mL	**13.08***	36	13
Negative HCD	6.11 ± 3.34	-	-
With metabolic activation; 4-hour exposure period			
Vehicle (DMSO)	4.36	27	100
Test item [µg/mL]			
31.3	4.80	64	84
62.5	2.15	38	83

Test group	Total mutant frequency (TMF) [per 10^{-6} cells]	Small mutant frequency[1] [%]	Relative survival (RS) [%]
125	2.91	27	91
250 [p]	5.81	21	97
CP			
µg/mL	**22.86***	61	17
Negative HCD	6.07 ± 3.22	-	-

[p] = precipitation occurred at the end of the exposure period

[1]= %SC (slowly growing (SG) mutant frequencies based on the early appearing and late appearing mutants)

* = $p < 0.05$

Table 6.8.2.1.4-8: TK gene mutation in mammalian cells - experiment without metabolic activation - 24-hour exposure period

Test group	Total mutant frequency [per 10^{-6} cells]	Small mutant frequency[1] [%]	Relative survival [%]
Without metabolic activation; 24-hour exposure period			
Vehicle (DMSO)	3.83	47	100
Test item [µg/mL]			
15.6	6.70	39	91
31.3	2.14	25	101
62.5	3.50	43	62
125	2.91	52	69
250 [p]	6.13	42	19
MMS			
µg/mL	**26.29***	36	68
Negative HCD	3.08 ± 1.96	-	-

[p] = precipitation occurred at the end of the exposure period

[1]= %SC (slowly growing (SG) mutant frequencies based on the early appearing and late appearing mutants)

* = $p < 0.05$

Table 6.8.2.1.4-9: TK gene mutation in mammalian cells - experiment with metabolic activation - 4-hour exposure period – Experiment II

Test group	Total mutant frequency [per 10^{-6} cells]	Small mutant frequency[1] [%]	Relative survival [%]
With metabolic activation; 4-hour exposure period			
Vehicle (DMSO)	5.24	43	100
Test item [µg/mL]			
120	6.89	35	107
140	6.29	45	94
160	4.49	42	82
180	4.65	34	84
200 [p]	5.29	24	104
CP			

Test group	Total mutant frequency [per 10^{-6} cells]	Small mutant frequency[1] [%]	Relative survival [%]
µg/mL	**25.27***	60	16
Negative HCD	6.07 ± 3.22	-	-

[p] = precipitation occurred at the end of the exposure period

[1]= %SC (slowly growing (SG) mutant frequencies based on the early appearing and late appearing mutants)

* = $p < 0.05$

Conclusion

In conclusion, it can be stated that under the experimental conditions reported, the test item [redacted]) did not induce gene mutations in the *in vitro* TK6 assay in the presence and absence of metabolic activation.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] (impurity of BAS 595 F, Triticonazole): Micronucleus test in human lymphocytes in vitro
Author(s), year:	Chang S., 2016
Report/Doc. number::	-/2016/1134747
Guideline(s):	OECD 487 (2014)
GLP:	Yes
Deviations from OECD 487 (2014):	No
Acceptability:	Yes

Material and Methods:

[redacted] ([redacted] batch: L76-158, purity 97.2%) was tested for its potential to induce micronuclei in human lymphocytes *in vitro* in the absence and presence of metabolic activation by S9 mix (phenobarbital/β-naphthoflavone induced rat liver). Two independent experiments were performed in duplicate cultures where the cells were exposed for 4 (±S9 mix) or 20 hours (-S9 mix) with the test substance at concentrations ranging from 1.5 to 2058µg/mL, of that three concentrations ranging from 43.6 to 686 µg/mL were evaluated. The vehicle DMSO (1% in culture medium) served as negative control, mitomycin C (4 h, pulse treatment) and demecolcin (20 h, continuous treatment) as positive controls in the absence of metabolic activation and cyclophosphamide as positive control in the presence of metabolic activation. Treatments started after a 48 hour stimulation period with phytohemeagglutinine. Thereafter cytochalasin B (4 µg/mL) was added and the cultures were fixed and stained finally after another 20 hours. Cytokinesis-block proliferation index and cytostasis were determined in 1000 binucleated cells per dose group as cytotoxicity parameters. The number of micronucleated cells was determined in 2000 and 1000 binucleated cells per dose group for evaluation of mutagenicity in solvent control or the treatment groups and positive control groups, respectively.

Results:

In Experiment I, precipitation of the test item in the culture medium was observed at 229 µg/mL and above in the absence of S9 mix and at 686 µg/mL and above in the presence of S9 mix at the end of treatment. In addition, precipitation occurred in Experiment II in the absence of S9 mix at 219 µg/mL and above and in the presence of S9 mix at 384 µg/mL and above at the end of treatment.

No relevant influence on osmolarity or pH was observed.

In the absence and presence of S9 mix, no cytotoxicity was observed up to the highest evaluated concentration, which showed precipitation.

In both independent experiments, neither a statistically significant nor a biologically relevant increase in the number of micronucleated cells was observed after treatment with the test item.

In both experiments, either demecolcin (75.0 ng/mL), MMC (1.5 µg/mL) or CPA (17.5 and 15.0 µg/mL) were used as positive controls and showed distinct increases in cells with micronuclei that were within the range of the positive historical control data, except the 4-hour treatment with MMS that slightly exceeded this range (4.15 – 24.00). However, this MMS-result is assessed not to compromise either the validity of the study or the study outcome.

In both experiments, the solvent control DMSO showed a low frequency of micronucleated cells that was within the range of the negative historical control data.

Thus, based on the results of the solvent and the positive controls, the suitability of the test system used to detect known clastogens and aneugens as well as the validity of the present study is demonstrated.

Table 6.8.2.1.4-10: Summary of results of the in vitro micronucleus test in human lymphocytes with [redacted]

Exp.	Exposure period [h]	Test item concentration [µg/mL]	Proliferation index CBPI	Cytostasis in %[a]	Micronucleated cells in %[b]
Without S9 mix					
I	4	Solvent control[1]	1.84		0.80
		Positive control[2c]	1.62	26.3	**28.50***
		43.6	1.85	n. c.	0.65
		76.2	1.93	n. c.	0.65
		229 [P]	1.77	8.5	0.80
II	20	Solvent control[1]	1.80		0.80
		Positive control[3]	1.72	9.5	**3.20***
		71.7	1.63	20.9	0.80
		125	1.56	29.8	0.50
		219 [P]	1.53	33.9	0.35
With S9 mix					
I	4	Solvent control[1]	1.99		0.95
		Positive control[4]	1.63	36.3	**10.25***
		76.2	1.99	0.4	1.10
		229	1.84	15.0	0.95
		686 [P]	1.89	9.9	0.70
II	4	Solvent control[1]	2.14		0.35
		Positive control[5]	1.62	45.8	**4.30***
		125	2.02	10.6	0.25
		219	2.03	10.4	0.30
		384 [P]	1.85	25.6	0.20

a: For the positive control groups and the test item treatment groups the values are related to the solvent controls
b: The number of micronucleated cells was determined in a sample of 2000 binucleated cells
c: The number of micronucleated cells was determined in a sample of 1000 binucleated cells
P Precipitation occurred at the end of the treatment by the unaided eye
CBPI: cytokinesis-block proliferation index
n. c. Not calculated as the CBPI is equal or higher than the solvent control value
*: The number of micronucleated cells is statistically significantly higher than corresponding control values ($p \leq 0.05$)
1 DMSO 1% (v/v)
2 MMC 1.5 µg/mL
3 Demecolcin 75.0 ng/mL
4 CPA 17.5 µg/mL
5 CPA 15.0 µg/mL

Conclusion:

Under the experimental conditions reported, the test item did not induce micronuclei as determined by the *in vitro* micronucleus test in human lymphocytes. Therefore, [redacted] is considered to be non-mutagenic in this *in vitro* micronucleus test when tested up to the highest analysable concentrations with and without metabolic activation.

B.6.8.2.2 [redacted]

- Negative AMES test with spiked [redacted] (new for renewal, study evaluation included in Volume 4 based on confidential information on content of impurities)
- QSAR analysis (new for renewal, included in Volume 4): negative for AMES and in vitro MNT

Based on the content of [redacted] in triticonazole and its toxicological profile, [redacted] is considered to be a non-relevant impurity. Detailed evaluation and conclusion according to Guidance document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) 1107/2009) (SANCO/10597/2003-rev.10.1, July 2012) is included in Volume 4.

B.6.8.2.3 [redacted]

- Negative AMES test with spiked [redacted] (new for renewal, study evaluation included in Volume 4 based on confidential information on content of impurities)
- QSAR analysis (new for renewal, included in Volume 4): negative for AMES and in vitro MNT

Based on the content of [redacted] in triticonazole and its toxicological profile, [redacted] is considered to be a non-relevant impurity. Detailed evaluation and conclusion according to Guidance document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) 1107/2009) (SANCO/10597/2003-rev.10.1, July 2012) is included in Volume 4.

B.6.8.2.4 [redacted]

- negative AMES test (new for renewal)

- negative AMES test with spiked [redacted] (new for renewal, study evaluation included in Volume 4 based on confidential information on content of impurities)
- QSAR analysis (new for renewal, included in Volume 4): negative for AMES and in vitro MNT

Based on the content of [redacted] in triticonazole and its toxicological profile, [redacted] is considered to be a non-relevant impurity. Detailed evaluation and conclusion according to Guidance document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) 1107/2009) (SANCO/10597/2003-rev.10.1, July 2012) is included in Volume 4.

B.6.8.2.4.1 Genotoxicity

Previous evaluation:	No
DRAR (2016)	New study (submitted for the purpose of renewal)

Reference:	[redacted] (technical impurity of BAS 595 F) - Salmonella typhimurium / Escherichia coli reverse mutation assay (Standard plate test and preincubation test)
Author(s), year:	Schulz M.,Landsiedel R., 2009
Report/Doc. number::	-/ 2008/1065134
Guideline(s):	OECD 471(1997)
GLP:	Yes
Deviations from OECD 471 (1997):	No
Acceptabilioty	Yes

Material and methods

Test Material	[redacted] (technical impurity of BAS 595 F)
Description:	Solid, white
Lot/Batch #:	MCD2641
Purity:	99.9% (diastereoisomer ratio 98:2)
Stability:	Stable under storage conditions.
Solvent used:	DMSO
Control Material	
Sterility control:	Additional plates are treated with soft agar, S9 mix, buffer, vehicle or the test substance but without the addition of tester strains.
Vehicle control:	The vehicle control with and without S-9 mix only contained the vehicle used for the test substance at the same concentration and volume for all tester strains.
Positive controls:	Positive control compounds tested without addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration µg/plate
TA 100	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5
TA 1535	N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)	DMSO	5
TA 1537	9-aminoacridine	DMSO	100
TA 98	4-nitro-o-phenylenediamine (NOPD)	DMSO	10
WP2 uvrA	4-nitroquinoline-N-oxide (4-NQO)	DMSO	5

Positive control compounds tested with addition of metabolic activation system:

Strain	Mutagen	Solvent	Concentration µg/plate
TA 100	2-aminoanthracene	DMSO	2.5
TA 1535	2-aminoanthracene	DMSO	2.5
TA 1537	2-aminoanthracene	DMSO	2.5
TA 98	2-aminoanthracene	DMSO	2.5
WP2 uvrA	2-aminoanthracene	DMSO	60

Activation

S9 was produced from the livers of male Wistar rats treated with 80 mg/kg bw phenobarbital (i.p.) and β-naphthoflavone (orally) for three consecutive days. The S9-mix was prepared freshly prior to each experiment. For this purpose, a sufficient amount of S9-fraction is thawed at room temperature and 1 volume of S9-fraction is mixed with 9 volumes of S9-supplement (cofactors). This preparation, the so-called S9-mix, was kept on ice until used.
The rat liver S9-mix was prepared immediately before use and had the following composition:

Component	Concentration
Phosphate buffer (pH 7.4)	15 mM
Glucose 6-phosphate	5 mM
NADP	4 mM
KCl	33 mM
$MgCl_2$	8 mM
S9	10 %

To demonstrate the efficacy of the S9 mix, the S9 batch was characterized with benzo(a)pyrene.

Test organisms :

S. typhimurium strains : TA98, TA100, TA1535, TA1537
E. coli: WP2 uvrA

Test concentrations:
Preliminary cytotoxicity test:

up to 5000 µg/plate

Standard plate test:

Concentrations of 0, 20, 100, 500, 2500, 5000 µg/plate were tested in all tester strains ±S9.

Preincubation test:

Concentrations of 0, 20, 100, 500, 2500, 5000 µg/plate (TA 1535, E. coli WP2 uvrA; ±S9).

Concentrations of 0, 10, 50, 250, 1250, 2500 µg/plate (TA 100, TA 1537, TA 98; ±S9).

Mutagenicity tests:

Standard plate test:

Test tubes containing 2-mL portions of soft agar (overlay agar), which consists of 100 mL agar (0.8% [w/v] agar + 0.6% [w/v] NaCl) and 10 mL amino acid solution (minimal amino acid solution for the determination of mutants: 0.5 mM histidine + 0.5 mM biotin (Salmonella strains); 0.5 mM tryptophan (E.Coli)) are kept in a water bath at about 42 – 45°C, and the remaining components are added in the following order:

- 0.1 mL test solution or vehicle (negative control)
- 0.1 mL fresh bacterial culture
- 0.5 mL S9 mix (**with metabolic activation**)

- **or**
- 0.5 mL phosphate buffer (**without metabolic activation**)

After mixing, the samples are poured onto minimal agar plates within 242pprox.. 30 seconds.

After incubation at 37°C for 48 – 72 hours in the dark, the bacterial colonies (his^+ revertants or trp^+ revertants) are counted.

Preincubation test:

1 mL test solution or vehicle, 0.1 mL bacterial suspension and 0.5 mL S9 mix (**with metabolic activation**) or phosphate buffer (**without metabolic activation**) are incubated at 37°C for the duration of about 20 minutes using a shaker. Subsequently, 2 mL of soft agar is added and, after mixing, the samples are poured onto the agar plates within 242pprox.. 30 seconds. After incubation at 37°C for 48 – 72 hours in the dark, the bacterial colonies are counted. After incubation at 37°C for 48 – 72 hours in the dark, the bacterial colonies (his^+ revertants or trp^+ revertants) are counted.

Statistics:

No special statistical tests were performed. Means and standard deviations were calculated for number of revertant colonies observed.

Evaluation criteria:

Toxicity

Toxicity detected by a

- decrease in the number of revertants
- clearing or diminution of the background lawn (= reduced his- or trp- background growth)
- reduction in the titer

is recorded for all test groups both with and without S9 mix in all experiments and indicated in the tables.

Acceptance criteria

Generally, the experiment is considered valid if the following criteria are met:

- The number of revertant colonies in the negative controls was within the range of the historical negative control data for each tester strain.
- The sterility controls revealed no indication of bacterial contamination.
- The positive control substances both with and without S9 mix induced a distinct increase in the number of revertant colonies within the range of the historical positive control data or above.
- The titer of viable bacteria was > 10^8/mL.

Assessment criteria

The test chemical is considered positive in this assay if the following criteria are met:

- A dose-related and reproducible increase in the number of revertant colonies, i.e. about

doubling of the spontaneous mutation rate in at least one tester strain either without S9 mix or after adding a metabolizing system.

A test substance is generally considered non-mutagenic in this test if:

- The number of revertants for all tester strains were within the historical negative control range under all experimental conditions in at least two experiments carried out independently of each other.

Results

A bacteriotoxic effect (reduced his- or trp- background growth, decrease in the number of his^+ or trp^+ revertants, reduction in the titer) was occasionally observed in the standard plate test depending on the strain and the conditions from about 2500 µg/plate onward. In the preincubation assay bacteriotoxicity (reduced his- or trp- background growth, decrease in the number of his^+ or trp^+ revertants, reduction in the titer) was observed from about 1250 µg/plate onward.

Test substance precipitation was found from about 1250 µg/plate onward with and without S9 mix.

According to the results of the present study, the test substance did not lead to an increase in the number of revertant colonies either without S9 mix or after adding a metabolizing system in two experiments carried out independently of each other (standard plate test and preincubation assay).

Besides, the results of the negative as well as the positive controls performed in parallel confirmed the validity of this study, since the values fulfilled the acceptance criteria of this study.

In this study with and without S9 mix, the number of revertant colonies in the negative controls was within the range of the historical negative control data for each tester strain.

In addition, the positive control substances both with and without S9 mix induced a significant increase in the number of revertant colonies within the range of the historical positive control data or above.

Table 6.8.2.4.1-1 : Bacterial gene mutation assay with [redacted] – Mean number of revertants (Standard Plate Test)

Bacteria	**Salmonella typhimurium**								**E. coli**	
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**uvrA**	
Metabol. Activation	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**
Neg. control (DMSO)	42 ± 5	23 ± 3	102 ± 13	104 ± 10	18 ± 1	17 ± 1	8 ± 2	9 ± 2	32 ± 6	43 ± 14
Test item (µg/plate)										
20	37 ± 15	24 ± 7	95 ± 20	114 ± 13	20 ± 4	17 ± 3	9 ± 1	7 ± 2	35 ± 2	38 ± 11
100	30 ± 2	19 ± 4	104 ± 18	110 ± 13	18 ± 4	16 ± 1	7 ± 2	8 ± 3	27 ± 3	29 ± 3
500	28 ± 1	19 ± 3	82 ±5	86 ± 11	18 ± 2	16 ± 2	8 ± 2	6 ± 1	30 ± 4	35 ± 7
2500	16 ± 9 P	17 ± 2 P	43 ± 8 P	28 ± 10 P	14 ± 4 P	14 ± 3 P	5 ± 1 P	5 ± 1 P	28 ± 3 P	36 ± 2 P
5000	8 ± 5 B/P	10 ± 5 B/P	25 ± 6 B/P	15 ± 5 B/P	6 ± 1 B/P	7 ± 2 B/P	2 ± 1 B/P	2 ± 1 B/P	25 ± 6 B/P	25 ± 2 B/P

Pos. control	570 ± 56	359 ± 56	686 ± 44	622 ± 49	104 ± 13	638 ± 60	172 ± 17	367 ± 34	206 ± 48	750 ± 92

P: Precipitation; B: Reduced Background Growth

Table 6.8.2.4.1-2: Bacterial gene mutation assay with [redacted] – Mean number of revertants (Preincubation Test)

Bacteria	**Salmonella typhimurium**								**E. coli**	
Strain	**TA 98**		**TA 100**		**TA 1535**		**TA 1537**		**uvrA**	
Metabol. Activation	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**	**+S9**	**-S9**
Neg. control (DMSO)	28 ± 3	24 ± 4	99 ± 6	98 ± 7	14 ± 2	14 ± 1	7 ± 3	7 ± 2	30 ± 4	34 ± 3
Test item (µg/plate)										
10	30 ± 3	21 ± 5	100 ± 12	104 ± 12	-	-	7 ± 2	8 ± 1	-	-
20	-	-	-	-	15 ± 3	17 ± 3	-	-	28 ± 3	34 ± 2
50	32 ± 9	22 ± 4	102 ± 11	93 ± 14	-	-	7 ± 3	7 ± 2	-	-
100	-	-	-	-	12 ± 3	15 ± 2	-	-	27 ± 2	33 ± 6
250	31 ± 6	25 ± 1	97 ± 6	93 ± 12	-	-	6 ± 3	5 ± 2	-	-
500	-	-	-	-	13 ± 2	16 ± 2	-	-	26 ± 3	37 ± 1
1250	16 ± 4 P	18 ± 3 P	54 ± 13 P	73 ± 3 P	-	-	4 ± 1 P	5 ± 2 P	-	-
2500	9 ± 4 B/P	15 ± 2 B/P	14 ± 0 B/P	19 ± 9 P	9 ± 4 P	11 ± 1 P	2 ± 1 P	4 ± 1 B/P	23 ± 6 P	34 ± 11 P
5000	-	-	-	-	2 ± 1 B/P	5 ± 4 B/P	-	-	16 ± 4 B/P	20 ± 6 B/P
Pos. control	662 ± 45	588 ± 26	810 ± 65	711 ± 73	136 ± 19	529 ± 192	155 ± 16	357 ± 11	211 ± 14	573 ± 54

P: Precipitation; B: Reduced Background Growth

Conclusion

According to the results of the present study, [redacted] is not mutagenic in the Salmonella typhimurium / Escherichia coli reverse mutation assay under the experimental conditions applied.

B.6.8.2.5 [redacted] ([redacted])

- Acute oral LD50 value > 2000 mg/kg bw (new for renewal)
- Acute dermal LD50 value (rats) > 2000 mg/kg bw (new for renewal)
- Negative skin irritation study (rabbits) (new for renewal)
- Negative eye irritation study (rabbits) (new for renewal)
- Negative skin sensitization study (M&K test) (new for renewal)
- 5 days toxicity study by oral route in rats (no NOAEL discussed, limited parameters) (new for renewal)
- 4-week toxicity study by oral route in rats (no NOAEL could be set, LOAEL = 50 mg/kg bw per day) (new for renewal)
- negative AMES test (new for renewal)
- negative mammalian cytogenetic test in human lymphocytes (new for renewal)
- QSAR analysis (new for renewal, included in Volume 4): negative for AMES and in vitro MNT

[redacted] is only a theoretical impurity. In the 4 week rat study, no NOAEL could be determined and the LOAEL was set at 50 mg/kg bw per day, based on hypersalivation in 3 from 12 animals, increase in relative liver weight > 10% in females and centrilobular hypertrophy in 1/6 females. For triticonazole NOAEL in 28 days rat study is set for males at 152.3 mg/kg bw per day, based on the decrease in body weight gain (-22.6%) at 513 mg/kg bw per day and at 52.4 mg/kg bw per day in females, since at 151 mg/kg bw per day statistically significant decrease in absolute uterus weight (without any accompanied histopathological findings) was observed.

Comparing [redacted] and triticonazole for their toxicity they appear to have qualitatively and quantitatively different profile. However, since [redacted] is not present in triticonazole technical, the toxicological difference does not have impact on conclusion on triticonazole technical material.

B.6.8.2.5.1 Acute oral toxicity

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] - Acute oral toxcity in rats
Author(s), year:	[redacted] 1992
Report/Doc. number::	C039792 / -
Guideline(s):	OECD 401 (1987)
GLP:	Yes
Deviations from OECD 401:	No
Acceptability:	Yes

Materials and methods

Groups of 5 male and 5 female Sprague-Dawley rats (ICO:OFA-SD; IOPS Caw) were administered 2000 mg/kg bw [redacted] (batch: 07/W, purity: 96.5%) in a 0.5% aqueous methylcellulose preparation (dose volume of 10 mL/kg bw) by gavage and were observed for a period of 14 days. Mortality was observed at least twice a day and clinical signs of toxicity were recorded daily. Body weight was determined just before application (day 1) and on days 5, 8 and 15 thereafter. Gross necropsy was performed at the end of the study period.

Results

No mortalities occurred and no signs of toxicity were observed during the study period. Body weight and body weight gain were unaffected by the test item. At necropsy, no pathological changes were observed that could be attributed to administration of the test material.

Conclusion

Under the conditions of the study and based on the information given in the study report, oral LD_{50} of [redacted] in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute oral toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.2.5.2 Acute dermal toxicity

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] - Acute dermal toxicity in rats
Author(s), year:	[redacted] 1992
Report/Doc. number::	C039798 / -
Guideline(s):	OECD 402 (1987)
GLP:	Yes
Deviations from OECD (402):	No
Acceptability:	Yes

Materials and methods

Groups of 5 male and 5 female Sprague-Dawley rats (ICO:OFA-SD; IOPS Caw) were administered 2000 mg/kg bw [redacted] (batch: 07/W, purity: 96.5%) as a water-moistened compress to the clipped trunk skin (~ 50 cm²) for 24 hours under semi-occlusive conditions. Thereafter, test item was removed with water and animals were observed for a period of 14 days. Mortality was observed at least twice a day and clinical signs of toxicity were recorded daily. Body weight was determined just before application (day 1) and on days 5, 8 and 15 thereafter. Gross necropsy was performed at the end of the study period.

Results

No mortalities occurred and no signs of toxicity were observed during the study period. Body weight and body weight gain were unaffected by the test item. At necropsy, no pathological changes were observed that could be attributed to administration of the test material.

Conclusion

Under the conditions of the study and based on the information given in the study report, dermal LD_{50} of [redacted] in male and female rats was above 2000 mg/kg bw. Therefore, no classification for acute dermal toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.2.5.3 Skin irritation

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] - Acute dermal irritation in rabbits
Author(s), year:	[redacted] 1992
Report/Doc. number::	C039799 / -
Guideline(s):	OECD 404 (1981)
GLP:	Yes
Deviations from OECD (2015):	No deviations in study design: OECD 404 (2015) describes how to integrate and use existing testing and non-testing data for the assessment of the skin irritation and skin corrosion potentials of chemicals and proposes an approach when further testing is needed. This is not considered applicable to studies conducted in 1990-ies.
Acceptability:	Yes

Materials and methods

Three male White New Zealand rabbits were exposed with 0.5 g [redacted] (batch: 07/W, purity: 96.5%) on the clipped trunk skin (patch size 6 cm²) for 4 hours under semi-occlusive conditions. Thereafter, test item was removed with water and animals were observed for 72 hours. Skin readings were performed at 1, 24, 48 and 72 hours after removal of the test item.

Results

No mortalities occurred during the study period. No systemic signs of toxicity were observed. A very slight erythema (grade 1) was observed in one animal 1 hour after patch removal that was resolved at the 24-hour reading time point. No oedema was noted in any animal at any observation time point. The average irritation score (24 – 72 hours) was 0.0 and 0.0 for erythema and oedema, respectively. Since all skin reactions were reversible within 24 hours after patch removal, the study was terminated after 72 hours.

Conclusion

Under the conditions of the study and based on the information given in the study report, rabbits exposed dermally to [redacted] for four hours did not develop any sign of skin irritation. Therefore, no classification for skin irritation according to Regulation (EC) 1272/2008 is necessary.

B.6.8.2.5.4 Eye irritation

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] - Acute eye irritation in rabbits
Author(s), year:	[redacted] 1992
Report/Doc. number::	C039800 / -
Guideline(s):	OECD 405 (1987)
GLP:	Yes
Deviations from OECD 405 (2012):	No deviations in study design: OECD 405 (2012). A preferred sequential testing strategy, which includes the performance of validated in vitro or ex vivo eye corrosion/irritation tests, is included as a Supplement to this Guideline (2012). It is recommended that this testing strategy be followed prior to undertaking in vivo testing. This is not considered applicable to studies conducted in 1990-ies.
Acceptability:	Yes

Material and methods

One eye of 3 male White New Zealand rabbits was instilled with 100 mg of [redacted] (batch: 07/W, purity: 96.5%), whereas the other eye remained untreated and served as control. The eyes were not rinsed after introduction of the test item. Animals were observed for 72 hours. Eye readings were performed at 1, 24, 48 and 72 hours after instillation.

Results

No mortalities occurred during the study period. No signs of toxicity were observed. Slight (grade 1 to 2) conjunctival reactions (chemosis and redness) were observed in all animals 1 hour after instillation, persisted in two rabbits after 24 hours and were completely resolved at 48-hour reading time point. Slight (grade 1) corneal opacity was noted in 2 animals after 24 hours that persisted in one of these animals until 48-hour reading time point. The average irritation score (24 – 72 hours) was 0.3, 0.0, 0.2 and 0.2 for corneal opacity, iris lesions, chemosis, and conjunctivae redness, respectively. Since all eye reactions were reversible within 72 hours, the study was terminated after the 72-hour reading.

Conclusion

Under the conditions of the study and based on the information given in the study report, [redacted] is not an eye irritant. Therefore, no classification for eye irritation toxicity according to Regulation (EC) 1272/2008 is necessary.

B.6.8.2.5.5 Skin sensitisation

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] - Skin sensitization test in guinea-pigs (Maximization method of Magnuson and Kligman)
Author(s), year:	[redacted] 1992
Report/Doc. number::	C039801 / -
Guideline(s):	OECD 406 (1981)
GLP:	Yes
Deviations from OECD (1992):	- No positive control included; no information on any positive control data from that time period
Acceptability:	Yes; limited reliability since no information on positive controls

Material and methods

[redacted] (batch: DA 696, purity: 95.6%) was tested in a maximization test in a group of 10 male and 10 female Dunkin Hartley guinea pigs according to Magnusson and Kligman. Intradermal induction (using the adjuvant technique, day 0) was performed with a 5% test item preparation in paraffin oil, and the percutaneous induction (day 7; pre-treatment with 10% sodium lauryl sulphate in Vaseline on the day 6) as well as the challenge (day 21) were performed with undiluted (100%) test item. For the dermal applications, the test item was incubated 48 and 24 hours under occlusive conditions for the percutaneous induction and the challenge, respectively. Skin readings were performed 1 hour after percutaneous inductions, and 24 and 48 hours after the patch removal upon the challenge application. A control group of 5 males and 5 females was treated with the vehicle in the induction phase, and was treated in the same manner as the test group animals in the challenge phase.

Results

No mortalities occurred and no signs of toxicity were observed during the study period. Body weights were not affected by the test item.

One hour after the patch removal upon the percutaneous induction, slight irritation in the control and irritation in the treatment groups were observed in the intradermal injection sites in the scapular area. The challenge resulted in very slight erythema in 1 and well-defined erythema in 1 out of 20 animals 24 hours after patch removal. Forty eight hours after patch removal, very slight erythema and dryness of the skin were noted in 1 out of 20 animals. No oedema was observed. Thus, the test item induced skin sensitising cutaneous reactions in 2 out of 20 (i.e. 10%) guinea-pigs. Treatment with the vehicle caused no dermal irritation. No cutaneous reactions were observed in the control group after challenge.

Conclusion

It was concluded that under the conditions of the Maximization test, [redacted] not cause delayed contact hypersensitivity in guinea pigs. The results do not indicate classification of the test material for skin sensitization according to Regulation (EC) 1272/2008, although the reliability of the study cannot be completely confirmed (no positive control included or information on positive historical controls available).

B.6.8.2.5.6 Repeated dose studies

Previous evaluation:	No
DRAR (2016)	New study

Reference:	5-day toxicity study by oral route in rats - [redacted]
Author(s), year:	[redacted] 1992
Report/Doc. number::	C039807/ -
Guideline(s):	OECD 407 (1981)
GLP:	Yes
Deviations from OECD 407 (2008):	Only few parameters measured (mortality, morbidity, clinical signs and body weight)
Acceptability:	Yes; limited information (range finding study)

Material and methods

[redacted](batch: DA 696, purity: 95.6%) was administered to groups of 3 male and 3 female Sprague-Dawley rats (Crl:CD (SD) BR) at concentrations of 0 (corn oil), 250, 500, 1000 and 2000 mg/kg bw per dayay orally by gavage for a period of 6 days. Mortality and signs of morbidity and clinical signs of toxicity were observed twice a day. Body weight was recorded on days 1, 3 and 6, and food consumption was determined in 3-day intervals. No macroscopic examinations were performed.

Results

No mortalities occurred during the study period. The daily administration of the test substance for 6 days was well-tolerated in all doses, inducing only hypersalivation in all treatment groups and piloerection in one male of the top dose.

This study was performed as a dose-finding study for an oral 4-week study in the rats of the same strain.

Conclusion

This study was performed as a dose-finding study for an oral 4-week study in the rats of the same strain. No NOAEL was discussed in the study report.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] 4-week toxicity study by oral route in rats
Author(s), year:	[redacted] 1993
Report/Doc. number::	C039805/ -
Guideline(s):	OECD 407 (1981)
GLP:	Yes
Deviations from OECD 407 (2008):	Lower number of animals per group and sex (6), not all organs according to OECD 407 (2008) weighed and investigated
Acceptability:	Yes

Materials and methods

[redacted] (batch: DA 696, purity: 95.6%) was administered to groups of 6 male and 6 female Sprague-Dawley rats (Crl:CD (SD) BR) at concentrations of 0 (corn oil), 50, 220 and 1000 mg/kg bw per dayay orally by gavage for

a period of 4 weeks. Mortality and signs of morbidity were observed twice a day and clinical signs of toxicity were recorded at least daily. Body weight and food consumption were determined in weekly intervals. At the end of the study period, haematological and clinical chemistry investigations as well as urinalysis were performed. Terminal investigations consisted of organ weight determinations, gross necropsy and histopathological evaluations of selected tissues.

Results

No mortalities occurred during the study period. The daily administration of the test substance for 4 weeks induced hypersalivation in all treatment groups (3/12 in 50 mg/kg bw per day, 12/12 in both 220 and 1000 mg/kg bw per day) and a lower body weight (91% and 93% of control) in males at 220 and 1000 mg/kg bw per day (no effects (even slight increase) in females).

Regarding haematological parameters, statistically significant decrease in haemoglobin and MCHC ($p < 0.05$) was measured in females at 220 and 1000 mg/kg bw per day, no effects were observed in males.

A slight but statistically significant lower inorganic phosphorus concentration was noted in females in 1000 mg/kg bw per day group. The individual values for 4 out of 6 females were below the lower limit of the laboratory and were considered treatment related.

Further treatment-related findings included increased relative liver weights ≥ 50 mg/kg bw per dayay in females (113% of control at 50, 123% of control in 220 and 136% of control in 10000 mg/kg bw per day) and at ≥ 200 mg/kg bw per dayay in males (116% of control in 220 and 132% of control in 1000 mg/kg bw per day group) with correlating liver enlargement (≥ 220 mg/kg bw per dayay in males) and dose-related histopathological changes (centrilobular hypertrophy, ≥ 50 mg/kg bw per dayay in females and at 1000 mg/kg bw per dayay in males).

Conclusion

Based on the effects observed at 50 mg/kg bw per day (hypersalivation in 3 from 12 animals, increase in relative liver weight > 10% in females and centrilobular hypertrophy in 1/6 females) no NOAEL can be set for [redacted] in this study.

Comparing [redacted] and triticonazole for their toxicity they appear to have qualitatively and quantitatively different profile. However, since [redacted] is not present in triticonazole technical, the toxicological difference does not have impact on conclusion on triticonazole technical material.

B.6.8.2.5.7 Genotoxicity

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] - Reverse mutation assay by the AMES test
Author(s), year:	Molinier B., 1993
Report/Doc. number::	C039809 / -
Guideline(s):	OECD 471 (1983)
GLP:	Yes
Deviations from OECD 471 (1997):	No
Acceptability:	Yes

Materials and methods

S. typhimurium strains TA 98, TA 100, TA 1535, TA 1537 and TA 1538 were exposed with [redacted] (batch: 07/W, purity: 96.5%) using DMSO as a solvent for 48 - 72 hours. Vehicle and positive controls were included in each experiment. The test item was tested via standard plate test (direct plate incorporation method) in concentrations ranged from 31.25 to 2500 µg/plate in two independent experiments in the presence and absence of metabolic activation (liver S9 mix of Aroclor 1254-induced Sprague Dawley rats).

Results

Precipitation of the test substance was observed at concentrations ≥ 1250 µg/plate with and without metabolic activation. Slight to moderate bacteriotoxicity of the test substance was observed at concentrations ≥ 250 µg/plate and ≥ 62.5 µg/plate with and without S9 mix, respectively. A significant increase in the number of his^+ revertants was not observed either without S-9 mix or after the addition of a metabolizing system. The positive controls induced the appropriate response in the corresponding strains, thus demonstrating the sensitivity of the test system.

Conclusion

According to the results of the study, [redacted] is not mutagenic in the Ames reverse mutation assay under the experimental conditions chosen.

Previous evaluation:	No
DRAR (2016)	New study

Reference:	[redacted] In vitro mammalian cytogenetic test in human lymphocytes
Author(s), year:	Molinier B., 1993
Report/Doc. number::	C039812 / -
Guideline(s):	OECD 473 (1983), EEC 84/449 B 10
GLP:	Yes
Deviations from OECD 473 (1997):	-the number of metaphases scored lower (200) than required (300) by the OECD guidelinefrom 1997
Acceptability:	Yes, but limited based on lower number of scored metaphases

Materials and methods

[redacted] (batch: 07/W, purity: 96.5%) was tested in vitro for its ability to induce structural chromosome aberrations in cultured human lymphocytes (from 2 heathy donors - man and woman). Based on previously assessed cytotoxicity (mitotic index, MI), [redacted] was tested at concentrations of 25, 50 and 100 µg/mL (solubility limit) with and without metabolic activation (liver S-9 mix from Aroclor 1254-induced rats). Without metabolic activation, experiments with 24 h exposure/24 h sampling times and 48 h exposure/48 h sampling times were performed, while in the presence of S9 mix 2 h exposure/24 h and 48 h sampling times were applied. For each independent experiment, negative (culture medium), vehicle (DMSO) and positive controls (cyclophosphamide (CPA) and mitomycine C (MMC) for the experiments with and without metabolic activation, respectively) were included to demonstrate the validity of the experiments and the sensitivity of the test system. Metaphase arrest was induced by colcemid treatment 2 hours prior cell harvest. Two hundred metaphases per dose group, negative and vehicle control, and 100 metaphases per positive control group were analysed for chromosomal aberrations.

Results

Cytotoxicity (MI ≤ 45 ± 5%) was observed in experiments with 24-hour sampling time at 100 µg/mL with S9 mix and with 48-hour sampling time at 100 µg/mL without S9. No significant increase in the number of structural chromosomal aberrations including and excluding gaps either without or with S9 mix were induced by the test item compared with the negative and vehicle control at any exposure and sampling times. The frequencies of aberration in the concurrent negative and vehicle controls were within the range of historical control data. The positive controls MMC and CPA induced substantial chromosome damage in any experiment that was within the historical range, demonstrating the ability of the system to detect known clastogens.

Conclusion

Based on the results of this study, [redacted] has no clastogenic properties in cultured human lymphocytes under the in vitro conditions chosen.

B.6.8.2.6 Methanol

According to *the Guidance Document on the assessment of the equivalence of technical materials of substances regulated under Regulation (EC) No 1107/2009* methanol is considered a toxicologically relevant impurity, contained in the technical active ingredient triticonazole up to a level of 0.3%. Although the content of methanol in triticonazole does not influence classification of triticonazole, methanol poses more severe intrinsich properties and is, therefore, considered to be a relevant impurity.

The harmonized classification (CLP regulation EC 1272/2008) regarding toxicological properties of methanol is acutely toxic by oral, dermal and inhalation route (Acute Tox 3, H301, H311 and H331) and specific target organ toxicity by single exposure (STOT SE 1, H370). Based on a recent evaluation of the risk assessment committee of ECHA adopted Sept. 12, 2014 no further classification for e.g. reproduction toxicity is warranted.

Methanol (CAS-No. 67-56-1) as a well-known high volume industrial chemical has been evaluated by ECHA. Based on the toxicological database as provided in the ECHA Co-RAP SUBSTANCE EVALUATION REPORT, September 2015, reference values are available both for worker and general population.

A reference value for long-term systemic exposure (DNEL) via dermal route of 6.66 mg/kg bw per dayay is proposed for the general population and a value of 40 mg/kg bw per dayay for worker. The respective values for acute exposure are the same as for long-term exposure. Local effects are assessed to be of low hazard for the dermal route and consequently no threshold is derived. Regarding inhalation, the DNEL for worker is concluded to be 260 mg/m³ and for the general population it is 43.3 mg/m³. Local effects via inhalation have been concluded to be of the same magnitude as systemic effects and thus are the same. Moreover, the respective values for acute exposure are the same as for long-term exposure. A DNEL for oral exposure is not proposed. However, the dermal DNEL is actually based on a recalculation of the systemic concentration after inhalation exposure and thus represents also a systemic reference values that would be obtained when exposed by the oral route. For methanol no matter which route of exposure is taken into account differences in absorption and thus systemic bioavailability are not considered neglecting the high volatility of the compound. Thus, absorption via all routes inhalation, dermal and oral is considered 100%.

For the non-dietary risk assessment (included in Vol 3, B6 CP) both exposure groups worker (during seed treatment operation and during sowing) and general population (bystander and resident) are to be considered. As a worst case expsoure assessment is based on the more conservative systemic long-term DNEL value for the general population of 6.66 mg/kg bw per dayay.

B.6.8.3. Supplementary studies on the active substance

B.6.8.3.1. Immunotoxicity

Previous evaluation:	No
DRAR (2016)	New study (conducted in order to support US registration)

Reference:	BAS 595 F (Triticonazole) - Immunotoxicity study in female Wistar rats - Administration via the diet for 4 weeks
Author(s), year:	[redacted] 2011
Report/Doc. number::	-/ 2011/1268148
Guideline(s):	OPPTS 870.7800
GLP:	Yes
Deviations from Guideline:	- Dosing was 28 instead of at least 30 days - The animals were immunised 6 days before of blood sampling instead of 4 days - Immunisation was by intraperitoneal route instead of intravenous injection - 8 females per dose instead of at least 10 - Only females included in the test - No non-specific immune system investigation included
Acceptability:	Yes, additional information; unfortunately the test was arranged for only 28 days (no effects on adrenals were seen in 28-days oral repeated rat study) and did not include investigations on non-specific immune system. By a study of longer duration with extended measurement of parameters maybe more information on the role of potential adrenal insufficiency on immune system could have been gained

Material and methods

Test Material:	Triticonazole (BAS 595 F)
Description:	solid/ white
Lot/Batch #:	COD-001440
Purity:	91.3%
Stability of test compound:	The test substance was stable over the study period (Expiry date February 11, 2014).
Vehicle control:	Rodent diet
Positive control:	Cyclophosphamide monohydrate (CPA)
Description:	Solid / white
Lot/Batch #:	1362353
Purity:	100% (according to supplier)
Stability of test compound:	According to the supplier (Sigma-Aldrich) the positive control substance was stable over the study period (Expiry date Sep. 2012).
Vehicle for CPA:	Drinking water
Test animals:	
Species:	Rat
Strain:	Crl:WI(Han)

Sex:	Female (it was stetd that females were more sensitive gender based on previously performed repeated dose studies, however, the NOAELs were always the same for males and females)
Age:	34 ± 1 days at delivery; approx. 42 ± 1 days at start of administration
Reason for the selection:	The rat is a frequently used laboratory animal, and there is comprehensive experience with this animal species. Moreover, the rat has been proposed as a suitable animal species by the OECD and the EPA for this type of study
Weight at dosing:	133.3 ± 1.2 g
Source:	[redacted]
Acclimation period:	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:	4 animals per cage in H-Temp (PSU, floor area about 2065 cm^2) cages (TECNIPLAST, Hohenpeißenberg, Germany). Dust-free wooden bedding was used in this study. Wooden gnawing blocks (Type NGM E-022; Abedd® Lab. and Vet. Service GmbH, Vienna, Austria) were used for environmental 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)
Dates of experimental work**:**	19-Apr-2011 - 28-Oct-2011 (In life dates: 27-Apr-2011 (start of administration) to 26-May-2011 (necropsy))

Triticonazole was administered to groups of 8 female rats at dietary concentrations of 0, 500, 1500 and 5000 ppm (corresponding to mean intake levels of 53, 162 and 462 mg/kg bw per dayay, respectively) over a period of 4 weeks. Control animals received the ground diet only. Additionally, 8 female mice were treated orally (gavage) with 4.5 mg/kg bw per dayay Cyclophosphamide monohydrate (CPA; positive control substance for immunotoxicity). CPA was administered as a solution in drinking water at a volume of 10 mL/kg. The administered volume was determined based on the most recently determined body weights.
Six days before necropsy (day 23), all animals received a single intraperitoneal injection (0.5 mL) of a sheep red blood cell (SRBC)-suspension containing 4 x 10^8 cells/mL for immunization.

The stability of the test substance triticonazole in the diet over a period of up to 32 days was proven before the start of the study. Homogeneity and concentration analyses of the diet preparations were performed at the beginning of the administration period for all concentrations.

Table 6.8.2.1-1: Results of homogeneity and concentration control analysis of triticonazole in rodent diet

Nominal dose level [ppm]	Sampling/ Analysis	Concentration Mean ± SD[#] [ppm]	Mean of nominal concentration [%]	Relative standard deviation [%]
500	Apr. 25, 2011/ May 09, 2011	556.9 ± 22.9	111.4	4.1
1500	"/"	1662.7 ± 88.6	110.8	5.3
5000	"/"	5467.3 ± 179.4	109.3	3.3

[#] = Values may not calculate exactly due to rounding of figures

Relative standard deviations of the homogeneity of the triticonazole samples were quite low in the range of 3.3 to 5.3%, which indicates the homogenous distribution of triticonazole in the diet preparations. The actual (mean) average test-substance concentrations were in the range of 109.3 to 111.4% of the nominal concentrations confirming the correctness of the concentrations. No test substance was found in the control samples.

The positive control substance preparation (CPA in drinking water) was prepared once at the beginning of the study, split in daily aliquots and deep frozen at -18°C. The mixtures were applied when reaching room temperature. The concentration control of the CPA solution was performed at the beginning of the study. Since the CPA formulation in drinking water was a solution a homogeneity analysis was redundant. The stability analysis conducted revealed the stability of the CPA solution for 7 and 32 days when stored at room temperature and deep frozen, respectively.
The actual CPA concentrations were in the range of 97.3 to 100.5% of the nominal concentration confirming the correctness of the concentration.

Table 6.8.2.1-2: Results of concentration control analysis of CPA in drinking water

Nominal Concentration [g/100 mL]	Sampling/ Analysis	Analytical concentration [g/100 mL]	Mean of nominal concentration [%]
0.045	Jan. 28, 2010/ Mar. 01, 2010	0.0435	100.5
0.045	Apr. 26, 2011/ Jul. 26, 2011	0.0438	97.3

Means and standard deviations of each test group were calculated for several parameters. Further statistical analyses were performed according to following tables:

Table 6.8.2.1-3: Statistics of clinical examinations

Parameter	Statistical test
body weight and body weight change	For the test substance and the vehicle control groups: A comparison of each group with the control group was performed using DUNNETT's test (two-sided) for the hypothesis of equal means For the vehicle and positive control groups: A comparison of the dose group with the control group was performed using the t-test (two-sided) for the hypothesis of equal means

Table 6.8.2.1-4: Statistics of clinical pathology

Parameter	Statistical test
Clinical pathology 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 Wilcoxon-test (two-sided) for the equal medians

Table 6.8.2.1-5: Statistics of pathology

Parameter	Statistical test
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 for the hypothesis of equal medians

The animals were examined for morbidity or mortality twice daily on working days and once daily on weekends and public holidays. Observation for overt clinical signs of toxicity was performed at least once daily. Detailed clinical observations were performed in all animals prior to the start of the administration period and weekly thereafter. The animals were transferred to a standard arena (50 x 37.5 cm with sides of 25 cm high). The findings were ranked according to the degree of severity, if applicable.

1. abnormal behavior during handling	10. abnormal movements
2. fur	11. impairment of gait
3. skin	12. lacrimation
4. body posture	13. palpebral closure
5. salivation	14. exophthalmus
6. respiration	15. feces (appearance/consistency)
7. activity/arousal level	16. urine
8. tremors	17. pupil size
9. convulsions	

Food consumption was determined weekly for each cage. The average food consumption per cage was used to estimate the mean food consumption in grams per animal per day.

Drinking water consumption was determined by daily visual inspection of the water bottles for any overt changes in volume.

The body weight of the animals was determined before the start of the administration period (in order to randomize the animals), at the start of the treatment (day 0), and twice weekly thereafter. The difference between the body weight on the respective day of weighing and the body weight on day 0 was calculated as body weight change.

Analysis of the primary immune response

Immunisation

- Sterile, heparinized sheep blood was washed with sterile 0.9% NaCl solution and adjusted to $4x10^8$ RBC/mL.
- On study day 23, each rat was immunized with 0.5 mL of the SRBC solution injected intraperitoneally.
- On study day 29 blood was taken in the morning from fasted, isoflurane anesthetized animals from the retro-orbital venous plexus. The blood sampling procedure and the subsequent analysis of the serum samples were carried out under internal laboratory quality control conditions and in a randomized sequence to assure reliable test results.

Anti-SRBC IgM ELISA:

- The Anti SRBC IgM ELISA of Life Diagnostics Inc. (cat no. 4200-2) was performed according to manufacturer instructions.
- Each serum sample was applied to the ELISA in two dilutions, i.e. 1:500 and 1:1000 (positive control group samples: 1:50 and 1:100).
- OD values of the sample dilutions outside of the linear range of the standard curve were repeated with an appropriate further dilution.
- Generally, two in-house controls were measured with each test run.
- The ELISA was measured with a Sunrise MTP-reader, Tecan AG, Maennedorf, Switzerland, and evaluated with the Magellan-Software of the instrument producer.

The animals were sacrificed by decapitation under isoflurane anesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. Animals that died within the test substance application period were necropsied as soon as possible after their death. The following weights were determined for all animals sacrificed at scheduled dates and the organs or tissues were fixed in 4% buffered formaldehyde solution:

1. Anesthetized animals (all gross lesions fixed in formaldehyde)
2. Liver
3. Spleen
4. Thymus

From the liver, each one slice of the Lobus dexter medialis and the Lobus sinister lateralis was fixed in Carnoy's solution and embedded in paraplast.
No histopathological examinations were performed.

Results

No clinical signs of toxicity were observed in animals treated with Triticonazole at any dose level. Two of eight animals (Nos. 35 and 36) treated with CPA showed signs of general poor condition like moderately labored respiration and slight piloerection.

Regarding mortality, animal No. 35 of the positive control group was found dead and cannibalized on study days 25. Animal No. 36 of the same test group was found dead on study day 28. Both animals showed clinical findings on the day before premature death. The premature deaths of these animals were likely due to treatment (gavage error). However, a relation to treatment with CPA was possible.

No test substance-related findings were observed for the food and water consumption.

Body weight in animals of 5000 ppm group was reduced from day 7 until day 28, with a maximum of 91.1% body weight compared to control on day 14.

Body weight gain in animals of 5000 ppm group was reduced after the first study week and reached a significant maximum reduction of about 68% of control on study days 10 and 14. These finding occurred in the absence of a similar pattern in mean food consumption, and therefore, were considered to be treatment-related, direct adverse systemic effects of triticonazole.

While at 500 ppm no effect on body weight was observed, body weight change was impaired by more than 10%. Since no comparable effect was observed at 1500 ppm, reduced body weight gain at 500 ppm was not considered treatment-related. The significant deviation of the mean body weight gain between study days 0-3 at 500 ppm was assessed as being incidental.

Body weights of animals treated with CPA were not significantly reduced from day 7 until day 28, with a maximum of 94.3% of control values on day 24. Maximal reduction in body weight gain was 84.3% of control on day 24.

Table 6.8.2.1-6: Mean body weight of rats administered Triticonazole or Cyclophosphamide for 28 days

Treatment	**Triticonazole**				**CPA**
Dose level	**0 ppm**	**500 ppm**	**1500 ppm**	**5000 ppm**	**4.5 mg/kg bw per dayay**
Body weight ± SD [g] (% control)					
Day 0	134.6 ± 6.4	134.3 ± 6.9 (99.8)	132.9 ± 6.7 (98.7)	131.8 ± 6.9 (97.9)	132.7 ± 6.0 (98.6)
Day 3	143.2 ± 7.1	133.8 ± 7.6 (93.4)	143.2 ± 8.6 (100)	141.6 ± 8.5 (98.9)	142.5 ± 9.3 (99.5)
Day 7	153.8 ± 7.1	147.6 ± 9.0 (95.9)	151.6 ± 11.0 (98.6)	146.8 ± 9.8 (95.4)	150.9 ± 9.9 (98.1)
Day 10	161.1 ± 9.8	157.0 ± 9.5 (97.2)	159.3 ± 11.8 (98.6)	149.9 ± 12.0 (93.1)	158.1 ± 8.2 (98.1)
Day 14	173.9 ± 10.1	168.2 ± 8.8 (96.8)	170.4 ± 10.0 (98.0)	**158.4*** ± 13.4 (91.1)	168.8 ± 9.4 (97.1)
Day 24	193.2 ± 12.9	184.5 ± 8.5 (95.5)	185.7 ± 9.0 (96.1)	177.4 ± 19.1 (91.8)	182.2 ± 12.7 (94.3)
Day 28	202.2 ± 11.5	194.3 ± 9.4 (96.1)	195.6 ± 9.8 (96.7)	187.5 ± 19.0 (92.7)	195.2 ± 12.1 (96.5)

*: $p \leq 0.05$

Table 6.8.2.1-7: Mean body weight gain of rats administered Triticonazole or Cyclophosphamide for 28 days

Treatment	**Triticonazole**				**CPA**
Dose level	**0 ppm**	**500 ppm**	**1500 ppm**	**5000 ppm**	**4.5 mg/kg bw per dayay**
Body weight gain ± SD [g] (% control)					
Day 0 →3	8.6 ± 2.2	**-0.5**** ±3.6 (-5.8)	10.3 ± 5.1 (119.8)	9.8 ± 3.4 (113.9)	9.7 ± 5.9 (112.7)
Day 0 →7	19.3 ± 4.1	13.3 ± 4.3 (68.9)	19.0 ± 7.1 (98.4)	15.0 ± 4.0 (77.7)	18.2 ± 6.0 (94.3)
Day 0 →10	26.5 ± 6.5	22.7 ± 3.8 (85.7)	26.4 ± 9.1 (99.6)	**18.2*** ± 5.8 (68.7)	25.3 ± 4.5 (95.5)
Day 0 →14	39.3 ± 7.4	33.9 ± 3.0 (86.3)	37.5 ± 7.1 (95.4)	**26.7**** ± 7.8 (67.9)	36.0 ± 6.7 (91.8)
Day 0 →24	58.6 ± 12.4	50.1 ± 4.9 (85.6)	52.8 ± 6.2 (90.1)	**45.6*** ± 13.3 (77.9)	49.4 ± 10.8 (84.3)
Day 0 →28	67.6 ± 10.7	60.0 ± 4.2 (88.7)	62.7 ± 6.5 (92.7)	**55.7*** ± 13.0 (82.4)	62.0 ± 9.9 (91.7)

*: p≤0.05, **: p≤0.01

Immunological analysis:

Analysis of the primary T-cell dependent antibody response (TDAR)

Six days after immunization, no changes in the SRBC IgM titres were found in female rats treated with the test substance, whereas the SRBC titres were significantly lower in rats of test group 4 (CPA, positive control group).

Table 6.8.2.1-8: Analysis of the specific primary (IgM) immune response to SRBC in rats treated with triticonazole or cyclophosphamide for 28 days

Treatment	**Triticonazole**				**CPA**
Dose [ppm]	0	500	1500	5000	
[mg/kg bw per dayay]		53	162	462	4.5
Specific IgM Titer (U/mL)					
Mean	42129 ± 16647	25692 ± 9511	40858 ± 27242	43540± 25949	**15456**** ± 5824
Median	38297	27973	39414	42298	16622

* p ≤ 0.05; ** p ≤ 0.01 (Kruskal-Wallis and Wilcoxon-test. two sided)

The absolute and relative mean weights of spleen and thymus of animals in test groups 50, 1500, and 5000 ppm showed no differences compared to the control group.

The positive control group (CPA) revealed significant decreases of spleen and thymus weights, which corresponded to the expected result.

The absolute and relative liver weight of animals treated with 5000 ppm was significantly increased by 17% (p<0.01) and 28% (p<0.01), respectively, and was regarded as treatment-related. In animals of the 1500 ppm dose group, the marginal (7%), but statistically significant increase in relative live weight was not regarded as adverse.

Table 6.8.2.1-9: Mean absolute and relative organ weights of female rats treated with triticonazole or cyclophosphamide for at 28 days

	Triticonazole				CPA
Dose [ppm]	**0**	**500**	**1500**	**5000**	
Terminal bodyweight ± SD (g) (% of control)	184.81 ± 12.12	176.83 ± 9.12 (96)	179.06 ± 9.47 (97)	169.63 ± 17.18 (92)	176.2 ± 10.36 (95)
Liver, absolute ± SD (g) (% of control)	5.13 ± 0.34	5.12 ± 0.33 (100)	5.30 ± 0.4 (103)	**6.02**** ± 0.74 (117)	5.15 ± 0.89 (100)
Liver, relative ± SD (% of control)	2.78 ± 0.11	2.90 ± 0.12 (104)	**2.96*** ± 0.18 (107)	**3.56****± 0.32 (128)	2.91 ± 0.36 (105)
Spleen, absolute ± SD (g) (% of control)	0.45 ± 0.05	0.45 ± 0.06 (99)	0.42 ± 0.05 (93)	0.45 ± 0.06 (99)	**0.27**** ± 0.02 (59)
Spleen, relative ± SD (% of control)	0.24 ± 0.02	0.25 ± 0.03 (103)	0.24 ± 0.02 (96)	0.27 ± 0.04 (109)	**0.15**** ± 0.01 (62)
Thymus, absolute ±SD (mg) (% of control)	475.25 ± 64.44	453.13± 91.38 (95)	477.63 ± 22.37 (100)	449.88 ± 36.3 (95)	**316.83****± 61.36 (67)
Thymus, relative ± SD (% of control)	0.26 ± 0.03	0.26 ± 0.05 (100)	0.27 ± 0.01 (104)	0.27 ± 0.04 (104)	**0.18**** ± 0.04 (70)

* p □ 0.05; ** p □ 0.01 (Kruskal-Wallis and Wilcoxon-test, two sided)

The enlarged liver of two animals in the 5000 ppm test group was regarded as treatment-related. All other gross lesions observed were considered incidental and not related to treatment.

Animal Nos. 35 and 36 of the positive control group (CPA) died spontaneously without any signs of relationship to the treatment. However, a treatment-relation could not be excluded totally.

Conclusion

Under the conditions of the study, triticonazole did not reveal any signs of immunotoxicity when administered via the diet over a period of 4 weeks to female Wistar rats. The NOAEL for the immunotoxicity was determined to be 5000 ppm (462 mg/kg bw per dayay; highest dose tested). The NOAEL for systemic toxicity was set to 1500 ppm (162 mg/kg bw per dayay), based on treatment-related changes (reduced body weight (gain) and increased absolute and relative liver weights) in the next higher dose group (5000 ppm).

The oral administration of the positive control substance Cyclophosphamide (4.5 mg/kg bw per dayay) led to findings indicative of immunotoxicity by TDAR assay. This was represented by significantly lower SRBC IgM antibody titres as well as reduced spleen and thymus weights.

RMS concludes that the study was conducted mostly according to guideline. However, although no effects on immune system were observed it is noted that a study of longer duration (no effects on adrenals were observed after oral 28 days range-finding study in rats) with inclusion of parameters for non-specific immune system could have provided more information on the effects of triticonazole on adrenal insufficiency and immune system

B.6.8.3.2. Pharmacology

Previous evaluation:	No

DRAR (2016)	New study (conducted in order to support Japanaese registration)

Reference:	General pharmacology study of BAS 595 F
Author(s), year:	[redacted] 2014
Report/Doc. number::	-/ 2014/8000175
Guideline(s):	JMAFF No 12 Nosan No 8147, Pharmacology studies (2-2-1) of Data Requirements for Supporting Registration of Pesticides, MAFF 25-Shouan-630 (May 2013)
GLP:	Yes
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Materials and methods

Test Material:	Triticonazole (BAS 595 F)
Description:	solid (powder) / white
Lot/Batch #:	COD-001440
Purity:	91.3%
Stability of test compound:	The test substance was stable over the study period (Expiry date February 11, 2014)
Vehicle control:	0.5% (w/v) methyl cellulose (MC) 400 solution
Test animals:	
Species:	Mouse / Rat
Strain:	Crlj:CD1(ICR) / Crl:CD(SD)
Sex:	Female mice (more sensitive gender based on results of a preliminary test) / Female rats (more sensitive gender based on previously performed acute oral toxicity study)
Age:	5 / 6 weeks at delivery; approx. 6 / 7 weeks at start of administration
Reason for the selection:	The rat and mouse are frequently used laboratory animals, and there is comprehensive experience with these animal species.
Weight at dosing:	19.3 to 22.4 g / 132.0 to 172.9 g
Source:	[redacted]
Acclimation period:	5 days
Diet:	commercial diet (CRF-1), ad libitum
Water:	Tap water, ad libitum
Housing:	animals were housed individually in metal mesh cages (mouse: 10 x 19.6 x 13 cm; rat: 19.7 x 26.3 x 18 cm (WDH)).
Environmental conditions:	
Temperature:	20 - 26 °C
Humidity:	35 - 70 %
Air changes:	at least 12/hour
Photo period:	12 h light / 12 h dark (07:00 - 19:00 / 19:00 - 07:00)

Animal assignment and treatment

The animals were assigned to the treatment groups by means of computer generated randomization list based on body weights.

Triticonazole was administered to groups of 5 female rats and mice in concentrations of 0, 200, 600 and 2000 mg/kg bw orally by gavage with an administration volume of 1 mL/100 g bw. Control animals received 0.5% MC solution.

After the experiments, the surviving and unused (rejected animals from the group assignment) animals were transferred to Animal Care Section.

Test substance preparation

Vehicle

0.5% (w/v) MC solution was prepared by weighing the required amount of methyl cellulose 400 (e.g. 2.5 g for 500 mL preparation) and dispersing in warm distilled water for injection (about 2/3 of final preparation volume). This water dispersion was stirred under room temperature to dissolve dispersoid, and was diluted to the required volume with distilled water for injection. The prepared vehicle was stored at 4°C and used within 14 days after preparation.

Test substance

The weighed amount of test substance for 200 mg/mL stock solution was corrected using the purity (correction factor: 1.10 [100%/91.3%]), and ground well in an agate mortar with agate pestle and mixed with a small volume of vehicle. Then, this suspension and the wash of the mortar were transferred into a graduated cylinder. The suspension was diluted to the required volume with the vehicle to give a stock solution of 200 mg/mL was serially diluted to make 60 and 20 mg/mL dosing solutions, for the 2000, 600 and 200 mg/kg bw dose groups, respectively for an application volume of 10 mL/kg.

Statistics

Means and standard deviations of each test group were calculated for several parameters. First, the raw data were analysed by Bartlett's test for comparison of homogeneity of variances. Homogeneous data were analysed by Dunnett's multiple comparison test for significant differences between the control group and each test substance-treated group. Heterogeneous data by Bartlett's test were subjected to Steel's test for significant differences between the control group and each test substance -treated group. Significance of differences were two-sided 5% in Bartlett's test, and two-sided 5% and 1% in other tests.

General behavior

The observation of general behavior was performed with mice as well as with rats.

According to the Irwin's Comprehensive Observation Assessment, the animals were observed for the presence and frequency of the symptoms listed below prior to the administration and at 0.5, 1, 2, 3, 6 and 24 hours after the administration of the dosing solution. At each observation time point, animals were observed for behavioral parameters for 2 minutes in the polycarbonate cage (34.5 x 40.3 x 17.7 cm (WDH)), then the items of reflex and muscle tone were examined.

The observed parameters were:

- Body position
- Behavior
- Autonomic nervous system
- Reflex and muscle tone

Effects on respiratory-cardiovascular system

The observations of the respiratory-cardiovascular systems were performed only with rats.

Respiratory rate

The respiratory movement was recorded on sampling/analyzing software (PowerLab/4S, ADInstuments) with a measuring device (Whole body plethysmography, emka Technologies), under food and water deprivation, for 30 minutes before administration and form just after to 6 hours after the administration.

Respiratory rate (resp. times/minute) and tidal volume (VT, mL) were measured prior to and 0.5, 1, 2, 3 and 6 hours after administration. The changes from the values before administration were calculated. These parameters were calculated from the 1-minute interval data at each measuring point using sampling/analyzing software (PowerLab).

Blood pressure and heart rate

The systolic blood pressure (SBP), diastolic blood pressure (DBP), mean blood pressure (MBP) and heart rate (HR) were measured with a non-invasive manometer (BP-98A, Softron) before and at 1, 2, 3 and 6 hours after administration of the dosing solutions. Rats were warmed for about 15 minutes before measurement using the warming equipment set at 38°C. The animals were measured 3-times at each time point, and the mean value was calculated. The changes from the values before administration were also calculated.

Results

General behaviour

Mice

Numbers of defecation in the 600 and 2000 mg/kg bw groups were significantly lower than that of the control at 6 hours after test item administration. However, this finding was not observed at 24 hours after administration and suppressed changes were not found in any time point of observation. In addition, there were no similar effects on the observation of general behavior in rats. Therefore, this change was judged to be not a test substance-related effect.
No other treatment-related effects in other parameters were observed in mice.

Rats

There were no abnormal clinical signs in any of the test substance-treated groups. Moreover, there were no significant differences in the numbers of appearance of grooming, yawn, urination and defecation between the test substance-treated groups and control groups.

Respiratory-cardiovascular system

Respiratory rate

There were no significant differences in the respiratory rate or the values of change from the value before administration of this parameter between the treatment groups and control group at each time point.

There were no significant differences in the tidal volume between the treatment groups and control group at each time point. The value of change from the value before administration of tidal volume in the 600 mg/kg group was significantly higher than that of control at 1-hour time point after administration.

Table 6.8.2.2-1:Effect of Triticonazole on tidal volume (VT) in rats

Test substance	**Vehicle (control)**	**Triticonazole**		
Dose (mg/kg bw)	10 mL/kg	200	600	2000
No. of animals used	5	5	5	5
Tidal volume (mL ± SD) **(change from before administration)**				
Before	1.00 ± 0.32	1.02 ± 0.15	1.00 ± 0.2	0.75 ± 0.31
0.5h	1.12 ± 0.36 (0.12 ± 0.12)	0.96 ± 0.12 (-0.06 ± 0.07)	1.42 ± 0.27 (0.42 ± 0.18)	1.32 ± 0.36 (0.57± 0.32)
1h	0.94 ± 0.30 (-0.06 ± 0.12)	0.95 ± 0.17 (-0.08 ± 0.14)	1.48 ± 0.32 (0.48 ± 0.16)*	1.11 ± 0.30 (0.37 ± 0.15)
2h	0.85 ± 0.24 (-0.15 ± 0.17)	1.13 ± 0.14 (0.11 ± 0.15)	1.16 ± 0.31 (0.16 ± 0.15)	1.12 ± 0.41 (0.37 ± 0.13)
3h	0.95 ± 0.26 (-0.05 ± 0.15)	1.06 ± 0.20 (0.04 ± 0.13)	1.24 ± 0.34 (0.23 ± 0.15)	0.98 ± 0.30 (0.23 ± 0.04)
6h	0.98 ± 0.26 (-0.02 ± 0.17)	0.93 ± 0.21 (-0.09 ± 0.12)	1.16 ± 0.21 (0.15 ± 0.08)	0.96 ± 0.29 (0.21 ± 0.11)

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

Blood pressure and heart rate

When compared with the control group, statistically significant higher systolic blood pressure (SBP) values and higher values of changes from the values before administration were noted in the 600 mg/kg bw group at 1 hour after administration and in the 2000 mg/kg group at 1 and 2 hours after administration.

Significant higher diastolic blood pressure (DBP) values were noted in the 600 mg/kg bw group at 2 hours after administration and in the 2000 mg/kg group at 1 and 2 hours after administration. Significant higher values of DBP from the values before administration were noted in the 200 and 2000 mg/kg groups at 1 hour after administration.

Significant higher values of changes of mean blood pressure (MBP) from the values before administration were noted in the 200, 600 and 2000 mg/kg bw groups at 1 hour after administration.

The differences of blood pressure in the 200 and 600 mg/kg bw groups were less than 10 mmHg, and there were no changes in MBP values throughout this study. Therefore, these changes were assessed to be not test substance-related effects.

In the 2000 mg/kg bw group, the SBP, DBP and MBP values were significantly higher or tended to be high at 1 and 2 hours after administration. These changes in the 2000 mg/kg bw group were suggested to be test substance-related effects. However, normal blood pressure was noted at 3 hours after administration, so that these high values were considered to be transient.

Table 6.8.2.2-2: Effect of Triticonazole on blood pressure in rats

Test substance	**Vehicle (control)**	**Triticonazole**		
Dose (mg/kg bw)	**10 mL/kg**	200	600	2000
No. of animals used	5	5	5	5
Systolic blood pressure (mmHg ± SD) (change from before administration)				
Before	101.9 ± 2.2	103.5 ± 2.2	102.1 ± 3.7	103.2 ± 4.6
1h	99.3 ± 3.7 (-2.7 ± 2.7)	106.2 ± 3.4 (2.7 ± 3.3)	111.7 ± 0.6 # (9.6 ± 3.4)*	116.2 ± 5.3 (13.0 ± 3.5)**
2h	95.7 ± 5.1 (-6.3 ± 4.2)	108.6 ± 6.0 (5.1 ± 6.5)	110.5 ± 2.3 (8.4 ± 5.9)	121.9± 7.0** (18.7 ± 6.2)*
3h	104.5 ± 7.7 (2.5 ± 7.3)	110.1 ± 5.0 (6.6 ± 5.6)	110.5 ± 3.1 (8.4 ± 4.7)	108.3 ± 4.6 (5.1 ± 4.9)

6h	101.7 ± 2.8 (-0.3 ± 4.9)	109.5 ± 4.0 (6.1 ± 4.3)	101.9 ± 3.3 (-0.1 ±5.2)	109.6 ± 3.5 (6.4 ± 1.7)
Diastolic blood pressure (mmHg ± SD) (change from before administration)				
Before	79.5 ± 4.8	77.5 ± 2.9	82.8 ± 3.2	80.6 ± 3.6
1h	77.0 ± 4.6 (-2.5 ± 3.4)	86.7 ± 1.7 (9.3 ± 2.8)*	89.2 ± 2.1 (6.4 ± 2.2)	91.3 ± 5.5* (10.7 ± 3.6)*
2h	77.3 ± 1.9 (-2.1 ± 3.2)	85.6 ± 3.8 (8.1 ± 4.4)	91.1 ± 1.8* (8.3 ± 4.6)	91.6 ± 5.4* (11.0 ± 7.2)
3h	83.5 ± 7.1 (4.0 ± 5.0)	90.0 ± 4.6 (12.6 ± 5.4)	91.8 ± 2.2 (8.9 ± 3.5)	86.3 ± 3.5 (5.7 ± 6.4)
6h	80.9 ± 3.5 (1.4 ± 6.7)	90.0 ± 4.3 (12.5 ± 4.8)	83.3 ± 1.7 (0.5 ± 3.6)	84.3 ± 3.9 (3.7 ± 2.5)
Mean blood pressure (mmHg ± SD) (change from before administration)				
Before	86.9 ± 3.7	86.1± 2.6	89.1± 3.3	88.1 ± 3.8
1h	84.2 ± 4.2 (-2.7 ± 2.5)	93.1 ± 2.1 (7.0 ± 2.3)*	96.6 ± 1.5 (7.5 ± 2.3)*	99.5 ± 5.2* (11.4 ± 2.9)**
2h	83.5 ± 2.8 (-3.4 ± 2.2)	93.2 ± 4.4 (7.1 ± 5.0)	97.4 ± 2.0 (8.3 ± 5.0)	101.6 ± 5.0* (13.5 ± 6.8)
3h	90.3 ± 7.2 (3.5 ± 5.5)	96.3 ± 4.6 (10.2 ± 5.4)	89.3 ± 2.5 (0.2 ± 3.8)	92.6 ± 3.5 (4.6 ± 5.8)
6h	87.7 ± 2.9 (0.8 ± 5.8)	96.3 ± 4.1 (10.2 ± 4.4)	89.3 ± 2.1 (0.2 ± 4.0)	92.6 ± 3.8 (4.6 ± 1.9)

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

There were no significant differences in the heart rate value or the value of change of it from the value before administration at each time point between test substance-treated groups and control group.

Conclusion

Based on the results of this study, triticonazole did not affect the general behavior of mice and rats. In the cardiorespiratory system, no effects were noted on the respiratory parameters in rats. Blood pressure was elevated transitory in rats treated with 2000 mg triticonazole/kg bw.

B.6.8.3.3. Hepatotoxicity

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by toxcast chemicals
Author(s), year:	Rotroff D.M. et al., 2010
Report/Doc. number::	-/ 2010/1233112
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This study is part of the EPA ToxCast program. Primary human hepatocyte cultures as model system were used to characterize the concentration- and time-response of the 320 ToxCast chemicals for changes in expression of genes regulated by nuclear receptors. Fourteen gene targets were monitored in quantitative nuclease protection assays: six

representative cytochromes P-450, four hepatic transporters, three Phase II conjugating enzymes, and one endogenous metabolism gene involved in cholesterol synthesis. These gene targets are sentinels of five major signalling pathways: AhR, CAR, PXR, FXR, and PPARalpha. Besides gene expression, the relative potency and efficacy of these chemicals to modulate cellular health and enzymatic activity were assessed. Results demonstrated that the culture system was an effective model of chemical-induced responses by prototypical inducers such as phenobarbital and rifampicin. Gene expression results identified various ToxCast chemicals that were potent or efficacious inducers of one or more of the 14 genes, and potent to interfere with the 5 nuclear receptor signalling pathways. Significant relative risk associations with rodent in vivo chronic toxicity effects are reported for the five major receptor pathways. These gene expression data are being incorporated into the larger ToxCast predictive modelling effort.

Triticonazole was active in the following NVS_ADME assays: rCYP1A1, hCYP2C18, rCYP3A1, rCYP2D2, rCYP2C11, rCYP3A2, rCYP2B1, hCYP2C19, hCYP1A1, rCYP2A2.

Expression of genes that were induced by triticonazole was linked to AhR, CAR, and PXR.T results. Together with the results of the transactivation studies (Shah et al., 2011a) this information is considered of less relevance since triticonazole did not induce liver tumors.

Material and methods

Test Material:	Triticonazole (CAS No. 131983-72-7)
Purity:	>90%

Gene expression analysis

ToxCast^{TM} chemicals were assessed for induction or suppression of xenobiotic metabolizing enzyme and transporter gene expression using primary human hepatocytes. The mRNA levels of 14 target and 2 control genes were measured: ABCB1, ABCB11, ABCG2, SLCO1B1, CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A4, UGT1A1, GSTA2, SULT2A1, HMGCS2, and control genes ACTB, GAPDH. These genes represent 5 nuclear receptor signaling pathways: AHR, CAR, PXR, PPARa and FXR. Gene expression was quantitatively measured by nuclease protection assays at 5 concentrations, 3 time points (6, 24, 48 hr), in 4 replicate wells. Hepatocytes from 2 male donors were isolated and cultured with 6 reference chemicals and 320 ToxCast phase I chemicals. CYP1A1/2 enzymatic activity and cell morphology were assessed in each well. Concentration-response curves were generated for 13813 chemical, time and gene combinations. EC_{50} (effective concentration 50%) and E_{max} (effective maximum) values from the curves determined nuclear receptor pathways modulated by ToxCast chemical exposures. Chemical potency and efficacy were determined relative to reference chemicals and correlated with in vivo chronic hepatotoxicity endpoints from EPA's ToxRefDB database. Using ≥40% efficacy and <40 µM EC_{50}, a preliminary analysis of the 5 nuclear receptor pathways at 48 hr was conducted based on expression of 5 representative genes for each pathway.

Results

Gene expression analysis

Triticonazole was active in 4 assays (CYP1A1/2, CYP3A4, and CYP2B6). These results indicate that triticonazole may induce induction of mRNA expression that is mediated by the CAR/PXR or AhR receptors. Triticonazole did not induce mRNA induction through the other investigated nuclear receptors.

Table 6.8.2.3-1: Summary of gene expression analysis for triticonazole (values extracted from ToxCast database)

Gene target	Primary receptor	AC_{50} [µM]	HitCall	AC_{50} [µM]	HitCall	AC_{50} [µM]	HitCall
		6 h		**24 h**		**48 h**	
CYP2C9	CAR/PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
CYP1A1	AhR	1000	Inactive	**4.26**	**Active**	**3.72**	**Active**
CYP1A2	AhR/Others	1000	Inactive	**3.47**	**Active**	**1.37**	**Active**
CYP2C19	CAR/PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
CYP3A4	CAR/PXR	1000	Inactive	1000	Inactive	**3.15**	**Active**
CYP2B6	CAR/PXR	1000	Inactive	**3.60**	**Active**	**3.97**	**Active**
GSTA2	CAR/PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
SULT2A1	CAR/PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
UGT1A1	CAR/AhR/PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
ABCB1	CAR/PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
HMGCS2	PPARα/Others	1000	Inactive	1000	Inactive	1000	Inactive
ABCB11 (BSEP)	FXR/Others	1000	Inactive	1000	Inactive	1000	Inactive
ABCG2 (BRCP)	CAR/PXR/PPARα/Others	1000	Inactive	1000	Inactive	1000	Inactive
SLCO1B1 (OATP-C)	PXR/Others	1000	Inactive	1000	Inactive	1000	Inactive

Conclusion

Triticonazole was active in 4 assays (CYP1A1/2, CYP3A4, and CYP2B6), indicating that triticonazole may induce expression of mRNA that is mediated by the CAR/PXR or AhR receptors.

As triticonazole did not induce liver tumors in vivo, these results are of minor relevance for the hazard assessment of triticonazole.

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Using nuclear receptor activity to stratify hepatocarcinogens
Author(s), year:	Shah I. et al., 2011
Report/Doc. number::	-/ 2011/1295091
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This study is part of the EPA ToxCast program. The authors investigated the effects of 309 environmental pesticides on nuclear receptors using primary human hepatocytes, HepG2 cells transfected with a multiple reporter transcription unit (MRTU) library consisting of 48 transcription factor binding sites, cis reporter gene assays and cell free and cell based cytochrome P450 assays. The resulting data was used to calculate an aggregate scaled activity score for different nuclear receptors (CAR, PXR, PPAR, AhR, SR, RXR), which was then correlated to lesion progression data extracted from EPA's pesticide database.

Triticonazole, together with Flutolanil and Oxyfluorfen, was grouped into category VII A; group A indicates, that the compound can activate the nuclear receptors AhR, CAR and PXR, but only produces mild or no lesions. The rationale for this grouping is largely based on the activation of the reporter genes.

Triticonazole was tested positive, amongst others, in the following activation assays: ATG_PXR_TRANS_up, ATG_PXRE_CIS, ATG_PPRE_CIS, Tox21_AR_LUC_MDAKB2_Agonist. However, as triticonazole did not induce liver tumors, these results are of minor relevance for the hazard assessment of triticonazole. Triticonazole was not associated with the activation in other assays targeting estrogen receptor.

Material and methods

Test Material:	Triticonazole (CAS No. 131983-72-7)
Purity:	>90%

Multiplexed Transcription Reporter Assays

A multiple reporter transcription unit (MRTU) library consisting of 48 transcription factor binding sites was transfected into the HepG2 human liver hepatoma cell line. In addition to the cis-acting reporter genes (CIS), a modification of the approach was used to generate a trans-system (TRANS) with a mammalian one-hybrid assay consisting of an additional 25 MRTU library reporting the activity of nuclear receptor super-family members. Based on an initial cytotoxicity screen, the maximum tolerated concentration (MTC) was derived as one-third the calculated IC_{50} or, if no IC_{50} was determined, the MTC was set to 100 mM. Chemicals were then tested in the CIS and TRANS assays at seven concentrations starting at the MTC and followed by threefold serial dilutions. These assays were performed by Attagene Inc. (Morrisville NC) under contract to EPA.

Cell-free HTS Assays

These are a collection of biochemical assays measuring binding constants and enzyme inhibition values. Chemicals were initially screened at a single concentration in duplicate wells at a concentration of 10 mM for cytochrome P-450 assays and 25 mM for all others. Chemicals that showed significant activity were then run in concentration response format, from which an AC50 value was extracted. For concentration response, 8 concentrations were tested in the ranges 0.00914-20 mM for cytochrome P-450 assays and 0.0229-50 mM for other assays. These assays were run by Caliper Life Sciences (Hanover, MD) under contract to EPA. Short assay descriptions are available at: http://www.caliperls.com/products/contract-research/in-vitro/.

Cell-based HTS Assays

These assays measure binding constants and enzyme inhibition values for nuclear receptors. The targets include AR, ER, FXR, LXR, PPARa, PPARd, PPARc, RXRa, RXRb and PXR. Each of the nuclear receptor targets was measured in agonist mode. Assays were run at the NIH Chemical Genomics Center (Rockville, MD).

<u>Tox21 Assay Overview:</u>

Androgen receptor (AR), a nuclear hormone receptor, plays a critical role in AR-dependent prostate cancer and other androgen related diseases. Endocrine disrupting chemicals (EDCs) and their interactions with steroid hormone

receptors like AR may cause disruption of normal endocrine function as well as interfere with metabolic homeostasis, reproduction, developmental and behavioral functions. To identify the compounds that activate AR signaling, GeneBLAzer AR-UAS-bla-GripTite cell line (Invitrogen, Carlsbad, CA, USA) containing a beta-lactamase reporter gene under the control of an upstream activator sequence (UAS) stably integrated into HEK293 cells was used to screen the Tox21 10K compound library. Compounds were also tested for auto fluorescence that may interfere with the biological target readout resulting in potential false positives and/or negatives.

Tox21 Assay Protocol Summary:

AR-bla cells were dispensed at 2000 cells/6 uL/well of assay medium into black wall/clear-bottom 1536-well plates using a Multidrop Combi (ThermoFisher Scientific, Waltham, MA) dispenser. After the assay plates were incubated at 37°C and 5% CO_2 for 4 h, 23 nL of compounds dissolved in DMSO, positive controls or DMSO only was transferred to the assay plate by a Pintool station. The plates were incubated at 37°C and 5% CO_2 for 16 h. Then 1 uL of LiveBLAzerTM B/G FRET substrate was added using a Bioraptr Flying Reagent Dispenser (FRD) workstation. The plates were incubated at room temperature for 2 h, and fluorescence intensity was measured by an Envision plate reader.

In vitro data

All data used in the publication from Shah et al. are publicly available from the ToxCast website (www.epa.gov/toxcast). The analysis was conducted using the R statistical language (www.r-project.org).

Aggregate Nuclear Receptor Activity

To summarize the activity of chemicals across the NR superfamily the ToxCast assays for genes and NR groups were aggregated as follows: retinoic X receptor-like (RXR; RXRa=b; NR2B); peroxisome proliferator-activated receptor-like (PPAR; PPARa=d=c;NR1C); constitutive androstane receptor (CAR; CAR1=2;NR1I3=4); pregnane X receptor (PXR; NR1I2); liver X receptor-like (LXR; LXRa=b, FXR; NR1H); and steroid receptor-like (SR; ERa=b, ERRa=d, AR). As there were differences in the number and types of assays for each group, aggregate activity was calculated as the average potency across the assays measured by the AC50 or LEC. This approach aggregated NR binding, activation, agonism or antagonism results into a single assessment of activity.

Combinatorial Nuclear Receptor Activity

The chemicals were clustered by similarity of aggregate NR activity into 7 putative groups (A-G).

Comparing NR Activity with Cancer Lesion Progression

In vivo rat and mouse long-term histopathology outcomes for chemicals were gathered from ToxRefDB and organized by severity of lesions progressing to cancer. Of the 309 ToxCast chemicals, 232 were tested in 2-year chronic feeding studies in both rat and mouse, and were characterized by liver histopathology as follows: 61 caused no observable effects and 171 chemicals caused a range of lesions of varying severity. Among the 61 negative substances administration at insufficient doses and thus false negatives cannot be excluded.

Results

Human NR activity for 309 environmental chemicals was obtained from in vitro high-throughput screening (HTS) experiments. Duplicates and triplicates for eight chemicals were included for quality control purposes. HTS data were collected for 10 out of the 48 human NR, selected based on availability of assays and potential relevance to toxicology, including: members of the NR1, NR2, NR3 and NR4 subfamilies. The aryl hydrocarbon receptor (AhR) data was also included because of its potential role in xenobiotic metabolism and non-genotoxic liver cancer. A total of 54 HTS assays were used to interrogate different facets of receptor activation including: ligand binding in a cell-free system (Cell-free HTS); reporter gene activation in HEK293 human cells (Cellbased HTS); multiplexed cis-activation and trans-activation assays for transcription factors in human HepG2 cells (Multiplexed Transcription Reporter); and, multiplexed gene expression assays of xenobiotic metabolizing enzymes regulated by specific NR in primary human hepatocytes (Multiplexed Gene Expression). Data for chemical-assay pairs were collected in concentration-response format and either the AC_{50} concentration or the Lowest Effective Concentration (LEC) were reported.

In the following chapter the detailed results from the underlying data for triticonazole, extracted from the EPA, ToxCast database are presented, although they were not reported in detail in the publication from Shah et al., but were used in the publication for further statistical comparison.

ATG assays (assays conducted by "Attagene" (ATG))

Triticonazole was active in two ATG assays, ATG_PXRE_CIS_up and ATG_PXR_Trans_up, showing an induction of mRNA expression mediated by the PXR receptor. Triticonazole did not induce mRNA induction through the other investigated nuclear receptors.

Table 6.8.2.3-2: Summary of ToxCast data for triticonazole as used in the publication for further statistical analysis (ATG assays)

Assay Endpoint Name	Gene symbol	Endpoint measured	Cell line	AC50 [µM]	HitCall
ATG_PXRE_CIS_up	**NR1I2**	**mRNA expression**	**HepG2**	**4.5**	**Active**
ATG_PXR_TRANS_up	**NR1I2**	**mRNA expression**	**HepG2**	**8.56**	**Active**
ATG_Ahr_CIS_up	Ahr	mRNA expression	HepG2	2.08	Inactive
ATG_DR4_LXR_CIS_up	LXR	mRNA expression	HepG2	1000	Inactive
ATG_ERE_CIS	ESR1	mRNA expression	HepG2	7.05	Inactive
ATG_PBREM_CIS_up	NR1I3	mRNA expression	HepG2	9.27	Inactive
ATG_AR_TRANS_up	AR	mRNA expression	HepG2	1000	Inactive
ATG_CAR_TRANS_up	NR1I3	mRNA expression	HepG2	1000	Inactive
ATG_ERRa_TRANS_up	ESRRA	mRNA expression	HepG2	1000	Inactive
ATG_ERRg_TRANS_up	ESRRG	mRNA expression	HepG2	1000	Inactive
ATG_ERα_TRANS_up	ESRRG	mRNA expression	HepG2	20.3	Inactive
ATG_FXR_TRANS_up	NR1H4	mRNA expression	HepG2	1000	Inactive
ATG_LXRa_TRANS_up	NR1H3	mRNA expression	HepG2	1000	Inactive
ATG_LXRb_TRANS_up	NR1H2	mRNA expression	HepG2	1000	Inactive
ATG_PPARa_TRANS_up	PPARA	mRNA expression	HepG2	1000	Inactive
ATG_PPARd_TRANS_up	PPARD	mRNA expression	HepG2	1000	Inactive
ATG_PPARg_TRANS_up	PPARG	mRNA expression	HepG2	5.8	Inactive
ATG_RXRa_TRANS_up	RXRA	mRNA expression	HepG2	1000	Inactive
ATG_RXRb_TRANS_up	RXRB	mRNA expression	HepG2	1000	Inactive

CLD assays (assays conducted by "CellzDirect" (CLD))

Triticonazole was active in one CLD assay, CLD_CYP2B6_6hr, showing an induction of CYP2B6. Triticonazole did not induce other mRNAs investigated in the CLD assays. The slight induction of CYP2B6 (PXR dependent) is not surprising, as triticonazole has shown to be a hepatic enzyme inducer, possibly acting through the CAR/PXR mediated transcription. This is in concordance with the results from the ATG assays, where triticonazole was found active for PXR receptor mediated transcription.

Table 6.8.2.3-3: Summary of ToxCast data for triticonazole as used in the publication for further statistical analysis (CLD assays)

Assay Endpoint Name	Target family	Endpoint measured	Cell line	AC50 [µM]	HitCall
CLD_CYP2B6_6hr	**Xenobiotic metabolism**	**mRNA expression**	**Human liver cells**	**1.9**	**Active**
CLD_ABCB1_6hr	ABC transporter	mRNA expression	Human liver cells	1000	Inactive
CLD_ABCB11_6hr	ABC transporter	mRNA expression	Human liver cells	1000	Inactive
CLD_CYP1A1_6hr	Xenobiotic metabolism	mRNA expression	Human liver cells	1000	Inactive
CLD_CYP1A2_6hr	Xenobiotic metabolism	mRNA expression	Human liver cells	1000	Inactive
CLD_CYP3A4_6hr	Xenobiotic metabolism	mRNA expression	Human liver cells	1000	Inactive
CLD_HMGCS2_6hr	HMG-CoA synthase	mRNA expression	Human liver cells	1000	Inactive

Tox21 assays

Triticonazole was inactive in all reported Tox21 assays. Triticonazole showed no induction or inhibition of the reporter gene through the nuclear receptors AR, ERα, FXR, PPARd and PPARg.

Table 6.8.2.3-4: Summary of ToxCast data for triticonazole as used in the publication for further statistical analysis (TOX21 assays)

Assay Endpoint Name	Target family	Endpoint measured	Cell line	AC50 [µM]	HitCall
Tox21_AR_BLA_Agonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_AR_BLA_Antagonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	36	Inactive
Tox21_ERα_BLA_Agonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_ERα_BLA_Antagonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_FXR_BLA_Agonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_FXR_BLA_Antagonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_PPARd_BLA_Agonist	Nuclear receptor	GAL4 BLAM Reporter Gene	Human kidney cells	1000	Inactive

		Assay	(HEK293T)		
Tox21_PPARd_BLA_Antagonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_PPARg_BLA_Agonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive
Tox21_PPARg_BLA_Antagonist	Nuclear receptor	GAL4 BLAM Reporter Gene Assay	Human kidney cells (HEK293T)	1000	Inactive

NVS assays (assays conducted by "Novascreen" (NVS))

Triticonazole was found to bind to the human and chimpanzee androgen receptor (AR) and thus may act as a (competitive) AR-receptor antagonist, although in the Tox21 assays triticonazole did not induce or inhibit AR-dependent gene expression (see above).

Table 6.8.2.3-5: Summary of ToxCast data for triticonazole as used in the publication for further statistical analysis (NVS assays)

Assay Endpoint Name	Target family	Endpoint measured	Cell line	AC50 [µM]	HitCall
NVS_NR_hAR		**Receptor binding**	**Human kidney prostate adenocarcinoma cell (LNCaP) lysate**	**0.91**	**Active**
NVS_NR_cAR		**Receptor binding**	**Chimpanzee ovarian cell (Sf9/Sf21) lysate**	**0.674**	**Active**
NVS_NR_hCAR_Agonist		Receptor binding	Cell free		Inactive
NVS_NR_hER		Receptor binding	Cell free		Inactive
NVS_NR_hFXR_Agonist		Receptor binding	Cell free		Inactive
NVS_NR_hPPARa_Agonist		Receptor binding	Cell free		Inactive
NVS_NR_hPPARg_Agonist		Receptor binding	Cell free		Inactive
NVS_NR_hPXR_Agonist		Receptor binding	Cell free		Inactive

Grouping by nuclear receptor activity and lesion progression

The resulting data from the nuclear receptor activity was used to calculate an aggregate scaled activity score for different nuclear receptors (CAR, PXR, PPAR, AhR, SR, RXR), which was then correlated to lesion progression data extracted from EPA's pesticide database.

Triticonazole, together with Flutolanil and Oxyfluorfen, was grouped into category VII A; group A indicates, that the compound can activate the nuclear receptors AhR, CAR and PXR, but only produces mild or no lesions. The rationale for this grouping is largely based on the activation of the reporter genes.

Conclusion

With regard to gene expression, triticonazole induced gene expression only via PXR receptor, no other active signal was seen in the other investigated nuclear receptors (among them AhR, AR, ER, CAR, and PPAR). Further

triticonazole showed a slight induction of Cyp2B6, inducing mRNA. This is in concordance to the activation of the PXR receptor. Triticonazole was inactive in all Tox21 assays and showed no induction or inhibition of the reporter gene through the nuclear receptors AR, Era, FXR, PPARd and PPARg. Triticonazole was found to bind to the human and the chimpanzee AR receptor, although no transactivation through the AR receptor was detected.

As triticonazole did not induce liver tumors, the results on PXR activation and Cyp2B6 induction are of minor relevance for the hazard assessment of triticonazole. Further, the ability of triticonazole to bind to the androgen receptor is of limited relevance as no gene expression via transactivation through the AR receptor was detected.

B.6.8.4. Studies on endocrine disruption

All endocrine-related mechanistic studies conducted by the notifier (*in vivo* and *in vitro*) or identified in the open literature are included in this chapter. The summary of all identified endocrine-related mechanistic level 2 and level 3/level 4 studies (according to the OECD Conceptual Framework for testing and assessment of endocrine discrupters; OECD TG 150) studies is followed by an assessment of treatment-related adverse effects with a potential endocrine mode of action.

Level 3/4 studies

In the toxicological data package conducted with triticonazole, degenerative histopathological effects on adrenals were seen in rats and dogs.

The primarily affected zone in several rat studies was zona fasciculata, which is the glucocorticoid producing zone of the adrenal. The lack of large increases in adrenals weight/hypertrophy supported the suggestion that there might be still adequate glucocorticoid competency; however, there was no definitive confirmation on functional competency of adrenals. Therefore, notifier commissioned an external laboratory to conduct an ACTH (adrenocorticotropic hormone) challenge assay, in order to obtain data on adrenocortical functional competency after treatment with triticonazole.

In the ACTH assay the adrenocortical response to a standardized, sufficiently large dose of ACTH can be assessed. Animals with regular adrenal function respond with a clear and appropriate increase in blood glucocorticoid levels, consistent with competent adrenocortical function, while animals with adrenocortical impairment will show lower or no response (Harvey and Sutcliffe, 2010[10]). In the figure below the pituitary-adrenocortical profiles of a normal and a toxicological inhibitory situation are depicted:

[10] Harvey PW and Sutcliffe C (2010). Adrenocortical Hypertrophy: Establishing Cause and Toxicological Significance. Journal of Applied Toxicology, 30: 617-626.

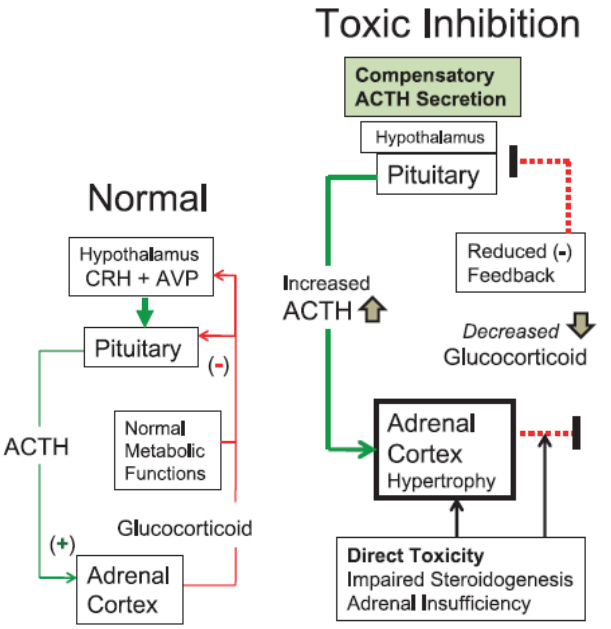


Fig. 6.8.3-1: Normal pituitary adrenocortical profile compared to toxic inhibition of steroidogenesis, causing adrenorcortical hypertrophy (taken from Harvey & Sutcliffe, 2010).

B.6.8.4.1. ACTH challenge assay

Previous evaluation:	No
DRAR (2016)	New study

Reference:	90-Day Oral Toxicity Study with BAS 595 F Triticonazole by Dietary Administration in the Rat
Author(s), year:	[redacted] 2016
Report/Doc. number::	-/ 2016/1296133
Referring to the following Guideline(s):	1) Commission regulation (EC) No 440/2008 Part B: Methods for the Determination of Toxicity and other Health Effects; B.26: "Sub-chronic Oral Toxicity Test: Repeated dose 90-day toxicity study in rodents". Official Journal of the European Union No. L142, May 2008. 2) OECD "Guidelines for Testing of Chemicals", Section 4, Health Effects, No. 408, "Repeated Dose 90-day Oral Toxicity Study in Rodents", Paris Cedex, September 1998. 3) United States Environmental Protection Agency Prevention, Pesticides and Toxic Substances (7101) EPA 712-C-98-199 "Health Effects Test Guidelines" OPPTS 870.3100 "90-Day Oral Toxicity in Rodents", August 1998.
GLP:	Yes
Deviations from OECD Guideline 408:	- Lower number of animals per sex and dose (6) than necessary (10) - Only limited number of parameters investigated, however, the study was designed to focus on adrenal findings and hormonal measurments; therefore this is not considered as deficiency - 38% injection failures at day 7 are considered to have compromised the results at this data point
Acceptability:	Yes; based on lower number of animals included per sex and dose and huge biological variation in hormone levels, as well as uncertainties regarding non-responders as technical shortcoming, the results of the study are considered sufficiently informative to draw a very general conclusion but not to give specific quantitative information

Material and methods

Test Material**:** BAS 595 F (Triticonazole)

Description:	solid / white
Lot/Batch #:	COD-001440
Purity:	91.3%
Stability of test compound:	The test substance was stable over the study period (Expiry date 01.01.2019).
Vehicle and/or positive control:	None
Test animals:	
Species:	Rat
Strain:	Crl:CD(SD) (outbred, SPF-Quality)
Sex:	Male and female
Age:	approx. 6 weeks at treatment start
Weight at dosing:	♂: 189 - 226 g, ♀: 142 - 182 g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Standard powder rodent diet (SM R/M-Z from SSNIFF® Spezialdiäten GmbH, Soest, Germany), ad libitum
Water:	Tap water, ad libitum
Housing:	Group housing of 3 animals per sex in Macrolon cages (MIV type, height 18 cm) with sterilized sawdust as bedding material (Lignocel S 8-15, JRS - J.Rettenmaier & Söhne GmbH + CO. KG, Rosenberg, Germany) and paper as cage-enrichment (Enviro-dri, Wm. Lilico & Son (Wonham Mill Ltd), Surrey, United Kingdom). During locomotor activity monitoring, animals were housed individually in a Hi-temp polycarbonate cage (Ancare corp., USA; dimensions: 48.3 x 26.7 x 20.3 cm) without cage-enrichment, bedding material, food and water.
Environmental conditions:	
Temperature:	18 - 24°C
Humidity:	40 - 70%
Air changes:	Fully air-conditioned rooms; at least 10 air-changes/hour
Photo period:	12 h light / 12 h dark
In life dates:	07-Mar-2016 – 07-Jun-2016

Animal assignment and treatment:

Triticonazole (BAS 595 F) was administered to groups of 6 male and 6 female Sprague Dawley rats at dietary concentrations of 0, 80, 750, and 5000 ppm for 90 days (equivalent to 0, 6, 59 and 412 mg/kg bw per day in males and 0, 7, 68 and 479 mg/kg bw per day in females). The animals were assigned to the treatment groups by means of a computer generated randomization lists based on body weights.

Test substance preparation and analysis:

The stability of the test substance in the diet at room temperature over a period of 14 days was given by the sponsor. Homogeneity analyses were performed for the low and the high dose levels in weeks 1, 7 and 13. Samples from the top (T), middle (M) and bottom (B) of the container were analysed. Additionally, accuracy of the diet preparations was proven for all dose groups at the same time-points.

Statistics:

The following statistical methods were used to analyse the data:

- If the variables could be assumed to follow a normal distribution, the Dunnett-test (many-to-one t-test) based on a pooled variance estimate was applied for the comparison of the treated groups and the control groups for each sex.
- The Steel-test (many-to-one rank test) was applied if the data could not be assumed to follow a normal distribution.
- The Fisher Exact-test was applied to frequency data.

All tests were two-sided and in all cases $p < 0.05$ was accepted as the lowest level of significance. Group means were calculated for continuous data and medians were calculated for discrete data (scores) in the summary tables. Test statistics were calculated on the basis of exact values for means and pooled variances. Individual values, means and standard deviations may have been rounded off before printing. Therefore, two groups may display the same printed means for a given parameter, yet display different test statistics values.

The study was conducted at Charles River Laboratories Den Bosch BV, Hambakenwetering 7, 5231 DD Den Bosch, The Netherlands, except the hormone determination in serum that was performed at BioVetim VetAgro Sup, 1 avenue Bourgelat, 69280 Marcy L'Etoile, France.
Doses and exposure duration were selected on the basis of the results seen in the regulatory 90-day rat study. This study is considered to be a supporting mechanistic study to specifically study adrenal functionality and not as a standard study conducted according to OECD 408. Thus, not the full pathological and clinicochemical and hematological parameters compared to OECD TG 408 were investigated.

Observations:

The animals were examined for mortality or evident signs of moribundity at least twice daily.
The clinical condition of the test animals was recorded at least once daily from start of treatment onwards; detailed clinical observations were made in all animals after dosing. The time of onset, grade and duration of any observed signs were recorded.

Body weight:

The body weight of the animals was determined once a week.

Food and water consumption as well as compound intake:

Food consumption was determined weekly. Therefore, remaining food was sieved (2 mm mesh diameter) before weighing to withdraw any sawdust/faeces.
Water consumption was observed regularily by visual inspections; however, no data are recorded, since no effect was observed.

Ophtalmoscopy:

Not performed in this study

Functional observation battery (FOB) and Motor activity:

Not performed in this study.

Clinicochemical and hematological parameters:

Not performed in this study

Corticosterone determination in serum after ACTH challenge:

The ACTH challenge was conducted as follows:

On days 7 and 90 all rats were intramuscularly injected (left hind limb) with ACTH (Adrenocorticotropic Hormone from porcine pituitary powder, (80-95 IU/mg (USP Corticotropin Ascorbic Acid Assay) Fragment 1-39, Corticotropin A, supplied by Sigma-Aldrich).

Approx. 0.1 mL/challenge/animal was given at Day 7 (5 IU/kg b.w.) and approximately. 0.2 mL/challenge/male and 0.1 mL/challenge/female at Day 90 (6 IU/kg b.w.), based on difference in weight, to challenge the adrenal gland.

One hour (+/- 10 minutes) after injection, blood samples of 0.3 mL each were collected from the jugular vein into serum tubes (Greiner Bio-One GmbH, Kremsmünster, Austria) from all animals (non-fasted). After clotting and centrifugation, serum was divided in two aliquots and stored in a labeled polypropylene tube (Greiner Bio-One GmbH, Frickenhausen, Germany) at ≤-20°C until hormone determination (stability of corticosterone in rat serum: at least 2 months at a temperature below -18°C) that was conducted at BioVetim VetAgroSup, Marcy L'Etoile, France.

Corticosterone analyses were carried out by a RadioImmunoAssay (RIA) using the ImmunoChem™ Double Antibody Corticosterone ^{125}I RIA kit according to manufacturer' instructions (Ref. 07-120102, supplied by MP Biomedicals, LLC – Diagnotics Division, Orangeburg, USA). Thereby the kit calibration range was 25 – 1000 ng/mL, the Limit Of Detection (LOD) was determined to be 3.0 ng/mL, the Low Limit Of Quantification (LLOQ) was determined to be 10.7 ng/mL, the intra-assay coefficient of variation was ranging from 6.8% to 13.8% and the inter-assay coefficient of variation was ranging from 8.9% to 11.5%. Detection was performed using a 2470 WIZARD^2 Automatic Gamma counter. No other clinicochemical or hematological parameters were investigated in this mechanistic study.

The literature range for basal corticosterone in unstressed rats is commonly considered to be 50-80 ng/mL plus 30% (trigger: 104 ng/mL) (Buckingham and Hodges 1976[11]; Mahl et al 2000[12]; Vahl et al 2005[13], Cole 2012[14]) with slight variations in sampling procedure, circadian rhythm and oestrous cycles as well as corticosterone analytical technique. The ACTH challenge by the dose and route used in this study was designed to increase corticosterone ≥5-fold (Cole 2012[13]) providing unequivocal responses.

Urinalysis:

Not performed in this study

[11] Buckingham JC and Hodges JR (1976). Hypothalamo-pituitary adrenocortical function in the rat after treatment with betamethasone. British Journal of Pharmacology 56: 235-239

[12] Mahl A, Heining P, Ulrich P, Jakubowski J, Bobadilla M, Zeller W, Bergmann R, Singer T and Meister L (2000). Comparison of clinical pathology parameters with two different blood sampling techniques in rats: retrobulbar plexus versus sublingual vein. Laboratory Animals 34: 351-361

[13] Vahl TP, Ulrich-Lai YM, Ostrander MM, Dolgas CM, Elfers EE, Seeley RJ, D'Alessio DA and Herman JP (2005). Comparative analysis of ACTH and corticosterone sampling methods in rats. American Journal of Physiology-Endocrinology and Metabolism 289: E823-E828

[14] Cole DC (2012). Non-invasive measurement of corticosterone in food restricted rats. MSc Thesis, University of Toronto

Sacrifice and pathology:

Fasted animals were sacrificed by decapitation under isoflurane (Abbott B.V., Hoofddorp, The Netherlands) anaesthesia. The exsanguinated animals were necropsied and assessed by gross pathology. The following organs were sampled, weighed and examined histopathologically:

Pathology:

The following organs were collected (column C), weighed (W) and examined histopathologically (H, ✓: all groups, #: control and top dose).

C	W	H		C	W	H		C	W	H	
✓	✓	✓	adrenals	✓			lachrymal glands (exorbital)	✓	✓		seminal vesicles[ß]
✓			aorta	✓			larynx				Skin
✓	✓		brain[%]	✓	✓		liver	✓			spinal cord (3 levels)[@]
			bone marrow	✓			lung (infused with formalin)	✓	✓		Spleen
✓			caecum	✓			lymph nodes[#]	✓			sternum with bone marrow
✓			colon	✓			mammary gland (♀)	✓			stomach
✓			duodenum				muscle, skeletal	✓	✓		testes[§]
✓	✓		epididymides[§]	✓			nerve, peripheral (sciatic n.)	✓	✓		Thymus
✓			esophagus				nose/nasal cavity	✓	✓		thyroid/parathyroid
✓			eyes[$]	✓	✓		ovaries	✓			Trachea
			femur (with joint)	✓			pancreas	✓			urinary bladder
			gall bladder	✓			Payer's patches	✓	✓		uterus (including cervix)
✓			gross lesions	✓			pituitary	✓			Vagina
✓	✓		heart				pharynx	✓			
✓			ileum	✓	✓		prostate		✓		body (anesthethized)
✓			jejunum	✓			rectum				
✓	✓		kidneys	✓			salivary glands*				

[%] cerebellum, mid-brain, cortex (7 levels); [§] fixed in modified Davidson's solution, prepared at [redacted] Formaldehyde 37-40%, Ethanol, Acetic acid - glacial (all Merck, Darmstadt, Germany) and Milli-Ro water (Millipore Corporation, Bedford, USA), tissues were transferred to formalin after fixation for at least 24 hours; [#] mandibular and mesenteric; [@] cervical, midthoracic, lumbar; *mandibular and sublingual, [$] with optic nerve and Harderian gland[§]; [ß] including coagulating glands

Pathology findings were subjected to an internal review conducted by an Dutch CRP/TP Certified Toxicologic Pathologist (WIL Research / Charles River Company, 's-Hertogenbosch, The Netherlands). Following the peer review, a consensus was reached between the study pathologist and the peer review pathologist with regard to diagnoses and interpretation. Histopathology data entries in PathData® and pathology data presented in the pathology report reflect this consensus.

Results

The stability of the test substance during the study period was guaranteed by the expiration date of Jan. 2019.

Sample preparations were taken at the 8th of March, 19th of April, and the 31st of May 2016, each three samples from the bottom the middle and the top of the container for the low and the high dose and the middle of the container for the mid dose. The diets are considered to be prepared sufficiently accurate and homogeneous for evaluation of dose levels in this study. A method validation has been performed and is summarized in DRAR B5.

The calculated test substance intakes are presented in the table below:

Table 6.8.3.1-1: Average Triticonazole intake by Sprague Dawley rats

Dose level	**80 ppm**	**750 ppm**	**5000 ppm**
Sex	**average intake [mg/kg bw per dayay]**		

Table 6.8.3.1-1: Average Triticonazole intake by Sprague Dawley rats

Dose level	**80 ppm**	**750 ppm**	**5000 ppm**
Sex	**average intake [mg/kg bw per dayay]**		
Males	6 (4 – 8)	59 (47 – 83)	412 (313 – 552)
Females	7 (6 – 9)	68 (54 – 89)	479 (398 – 589)

Clinical signs of toxicity

No clinical signs during daily observations and no abnormalities during weekly arena observations were noted during the observation periods that were considered to be related to treatment.

The individual clinical signs noted during the treatment period were of isolated occurrence and within the range of background findings to be expected for rats of this age and strain which are housed and treated under the conditions in this study and did not show any apparent dose-related trend. At the incidence observed, these were considered to be unrelated to treatment.

Table 6.8.3.1-2: Clinical signs of toxicity in Sprague Dawley rats administered Triticonazole for 90 day

Dose Level [ppm]	**Sex**	**0**	**80**	**750**	**5000**
[mg/kg bw ♂ / ♀]		**0**	**6 / 7**	**59 / 68**	**412 / 479**
Secretion / Excretion	♂	0	0	1 (w3)	0
- nasal discharge	♀	0	0	0	0
Skin / Fur	♂	0	0	0	1 (w1-2)
- scabs on tail	♀	0	0	0	0

n = number of animals affected (w = week of occurrence)

Mortality

No treatment related mortality occurred during the study.

However, one high dose females (animal no.44, treated on day 1 (9 March) only) was found dead on Day 2 (10 March). This death was considered to be accidental and not treatment related, therefore this animal was not macroscopically examined on day of death and no organs were fixed. The available data of this animal will be kept in the raw data and will not be reported.

This animal was replaced by a spare animal starting treatment Day 2 (10 March) and was identified as Animal 44 from 10 March onwards.

Body weight and body weight gain:

Body weight of males was not affected during the treatment. In the treated groups, either slightly lower, comparable or slightly higher body weight (up to max. 6%) was measured, compared to control males (table 6.8.3.1-3).

Body weight of the high dose females was affected by the treatment, from week 1 up to the end. However, neither statistical significance was reached nor was the difference higher than 10% comparing to controls (table 6.8.3.1-3).

Body weight gain of males was not reduced during the treatment. At the end of the study, males treated with 80 and 5000 ppm showed body weight gain at approximately 10% higher, while males treated with 750 ppm showed same body weight gain compared to controls (table 6.8.3.1-4). No statistical significance was reached at any timepoint.

Body weight gain of the high dose females was affected by the treatment. Body weight gain of high dose females gained statistical significance from week 1 to 7, revealing a reduction of > 50% as compared with the controls during the first two application weeks. For the rest of the study period, the body weight gain reduction was ranging from 40% to 10% (at the end of the study) as compared with the respective control groups (table 6.8.3.1-4).

Table 6.8.3.1-3: Body weight of Sprague Dawley rats administered Triticonazole for 13 weeks

Dose level [ppm]	0		80		750		5000	
	mean	SD	mean	SD	mean	SD	mean	SD
Body weight [g]								
Males								
Day 1	210	9.2	208	10.8	209	9.8	209	6.1
Δ%	-		-1.0		-0.5		-0.5	
Week 1	264	11.5	264	14.6	262	11.1	257	9.7
Δ%	-		0.0		-0.8		-2.7	
Week 2	270	12.5	271	15.0	271	11.6	266	12.3
Δ%	-		+0.4		+0.4		-1.5	
Week 3	316	12.9	321	22.3	316	18.4	322	13.9
Δ%	-		+4.7		0.0		+1.9	
Week 4	353	22.3	361	28.5	352	21.5	368	14.7
Δ%	-		+2.3		-0.3		+4.2	
Week 5	388	26.1	395	26.1	380	27.1	401	20.2
Δ%	-		+1.8		-2.1		+3.4	
Week 6	415	27.5	426	38.3	410	31.5	434	24.4
Δ%	-		+2.7		-1.2		+4.6	
Week 7	438	31.4	453	41.5	437	35.4	459	31.6
Δ%	-		+3.4		-0.2		+4.8	
Week 8	452	33.2	473	40.5	458	42.8	480	34.5
Δ%	-		+4.6		+1.3		+6.2	
Week 13	535	32.7	566	51.7	533	49.9	567	51.3
Δ%	-		+5.8		-0.4		+6.0	
Females								
Day 1	160	10.4	156	4.8	162	9.1	162	12.9
Δ%	-		-2.5		+1.3		+1.3	
Week 1	185	12.5	176	10.9	184	10.8	173	14.2
Δ%	-		-4.9		-0.5		-6.5	
Week 2	184	12.6	179	7.5	186	13.4	171	13.8
Δ%	-		-2.7		+1.1		-7.1	
Week 3	208	11.9	201	13.2	210	13.2	192	16.0
Δ%	-		-3.4		+1.0		-7.7	
Week 4	220	13.0	217	12.2	226	19.0	206	15.9
Δ%	-		-1.4		+2.7		-6.4	
Week 5	234	18.0	225	12.5	239	19.8	217	20.2
Δ%	-		-3.8		+2.1		-7.3	
Week 6	243	13.5	241	14.7	253	17.8	226	23.3
Δ%	-		-0.8		+4.1		-7.0	
Week 7	257	14.8	244	17.4	262	17.2	242	22.1
Δ%	-		-5.1		+1.9		-5.8	
Week 8	260	19.3	255	17.0	269	20.4	243	20.0
Δ%	-		-1.9		+3.5		-6.5	
Week 13	283	22.9	288	28.4	299	25.4	275	28.3
Δ%	-		+1.8		+5.7		-2.8	

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

Δ% = difference to the respective control group in percent

Table 6.8.3.1-4: Body weight gain of Sprague Dawley rats administered Triticonazole for 13 weeks

Dose level [ppm]	0		80		750		5000	
	mean	SD	mean	SD	mean	SD	mean	SD
Body weight gain [% of initial body weight]								
Males								
Week 1 (day 7)	25	3.1	27	1.7	25	2.9	23	3.4
Δ%	-		+8.0		0.0		-8.0	
Week 2 (day 8)	28	4.0	31	2.3	30	2.4	27	4.6
Δ%	-		+10.7		+7.1		-3.6	
Week 3	50	7.9	54	5.8	51	6.2	54	6.7
Δ%	-		+8.0		+2.0		+8.0	
Week 4	68	12.4	74	7.4	68	8.4	76	8.0
Δ%	-		+8.8		0.0		+11.8	
Week 5	85	14.3	90	10.3	82	9.6	92	9.5
Δ%	-		+5.9		-3.5		+8.2	
Week 6	98	15.6	105	10.9	97	12.2	107	11.1
Δ%	-		+7.1		-1.0		+9.2	
Week 7	108	17.2	118	12.1	109	13.5	119	14.6
Δ%	-		+9.3		+0.9		+10.2	
Week 8	115	18.4	128	11.3	119	17.1	130	16.1
Δ%	-		+11.3		+3.5		+13.0	
Week 13	155	17.9	172	15.1	155	20.5	171	23.4
Δ%	-		+11.0		0.0		+10.3	
Females								
Week 1	16	2.4	13	5.7	14	0.7	**7****	4.2
Δ%	-		-18.8		-12.5		-56.3	
Week 2	15	2.6	15	4.1	15	2.2	**5****	2.1
Δ%	-		0.0		0.0		-66.7	
Week 3	30	3.2	29	5.9	30	2.1	**18****	5.6
Δ%	-		-3.3		0.0		-40.0	
Week 4	38	6.8	39	5.8	40	5.9	**27***	4.5
Δ%	-		+2.6		+5.3		-28.9	
Week 5	46	7.3	45	5.0	48	5.6	**34****	4.9
Δ%	-		-2.2		+4.3		-26.1	
Week 6	52	6.0	55	8.2	56	4.1	**39***	7.7
Δ%	-		+5.8		+7.7		-25.0	
Week 7	61	7.3	57	9.2	62	5.2	**49***	5.4
Δ%	-		-6.6		+1.6		-19.7	
Week 8	63	9.9	64	10.7	67	8.7	50	3.6
Δ%	-		+1.6		+6.3		-20.6	
Week 13	78	13.7	85	19.3	85	11.6	70	10.5
Δ%	-		+9.0		+9.0		-10.3	

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

Δ% = difference to the respective control group in percent

Food consumption:

Food consumption by male Sprague Dawley rats was higher in all treated groups during the period of 90 days. In high dose males this even reached a statistical significance in 3 weeks. While for low and high dose males higher food consumption somehow correlated with higher body weights/body weight gains compared to control, no such correlation was visible for 750 ppm males (table 6.8.3.1-5).

Food consumption by female Sprague Dawley rats was higher in all treated groups during the period of 90 days and transiently gained statistical significance (table 6.8.3.1-5). While higher food consumption of females treated with 80 and 750 ppm somehow correlated with higher body weights/body weight gains compared to control, the higher food consumption of high dose females did not correlate at all with their (reduced) body weight/body weight gain. It is concluded that females treated with 5000 ppm had an insufficient food utilisation.

Table 6.8.3.1-5: Food consumption by Sprague Dawley rats administered Triticonazole for 13 weeks

Dose level [ppm]	0		80		750		5000	
	mean	SD	mean	SD	mean	SD	mean	SD
Food consumption [g/animal/day]								
Males								
Week 1-2 (Days 1-8)	28	0.8	28	0.4	29	0.4	28	1.9
Week 2-3 (Days 8-15)	28	3.8	29	0.3	31	0.1	34	2.0
Week 3-4 (Days 15-22)	28	4.5	30	0.7	34	3.2	36	1.0
Week 4-5 (Day 22-29)	28	3.7	30	0.8	32	1.6	**38***	2.8
Week 6-7 (Day 36-43)	29	5.6	30	0.6	32	0.6	38	2.7
Week 8-9 (Day 50-57)	29	3.4	30	1.0	35	0.9	**37***	0.5
Week 9-10 (Day 57-64)	30	4.0	31	0.1	35	0.2	36	2.3
Week 10-11 (Day 64-71)	29	2.3	30	0.0	34	0.6	**37***	3.1
Week 13 (Day 85-90)	27	2.7	31	0.8	33	4.5	36	3.3
Overall (mean of means)	29	1.0	30	1.4	33	2.4	36	3.0
Δ%	-		+5.6		+16.1		+26.0	
Females								
Week 1-2 (Days 1-8)	21	2.2	20	1.1	22	0.7	20	2.3
Week 2-3 (Days 8-15)	21	2.5	20	0.5	22	0.1	22	1.0
Week 3-4 (Days 15-22)	20	1.1	20	0.1	22	0.6	22	0.2
Week 4-5 (Day 22-29)	21	0.9	20	0.0	22	1.2	22	0.1
Week 6-7 (Day 36-43)	20	1.2	20	0.4	**23***	0.6	21	0.7
Week 8-9 (Day 50-57)	19	0.5	21	0.7	21	1.4	22	1.7
Week 9-10 (Day 57-64)	19	0.1	21	1.5	**23***	0.1	**22***	0.2
Week 10-11 (Day 64-71)	18	1.3	21	1.6	24	3.1	21	1.1
Week 13 (Day 85-90)	17	0.2	**23***	2.2	**24***	1.7	22	0.8
Overall (mean of means)	20	2.2	21	1.6	23	2.1	22	1.6
Δ%	-		+5.4		+14.7		+11.6	

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

Δ% = difference to the respective control group in percent

Corticosterone determination after ACTH challenge

Serum corticosterone values showed high variability.

BASF contracted external expert Phil Harvey, expert in adrenal toxicology[15], for his opinion concerning adrenal findings in investigative studies (BASF DocID 2016/1227736) with triticonazole. One part of this was also his opinion on results of the conducted ACTH assay. Mr Harvey elaborated in detail the results and interpreted them as follows:

There was evidence in animals across all treatment groups that at day 7 the ACTH injection was ineffective in a proportion of animals (no response above the basal level, even in controls). This has been attributed to the injection technique. Therefore, the corticosterone results (mean values plus standard deviations) both including and excluding affected animals are presented (table 6.8.3.1-6). Individual data analysis where ACTH was considered effective showed that triticonazole treated rats were able to respond to the challenge with increased corticosterone excretion (Remark: above basal level of 104 ng/mL trigger) across all dose levels. However, the magnitude at the high dose appeared slightly lower compared to other groups. This may have been confounded due to the challenge injection failure (Remark RMS: especially in females, where at day 7 four animals in control group did not respond, two animals at 80 ppm, four animals at 750 ppm and three animals at 5000 ppm) and subsequent lower number of animals/data points and high variability typical of endocrinology data. However, even in animals that were sucessfuly challenged the lowest corticosterone values clustered in the high dose animals.

Regarding findings at day 90, number of animals receiving an effective ACTH challenge markedly improved (only females Nr. 44 and 48 of 5000 ppm group did not respond above the non-stressed basal concentration, figure 6.8.3.1-1). Results in males showed that they had an unimpaired ACTH corticosterone response. Regarding females although the mean corticosterone values in females treated with 5000 ppm appeared slightly reduced, this group showed adequate increases in responses once the injection failures were removed from the analysis (the lower number of animals/data points may have counfounded the results). In addition, evaluation of individual data showed that all females treated with 5000 ppm (and responded) had higher corticosterone values than the lowest control and were therefore within the control range (the lowest plasma corticosterone value was in control group female Nr. 26 with a value of 108.8 ng/mL). At day 90 all animals receiving an effective ACTH challenge showed generally unimpaired adrenocortical function and were able to respond with significantly increased corticosterone.

Phil Harvey concluded that the results suggest that high dose animals showed a slight initial decrease of the ACTH stimulated corticosterone response (maybe also compromised by lower number of animals/data points) and that there was full recovery and compensation indicating physiological accommodation and adaptation.

Statistical evaluation (Jonkheere-Tepstra trend test) of the not-adjusted values showed the only trend in males of the high dose group after 7 days triticonazole treatment, while no effects were demonstrated for females and after 90 days in both sexes. Since effects on adrenals relevant for corticosterone production are not expected after 7 days of triticonazole treatment but could potentially rather occur after 90 days, the lower corticosterone level in males after 7 days of triticonazole treatement (but not observed after 90 days) is not considered treatment-related finding.

Table 6.8.3.1-6: Serum corticosterone levels one hour after adrenocorticotropic Hormone (ATCH) challenge in Sprague Dawley rats administered triticonazole for 7 or 90 days

Group	Day 7 (ng/mL) (± SD)	Day 7 adjusted* (ng/mL)	Day 90 (ng/mL)	Day 90 adjusted* (ng/mL)
Males				

[15] Harvey, P.W., Everett, D.J., Springall, C.J. Target organ toxicology series: Adrenal Toxicology. Informa Healthcare USA, Inc. 2009.

Group	Day 7 (ng/mL) (± SD)	Day 7 adjusted* (ng/mL)	Day 90 (ng/mL)	Day 90 adjusted* (ng/mL)
0 ppm	247.6 ± 102.11	278.8 ± 75.6	321.7 ± 91.3	NA
80 ppm	304.2 ± 97.14	NA	230.1 ± 96.5	NA
750 ppm	234.7 ± 152.29	307.8 ± 131.5	342.9 ± 50.41	NA
5000 ppm	144.4** ± 107.41	192.5 ± 99.7	348.3 ± 176.04	NA
Females				
0 ppm	151.1 ± 170.29	334.3 ± 208.0	387.4 ± 249.70	NA
80 ppm	163.9 ± 86.89	203.7 ± 77.9	380.4 ± 107.54	NA
750 ppm	187.4 ± 198.63	432.0 ± 113.6	350.8 ± 154.36	NA
5000 ppm	121.8 ± 108.46	205.1 ± 92.4	200.4 ± 155.99	270.0 ± 145.5

*data indicated that ATCH injection was ineffective in a proportion of animals and those with <104 ng/mL corticosterone are excluded (typical basal range 50-80 ng/mL plus 30%). NA = not applicable (all ACTH injections were successful). ACTH challenge was designed to increase corticosterone $\geq$ 5-fold.

no statistical significance for none of the values was determined using the Kruskal-Wallis test;

** in the Jonkheere-Tepstra trend test the only trend was seen in males of the high dose group after 7 days, while no effects were demonstrated for females and after 90 days in both sexes; all statistic conducted for not-adjusted values.

Figure 6.8.3.1-1. Corticosterone levels, measured one hour after ATCH challenge in Sprague Dawley rats administered Triticonazole for 7 or 90 days – values per animal

Corticosterone (ng/mL)

Group 1 (0 ppm)

		Day 7	Day 90			Day 7	Day 90
M	**1**	268.3	239.3	**F**	**25**	187.2	760.5
M	**2**	248.1	380.8	**F**	**26**	481.4	107.8
M	**3**	236.2	334.6	**F**	**27**	**60.6**	422.2
M	**4**	411.5	468.4	**F**	**28**	**85.1**	576.4
M	**5**	**91.4**	267.1	**F**	**29**	**45.2**	169.4
M	**6**	229.9	239.7	**F**	**30**	**47.0**	287.8
	Mean	247.6	321.7		Mean	151.1	387.4
	S.D.	102.11	91.30		S.D.	170.29	249.70
	†Adjusted Mean	**278.8**	NA		†Adjusted Mean	**334.3**	NA

Group 2 (80 ppm)

		Day 7	Day 90			Day 7	Day 90
M	**7**	205.2	190.6	**F**	**31**	**66.8**	486.9
M	**8**	197.9	213.1	**F**	**32**	304.5	404.4
M	**9**	353.3	374.8	**F**	**33**	225.5	183.9
M	**10**	260.2	159.7	**F**	**34**	145.3	433.9
M	**11**	433.8	124.6	**F**	**35**	**102.2**	340.1
M	**12**	374.5	317.8	**F**	**36**	139.3	433.4
	Mean	304.2	230.1		Mean	163.9	380.4
	S.D.	97.14	96.50		S.D.	86.89	107.54
	†Adjusted Mean	NA	NA		†Adjusted Mean	**203.7**	NA

Group 3 (750 ppm)

		Day 7	Day 90			Day 7	Day 90
M	**13**	201.0	361.1	**F**	**37**	**21.4**	296.5
M	**14**	191.4	294.9	**F**	**38**	512.3	582.8
M	**15**	**87.2**	317.3	**F**	**39**	**39.8**	410.7
M	**16**	451.1	380.2	**F**	**40**	**99.2**	273.5
M	**17**	387.5	414.6	**F**	**41**	**100.1**	129.3
M	**18**	**89.7**	289.2	**F**	**42**	351.6	411.9
	Mean	234.7	342.9		Mean	187.4	350.8
	S.D.	152.29	50.41		S.D.	198.63	154.36
	†Adjusted Mean	**307.8**	NA		†Adjusted Mean	**432.0**	NA

Group 4 (5000 ppm)

		Day 7	Day 90			Day 7	Day 90
M	**19**	**42.0**	448.8	**F**	**43**	**29.4**	324.0
M	**20**	341.3	602.2	**F**	**44**	120.3	**54.7**
M	**21**	152.4	309.8	**F**	**45**	**43.9**	133.9
M	**22**	146.6	403.9	**F**	**46**	**42.1**	172.0
M	**23**	**54.2**	221.3	**F**	**47**	303.5	450.1
M	**24**	129.6	104.0	**F**	**48**	191.4	**67.8**
	Mean	144.4	348.3		Mean	121.8	200.4
	S.D.	107.41	176.04		S.D.	108.46	155.99
	†Adjusted Mean	**192.5**	NA		†Adjusted Mean	**205.1**	**270.0**

Adjusted Mean†: re-calculated mean omitting values where the ACTH injection was ineffective (identified as bold) and those with <104 ng/mL corticosterone are excluded (typical basal range 50-80 ng/mL plus 30% for experimental and strain variability compared with the literature). Corticosterone values where the ACTH injection was ineffective equate to typical basal levels across all groups including controls. NA not applicable.

Based on significantly lower number of animals successfully challenged at day 7, RMS asked for additional information on some details of the test conditions and received following clarification from the laboratory and the notifier:

- The same technician was responsible for ACTH challenge and blood sampling at day 7 and 90
- The commissioned facility site conducted two ACTH assays in rat (the current one included) and three ACTH challenge studies in dogs during the last few years
- The data for control animals of the second study, conducted previously to this in the same test facility ([redacted] showed high variability in corticosterone levels at day 90, *prior* to ACTH challenge (8 males: 10.8 – 222.1 ng/mL, 8 females: 136.2 – 789.4 ng/mL)
- Control data from a different test site ([redacted]), although coming from Wistar strain (no obvious strain variability assumed), support the high variability in basal corticosterone level in non-treated rats (68 males: 9.7 – 608 ng/mL, mean±SD = 166.7 ± 114.8; 81 females: 20 – 889.6 ng/mL, mean±SD = 347.8 ± 211.87)
- No randomisation was done for ACTH challenge and blood sampling, the animals were treated with ACTH and blood samples were taken clustered according to the treatment groups

Based on the available results (low ACTH challenge success even in control animals) the RMS has doubts that ACTH challenge at day 7 was conducted with sufficient proficiency.

Organ weights

In males, no treatment-related weight alterations of any organ examined were observed.

In high dose females, absolute and relative liver weight was statistically significantly increased, and was also 26.3% higher compared to control animals (table 6.8.3.1-7). No corresponding macroscopic findings were observed by gross necropsy (table 6.8.3.1-8). As this study was a specific mechanistic study to investigate adrenal toxicity/functionality, no histopathologic evaluation of the liver was performed.

Statistically significantly decreased absolute thyroid weight was seen in females receiving 5000 ppm. Regarding relative thyroid weight, this was decreased in all treated groups by approximately 17% compared to controls.

No effects on the weight of adrenals were noted in animals of both sexes.

Additionally, high dose females revealed increased (> 20% of the control) absolute and relative spleen weight, the later parameter attaining statistical significance. No corresponding macroscopic finding was observed by gross necropsy (table 6.8.3.1-8). As no haematological and clinico-chemistry investigations or histopathological evaluations of the spleen were performed in this study, no further conclusion can be made.

Table 6.8.3.1-7: Organ weights of Sprague Dawley rats administered Triticonazole for 90 days

Sex			**Males**				**Females**			
		Dose [ppm]	**Absolute weight [g]**	**Δ%**	**Relative weight [% of bw]**	**Δ%**	**Absolute weight [g]**	**Δ%**	**Relative weight [% of bw]**	**Δ%**
Terminal weight	[g]	0	498				264			
		80	532	+6.8			259	-1.9		
		750	500	+0.4			282	+6.8		

Table 6.8.3.1-7: Organ weights of Sprague Dawley rats administered Triticonazole for 90 days

Sex		Dose [ppm]	Males Absolute weight [g]	Δ%	Males Relative weight [% of bw]	Δ%	Females Absolute weight [g]	Δ%	Females Relative weight [% of bw]	Δ%
		5000	527	+5.8			249	-5.7		
Liver	[g]	0	11.54		2.32		6.34		2.40	
		80	12.69	+10.0	2.37	+2.2	6.45	+1.7	2.50	+4.2
		750	11.93	+3.4	2.38	+2.6	7.02	+10.7	2.49	+3.8
		5000	12.87	+11.5	2.45	+5.6	**7.55***	+19.1	**3.03****	+26.3
Thyroid	[g]	0	0.017		0.003		0.016		0.006	
		80	0.020	+17.6	0.004	+33.3	0.013	-18.8	0.005	-16.7
		750	0.017	0.0	0.003	0.0	0.014	-12.5	0.005	-16.7
		5000	0.021	+23.5	0.004	+33.3	**0.012***	-25.0	0.005	-16.7
Adrenals	[g]	0	0.062		0.012		0.074		0.028	
		80	0.067	+8.1	0.013	+8.3	0.069	-6.8	0.027	-3.6
		750	0.062	0.0	0.012	0.0	0.078	+5.4	0.028	0.0
		5000	0.063	+1.6	0.012	0.0	0.067	-9.5	0.027	-3.6
Spleen	[g]	0	0.700		0.141		0.456		0.172	
		80	0.777	+11.0	0.145	+2.8	0.488	+7.0	0.188	+9.3
		750	0.669	-4.4	0.134	-5.0	0.518	+13.6	0.183	+6.4
		5000	0.773	+10.4	0.148	+5.0	0.549	+20.4	**0.222****	+29.1

* $p \leq 0.05$, ** $p \leq 0.01$ (Dunnett-test (two-sided))
Δ% = difference to the respective control group in percent

Gross lesions

Macroscopic examinations revealed no test substance related changes in the organs examined.

All findings observed were of isolated occurrence or lacking a dose-response relationship and therefore, considered to be incidental in nature and not treatment related (table 6.8.3.1-8).

Table 6.8.3.1-8: Selected gross necropsy findings in Sprague Dawley rats administered Triticonazole for 90 days

Sex	Males				Females			
Dose [ppm]	**0**	**80**	**750**	**5000**	**0**	**80**	**750**	**5000**
Animals examined **[n]**	6	6	6	6	6	6	6	6
Animals affected **[n (%)]**	3 (50)	3 (50)	3 (50)	4 (67)	2 (33)	1 (17)	1 (17)	3 (50)
Liver								
- Accentuated lobular pattern	1	1	0	0	0	0	0	0
- Accessory liver	1	0	0	0	0	0	0	0
Thyroid								
- Enlarged	1	0	0	0	0	0	0	0
Adrenals								
- Focus/foci	0	1	0	0	0	0	1	0
Spleen								
- Reduced in size	0	0	1	0	0	0	0	1
Lymph node (mandibular)								
- Discolouration	0	0	2	2	0	0	0	0

Histopathology

Due to the special issue of the present study, histopathology was only performed on the adrenal glands of the study animals.

Test item-related microscopic findings were noted in males and females of all treatment groups.

In males, an increased incidence and severity of vacuolation of the zona fasciculata (up to moderate) was observed with increasing doses.

Minimal vacoulation of zona fasciculata (grade 1) was observed in a single control male, and in a few low and mid dose males, as well as in a single high dose female. Slight vacuolation of zona vasciculata (grade 2) was observed in 3 males of 80 and 5000 ppm group each, but not in control or in middle dose group. In 5000 ppm group, two out of 6 animals showed also moderate (grade 3) vacuolation of zona fasciculata, while no findings of this grade were observed in other treated groups (table 6.8.3.1-9).

Degeneration/regeneration of the zona fasciculata/reticularis was observed in all high dose females, but not in other treated groups. This finding was characterised by a mixture of one or more of the following observations in the zona fasciculata/reticularis, including loss of normal architecture, basophilic staining of cells, "fibrillary" morphology of the cytoplasm, and hypertrophy / swollen cells.

Additionally, an increased incidence of inflammatory cell infiltrate was observed in high dose females, since all females of this group were affected. The majority of this infiltrate was mononuclear and surrounded/intermingled the affected zona fasciculata/reticularis cells. The few infiltrates noted at lower doses were mostly small foci with lymphocytes and were regarded to be within background.

The remainder of the recorded microscopic findings were within the range of background pathology encountered in rats of this age and strain. There was no test item-related alteration in the prevalence, severity, or histologic character of those incidental tissue alterations.

Table 6.8.3.1-9: Microscopic findings in adrenals of Sprague Dawley rats administered Triticonazole for 90 days

Sex		**Males**				**Females**			
Dose [ppm]		**0**	**80**	**750**	**5000**	**0**	**80**	**750**	**5000**
Animals examined [n]		**6**	**6**	**6**	**6**	**6**	**6**	**6**	**6**
Organ	***Grade***								
Adrenal cortex									
	1	1	1	3	1	0	0	0	1
- Vacuolation (zona fasciculata)	*2*	0	3	0	3	0	0	0	0
	3	0	0	0	2	0	0	0	0
Animals affected		**1**	**4**	**3**	**6**	**0**	**0**	**0**	**1**
- Degeneration / regeneration	*1*	0	0	0	0	0	0	0	3
(zona fasciculate/reticularis)	*2*	0	0	0	0	0	0	0	3
Animals affected		**0**	**0**	**0**	**0**	**0**	**0**	**0**	**6**
- Inflammatory cell infiltration	*1*	**0**	**1**	**0**	**0**	**0**	**2**	**2**	**6**

Gradings: 1 = minimal, 2 = slight; 3 = moderate

The individual relative adrenal weights of male and female control and high dose animals are summarized in table 6.8.3.1-10, in order to compare it with the individual corticosterone values. No correlation between highest grade in morphological changes and lowest corticosterone level could be observed.

Table 6.8.3.1-10: Individual adrenal weights, histopathological gradings of vacuolation and degeneration/regeneration and corticosterone levels after ACTH challenge in controls and high dose males and females

Animal no.	Relative adrenal weight [%]	Vacuolation zona fasciculata [grade]	Degeneration/ regeneration zona fasciculata / regularis [grade]	Corticosterone values after ACTH challenge day 7 [ng/mL]	Corticosterone values after ACTH challenge day 90 [ng/mL]
Males					
Control					
1	0.011	-	-	268.3	239.3
2	0.011	-	-	248.1	380.8
3	0.012	-	-	236.2	334.6
4	0.012	-.	-	411.5	468.4
5	0.014	-	-	**91.4**	267.1
6	0.011	1	-	229.9	239.7
5000 ppm					
19	0.012	1	-	**42.0**	448.8
20	0.010	2	-	341.3	602.2
21	0.012	3	-	152.4	309.8
22	0.013	3	-	146.6	403.9
23	0.009	2	-	**54.2**	221.3
24	0.011	2	-	129.6	104.0
Females					
Control					
25	0.026	-	-	187.2	760.5
26	0.026	-	-	481.4	107.8
27	0.025	-	-	**60.6**	422.2
28	0.026	-	-	**85.1**	576.4
29	0.021	-	-	**45.2**	169.4
30	0.021	-	-	**47.0**	287.8
5000 pmm					
43	0.023	-	1	**29.4**	324.0
44	0.020	-	2	120.3	**54.7**
45	0.024	1	1	**43.9**	133.9
46	0.019	-	2	**42.1**	172.0
47	0.028	-	2	303.5	450.1
48	0.024	-	1	191.4	**67.8**

-Not detected; **bold**: animals that did not respond based on ACTH injection failure

Conclusion

Based on the mechanistic character of the study, only a limited subset of parameters has been investigated.

According to the available information, the NOAEL could be proposed at 750 ppm (59 and 68 mg/kg for males and females, respectively), based on decreased food utilization and body weight/gain in females, more pronounced histopathological findings (grade “moderate”) in adrenals in both males and females, > 10% increased relative spleen and liver weight in females (without information on haematological or clinic-chemical parameters), all observed at 5000 ppm (412 mg/kg bw per day in males and 479 mg/kg bw per day in females). The NOAEL in the main 90-days rat study was set at 250 ppm, while the dose spacing was huge (LOAEL at 12500 ppm).

RMS understands that the study has been conducted with the aim to prove functionality of adrenals in rat after 90 days treatment with triticonazole, in presence of morphological alterations. Regarding this, RMS concluded that the results at day 7 can be hardly interpreted since 38% of animals (18 out of 48) were not sucesussfully challenged due to technical failure for ACTH challenge and this lowered dramatically the number of data.

At day 90, however, much better challenge rate was achieved than on day 7. Data measured at day 90 might indicate that at 5000 ppm, slightly lower (but not statistically different) corticosterone levels were measured in females, but not in males, compared to control animals. The interpretation of data for two females at 5000 ppm, showing no increase of corticosterone above basal level, is difficult – the reason might be, again, in technical failure during ACTH challenge, but it cannot be ruled out that these females were successfully challenged but did not respond. However, all corticosterone values from successfully challenged females at 5000 ppm were within values of control animals.although the mean was slightly lower than in control group. No correlation between highest grade in morphological changes in adrenals and lowest corticosterone level could be observed.

Summing up all information the RMS concluded that this mechanistic study could show that animals treated with triticonazole at 5000 ppm and suffering morphological changes in zona fasciculata of adrenals were still able to excrete corticosterone after a successful ACTH challenge, and therefore the functional capacity of adrenals generally remained. The results of the study are considered sufficiently informative to draw a general conclusion but not to give specific quantitative information (high variability in hormonal level, values within historical controls, technical failure, etc.)

Level 2 studies

B.6.8.4.2. YAS assay

Previous evaluation:	No
DRAR (2016)	New study

Reference:	a) Amendment No. 1 to the Summary of Results: BAS 595 F (Triticonazole) - Testing for potential androgenic and antiandrogenic activity using the YAS-assay [AR] (Yeast androgen screening) b) In house validation of recombinant yeast estrogen and androgen receptor agonist and antagonist screening assays
Author(s), year:	Woitkowiak C., 2012 (main study) and 2016 (amendment) [redacted] 2010 (validation)
Report/Doc. number::	-/ 2012/1276019 and 2016/1126702
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

The test is based on genetically modified yeast cells (*Saccharomyces cerevisiae*), which contain the gene for the human androgen receptor coupled to a reporter gene lacZ. Activation of the receptor initiates a cascade of events leading to the expression of the reporter gene product (β-galactosidase) which converts a substrate (CPRG, chlorophenol red-β-D-galactopyranoside) which leads to a measurable color change from yellow to red in the medium.

Material and methods

Test Material	BAS 595 F (Triticonazole)
Description:	Solid, white

Lot/Batch #:	COD-001440
Purity:	91.3% (tolerance ± 1.0%)
Stability of test compound:	NA
Solvent used:	Dimethylsulfoxide (DMSO)
Control Materials:	
Vehicle control:	DMSO 1% (v/v)
Positive control compounds:	Androgenic control: 5α-dihydrotestosterone Antiandrogenic control: 5α-dihydrotestosterone combined with hydroxyflutamide
Test organisms:	Yeast cells (*Saccharomyces cerevisiae*) have been stably transformed with a gene encoding the human androgen receptor (hAR), which is constitutively expressed. Additionally, these cells are stably transformed with a reporter gene plasmid, containing an androgen response element and the *LacZ* gene, which encodes the reporter enzyme β-galactosidase.
Final test substance concentrations:	10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} mol/L

A deep-frozen (-80°C) yeast stock culture was thawed at room temperature, inoculated in growth medium and incubated for pre-culture (24-72 h) and growth medium was exchanged after 72 h before use. Of the preculture, optical density (OD) was determined at 690 nm. For preparation of the test culture, 0.50 mL of the pre-culture with an OD of 1.0 was transferred into 50 mL fresh culture medium including 0.5 mL chromogenic substrate CPRG (chlorophenol red-β-D-galactopyranoside).

The study was carried out in 96-well microtiter plates in which 2 µL of different test substance solutions had been pipetted. 200 µL of the test culture was added to each well. The plates was sealed with breathable tape and incubated until measurement of the OD.

Controls:

Each experiment includes a negative control (vehicle control) and positive controls for the verification of the detection of androgenic and anti-androgenic activity in the yeast cells.

Negative controls / Vehicle controls

The vehicle control contains 2 µL of the vehicle used for the test substance. The final concentration of the vehicle in the culture medium will be 1% (v/v).

Positive controls

Androgenic control:

5α-dihydrotestosterone

Final concentrations: 10^{-11}, 10^{-10}, $5*10^{-9}$, 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} mol/L

Anti-androgenic control:

5α-dihydrotestosterone combined with hydroxyflutamide

Final concentrations:

$5x10^{-9}$ mol/L (5α-dihydrotestosterone)/ $1x10^{-5}$ mol/L (hydroxyflutamide)

The stability of the selected positive controls is well-defined under the chosen culture conditions since they are well established reference endocrine disruptors.

Endocrine activity

After 48 h (±4 h) incubation, absorbance of the plates is measured at 570 nm (colour development, androgen receptor dependent enzyme expression) and 690 nm (turbidity due to growth of the yeast). Evaluation is performed by calculating the difference of the measured ODs at the two wavelengths (absorption at 570 nm - absorption at 690 nm). For the 4 replicates per concentration the median, minimum and maximum value are presented in a diagram. The findings of the 1st experiment were confirmed in an additional assay. The concentrations and test conditions were the same as in the first investigation.

Cytotoxicity

Within an experiment, a decrease in cell density of ≥ 50% is considered as a cytotoxic effect.

Acceptance criteria

Generally, the experiment is considered valid, if the following criteria are met:

- The positive controls induced an agonistic / antagonistic effect within the range of the historical control data.
- The concentration $5x10^{-9}$ mol/L 5α-dihydrotestosterone achieved at least 40 percent of the maximum effected androgen receptor dependent enzyme expression of the positive control (colour development) based on the experiment.
- The vehicle control did not show colour development at 570 nm.

Assessment criteria

A test substance is considered to have agonistic effect on the androgen receptor in the YAS assay, if it induces a relative increase on at least one concentration exceeding 15% absorption, calculated as follows:

$$\frac{\text{median optical density (OD) (test concentration) – median OD (vehicle control)}}{\text{median OD (positive control at 1E-07 mol/L) – median OD (vehicle control)}} * 100\ [\%]$$

A test substance is considered to have antagonistic/positive activity in the YAS assays, if the relative decrease for at least one concentration exceeds 20%, calculated as follows:

$$\frac{\text{median OD (test concentration) – median OD (vehicle control)}}{\text{median OD (positive control) – median OD (vehicle control)}} * 100\ [\%] \quad -100$$

where the positive control is the test substance concentration at 1E-10 mol/L with 5 nmol/L dihydrotestosterone. Concentrations, for which cytotoxicity is concluded, are excluded from the assessment of antagonistic activity.

Results

No analytical determination of the test substance solutions was performed. No test substance precipitation was found.

Androgenicity:

An increase in the androgen receptor dependent enzyme expression (colour development) was not observed.

Anti-androgenicity:

Inhibition of the androgen effect in comparison to hydroxyflutamide was not observed.

Cytotoxicity

Due to clear cytotoxicity observed at a concentration of 10^{-6} mol/L, an evaluation of the androgenic/anti-androgenic potential of the test substance at this concentration was not possible.

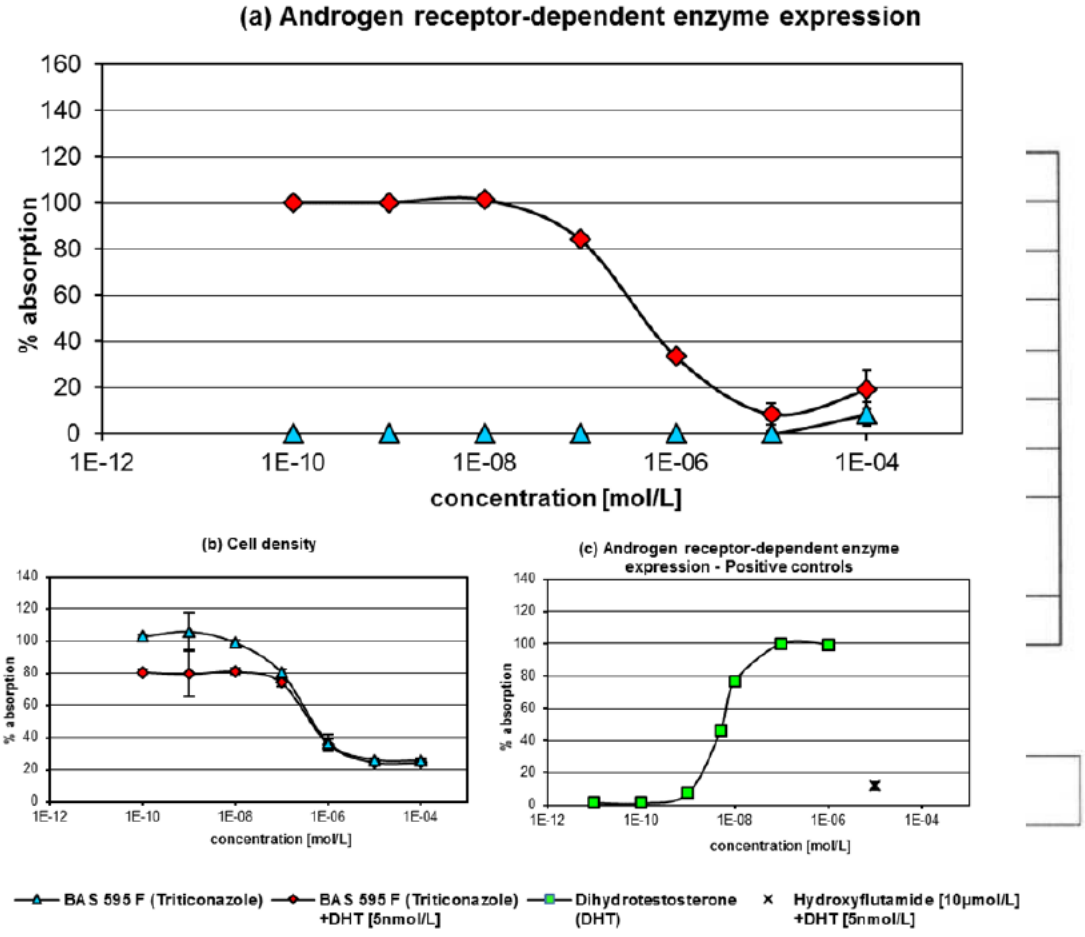


Figure 6.8.3.2-1: Androgen receptor-dependent enzyme expression; Graph a) shows the absence of an androgenic (triticonazole: triangles) and an antiandrogenic response (triticonazole + dihydrotestosterone: diamonds) compared to the responses of the positive controls (graph c). The results of the cell viability are given in graph b).

Table 6.8.3.2-1 gives overview over the calculated responses for an agonistic or antagonistic activity of triticonazole

Table 6.8.3.2-1: Overview of agonistic and antagonistic effects induced by triticonazole in the YAS assay

	agonistic activity of	antagonistic activity of	cell density of	
Concentration [mol/l]	BAS 595 F (Triticonazole) [%]	BAS 595 F (Triticonazole) +DHT [5nmol/L] [%]	BAS 595 F (Triticonazole) [%]	BAS 595 F (Triticonazole) +DHT [5nmol/L] [%]
1.00E-10	0.1	0.0	102.8	80.7
1.00E-09	0.0	0.2	105.9	79.6
1.00E-08	0.0	1.3	99.0	81.0
1.00E-07	0.0	-15.9	80.3	74.1
1.00E-06	0.0	no evaluation*	36.6	36.0
1.00E-05	0.0	no evaluation*	26.0	24.5
1.00E-04	8.4	no evaluation*	25.4	24.4

* cytotoxicity (decrease in cell density more than 50%)

Conclusion

In this Yeast Androgen Screening (YAS) assay using the hAR yeast strain, the test substance triticonazole showed no androgenic activity in comparison to dihydrotestosterone. The test substance triticonazole showed no antiandrogenic activity when compared to the effects induced by 10 µmol/L hydroxyflutamide. Due to clear cytotoxicity observed at a concentration of 1E-06 mol/L and higher, an evaluation of the androgenic/antiandrogenic potential of the test substance at these concentrations was not possible.

B.6.8.4.3. YES assay

Previous evaluation: DRAR (2016)	No New study

Reference:	a) Amendment No. 1 to the Summary of Results: BAS 595 F (Triticonazole) - Testing for potential estrogenic and antiestrogenic activity using the YES-assay [ERa] (Yeast estrogen screening) b) In house validation of recombinant yeast estrogen and androgen receptor agonist and antagonist screening assays
Author(s), year:	Woitkowiak C., 2012 (main study) and 2016 (amendment) [redacted] 2010 (validation)
Report/Doc. number::	-/ 2012/1276018 and 2016/1126368
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

The test is based on genetically modified yeast cells (*Saccharomyces cerevisiae*), which contain the gene for the human estrogen receptor coupled to a reporter gene lacZ. Activation of the receptor initiates a cascade of events leading to the expression of the reporter gene product (β-galactosidase) which converts a substrate (CPRG, chlorophenol red-β-D-galactopyranoside) which leads to a measurable color change from yellow to red in the medium.

Materials and methods

Test Material	BAS 595 F (Triticonazole)
Description:	Solid, white
Lot/Batch #:	COD-001440
Purity:	91.3% (tolerance ± 1.0%)
Stability of test compound:	NA
Solvent used:	Dimethylsulfoxide (DMSO)
Control Materials:	
Vehicle control:	DMSO 1% (v/v)
Positive control compounds:	Estrogenic control: 17β-estradiol Anti-estrogenic control: 17β-estradiol combined with 4-hydroxytamoxifen
Test organisms:	Yeast cells (*Saccharomyces cerevisiae*) have been stably transformed with a gene encoding the human estrogen receptor α (hERα), which is constitutively expressed. Additionally, these cells are stably transformed with a reporter gene plasmid, containing an estrogen response element and the *LacZ* gene, which encodes the reporter enzyme β-galactosidase. The hERα yeast strain was obtained from "Technische Universität Dresden", Prof. Dr. G. Vollmer on 11 Feb 2010.

Final test substance concentrations: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} mol/L

Test method:

A deep-frozen (-80°C) yeast stock culture was thawed at room temperature, inoculated in growth medium and incubated for pre-culture (24-72 h) and growth medium was exchanged after 72 h before use. Of the pre-culture, optical density (OD) was determined at 690 nm. For preparation of the test culture, 0.50 mL of the pre-culture with an OD of 1.0 was transferred into 50 mL fresh culture medium including 0.5 mL chromogenic substrate CPRG (chlorophenol red-β-D-galactopyranoside).

The study was carried out in 96-well microtiter plates in which 2 µL of different test substance solutions had been pipetted. 200 µL of the test culture was added to each well. The plates was sealed with breathable tape and incubated until measurement of the OD.

Controls:

Each experiment includes a negative control (vehicle control) and positive controls for the verification of the detection of estrogenic and anti-estrogenic activity in the yeast cells.

Negative controls / Vehicle controls

The vehicle control contains 2 µL of the vehicle used for the test substance. The final concentration of the vehicle in the culture medium will be 1% (v/v).

Positive controls

Estrogenic control:

17β-estradiol (dissolved in ethanol)
Final concentrations: 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} mol/L

Anti-estrogenic control:
17β-estradiol combined with 4-hydroxytamoxifen (dissolved in DMSO)
Final concentrations:
$1x10^{-9}$ mol/L (17β-estradiol)/ $1x10^{-6}$ mol/L (4-hydroxytamoxifen)

The stability of the selected positive controls is well-defined under the chosen culture conditions since they are well established reference endocrine disruptors.

Evaluation/Assessment

Endocrine activity

After 48 h (±4 h) incubation, absorbance of the plates is measured at 570 nm (colour development, estrogen receptor dependent enzyme expression) and 690 nm (turbidity due to growth of the yeast). Evaluation is performed by calculating the difference of the measured ODs at the two wavelengths (absorption at 570 nm - absorption at 690 nm). For the 4 replicates per concentration the median, minimum and maximum value are presented in a diagram. The findings of the 1st experiment were confirmed in an additional assay. The concentrations and test conditions were the same as in the first investigation.

Cytotoxicity

Within the experiment, a decrease in cell density of ≥ 50% is considered as a cytotoxic effect.

Acceptance criteria

Generally, the experiment is considered valid, if the following criteria are met:

- The positive controls induced an agonistic / antagonistic effect within the range of the historical control data.
- The concentration $1x10^{-9}$ mol/L 17β-estradiol achieved at least 40 percent of the maximum effected estrogen receptor dependent enzyme expression of the positive control (colour development) based on the experiment.
- The vehicle control did not show colour development at 570 nm.

Assessment criteria

A test substance is considered to have agonistic effect on the estrogen receptor in the YES assay, if it induces a relative increase on at least one concentration exceeding 15% absorption, calculated as follows:

$$\frac{\text{median optical density (OD) (test concentration) – median OD (vehicle control)}}{\text{median OD (positive control at 1E-08 mol/L) – median OD (vehicle control)}} * 100\ [\%]$$

Otherwise, it is considered non-agonistic/negative.

A test substance is considered to have antagonistic/positive activity in the YES assays, if the relative decrease for at least one concentration exceeds 20%, calculated as follows:

$$\frac{\text{median OD (test concentration)} - \text{median OD (vehicle control)}}{\text{median OD (positive control)} - \text{median OD (vehicle control)}} * 100\ [\%] - 100$$

where the positive control is the test substance concentration at 1E-10 mol/L with 1E-09 mol/L 17ß-estradiol.

Concentrations, for which cytotoxicity is concluded, are excluded from the assessment of antagonistic activity.

Results

No analytical determination of the test substance solutions was performed. No test substance precipitation was found.

Estrogenicity:

An increase in the androgen receptor dependent enzyme expression (colour development) was not observed.

Anti-estrogenicity:

A reproducible inhibition of the androgen effect compared to $1x10^{-9}$ mol/L 17β-estradiol (partly or total suppression of expected colour development) was not observed.

At 10^{-6} mol/L a moderate reduction of optical density compared to the control value was observed, and clear cytotoxicity of the test substance was noticed at a concentration of 10^{-5} mol/L onwards.

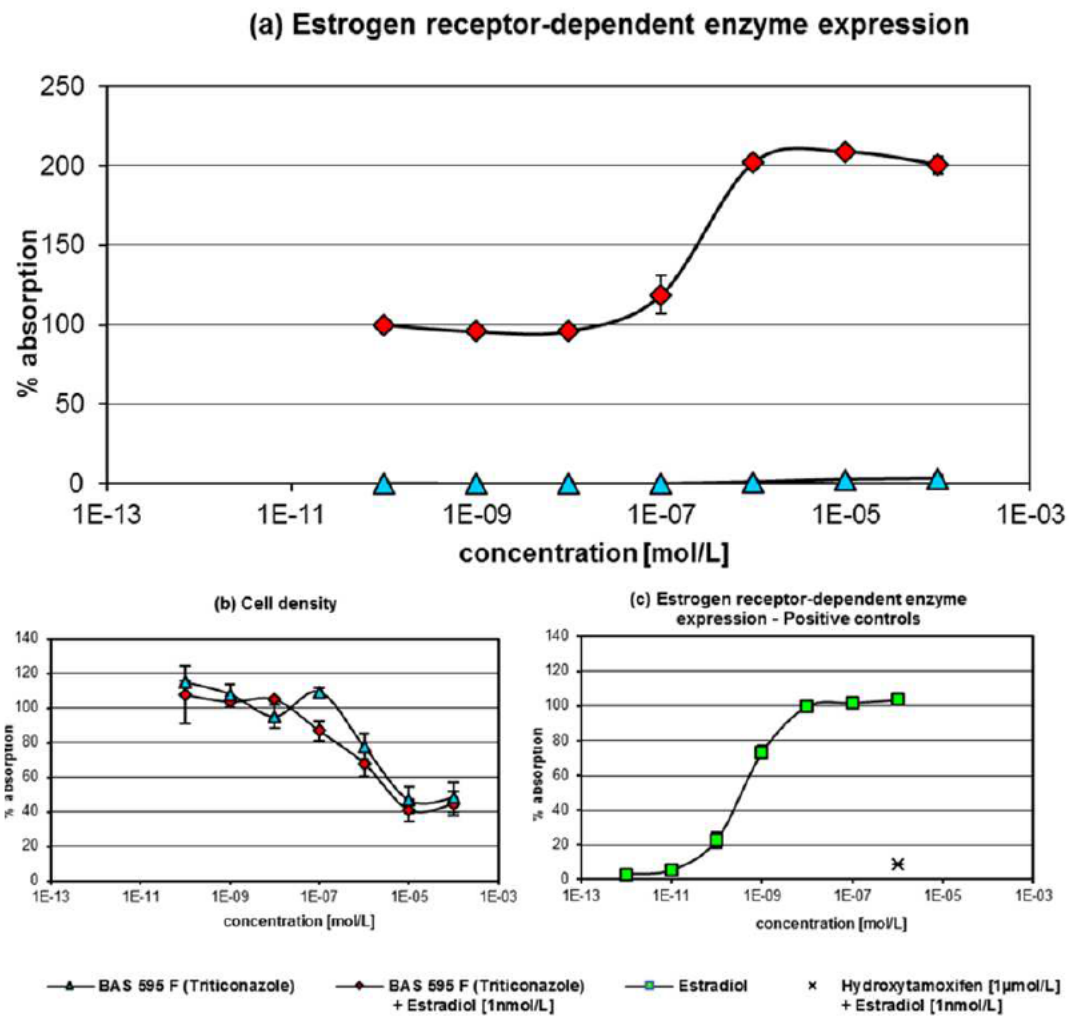


Figure 6.8.3.3-1: Estrogen receptor-dependent enzyme expression; Graph a) shows the absence of an estrogenic (triticonazole: triangles) and an antiestrogenic response (triticonazole + hydroxytamoxifen: diamonds) compared to the responses of the positive controls (graph c). The results of the cell viability are given in graph b).

Table 6.8.3.3 -1 gives overview over the calculated responses for an agonistic or antagonistic activity of triticonazole.

Table 6.8.3.3-1: Overview of agonistic and antagonistic effects induced by triticonazole in the YES assay

	agonistic activity of	antagonistic activity of	cell density of	
Concentration [mol/l]	BAS 595 F (Triticonazole) [%]	BAS 595 F (Triticonazole) + Estradiol [1nmol/L] [%]	BAS 595 F (Triticonazole) [%]	BAS 595 F (Triticonazole) + Estradiol [1nmol/L] [%]
1.00E-10	0.1	0.0	115.0	107.8
1.00E-09	0.0	-4.2	108.0	103.8
1.00E-08	0.0	-3.9	95.2	105.1
1.00E-07	0.1	18.9	109.3	86.9
1.00E-06	1.0	101.9	77.8	68.0
1.00E-05	2.5	no evaluation*	46.8	40.8
1.00E-04	3.1	no evaluation*	48.2	44.6

* cytotoxicity (decrease in cell density more than 50%)

Triticonazole alone did not enhance the estrogenic activity of estradiol at concentrations >1E-07 mol/L (see triangles in graph (a)). Although triticonazole did not induce agonistic effects when tested alone, it enhanced the estrogenic activity of estradiol at concentrations >1E-07 mol/L. This effect could be indicative of potential estradiol-receptor-complex stabilization, which could lead to prolonged reporter-gene translater. However, the mechanism underlying this effect is unclear.

Conclusion

In this Yeast Estrogen Screening (YES) assay using the hERα yeast strain, triticonazole showed no estrogenic activity in comparison to 17ß-estradiol. Triticonazole showed also no antiestrogenic activity when compared to the effects induced by 1 µmol/L hydroxytamoxifen. Although triticonazole did not induce agonistic effects when tested alone, it enhanced the estrogenic activity of estradiol at concentrations >1E-07 mol/L. Clear cytotoxicity of the test substance was noticed at concentrations of 1E-05 and 1E-04 mol/L.

B.6.8.4.4. Aromatase inhibition

Previous evaluation:	No
DRAR (2016)	New study

Reference:	Triticonazole (BAS 595 F) - Human and rat recombinant aromatase assay
Author(s), year:	Mentzel T., 2015
Report/Doc. number::	-/ 2015/1197309
Guideline(s):	EPA 890.1200
GLP:	No
Deviations from Guideline:	The method modified according to Stresser et al., 2000. - Instead of human and rat placental tissue as a source of aromatase enzyme and radiolabeled C19 androgens as substrate, reeecombinant aromatase and the fluorometric substrate O-benzyl fluorescein benzyl ester (DBF) were used, in order to avoid the use of radioactive substances and to increase the safety of lab procedure -Fenarimol, letozole and econazol nitrate were included as additional positive controls to ASDN - Bis(2-ehtylhexyl)phthalate was included as additional negative control - Reference compound, control substances and triticonazole were tested in each test run in parallel - Seven instead of three test runs were conducted

Acceptability:	Yes; additional information

Material and methods

Test Material	
Test item:	Triticonazole
Description:	Solid, powder
Lot/Batch #:	SZBB349XV
Purity:	98.8%
Vehicle control	DMSO (final concentration 1%)
Negative control	
Test substance 1	Atrazine
CAS No.:	1912-24-9
Description:	Solid, powder
Lot/Batch #:	SZBD158XV
Purity:	99.1%
Supplier:	Sigma-Aldrich #45330
Test substance 2	Bis(2-ehtylhexyl)phthalate
CAS No.:	117-81-7
Description:	Liquid
Lot/Batch #:	SZBB167XV
Purity:	99.7%
Supplier:	Fluka #36735
Positive control	
Test substance 1	4-OH ASDN
CAS No.:	566-48-3
Description:	Solid, powder
Lot/Batch #:	081k2133V
Purity:	99.6%
Supplier:	Sigma-Aldrich F25525
Test substance 2	Fenarimol
CAS No.:	60168-88-9
Description:	Solid, powder
Lot/Batch #:	SZBD071XV
Purity:	99.9%
Supplier:	Fluka #45484
Test substance 3	Econazol nitrate
CAS No.:	24169-02-6
Description:	Solid, powder
Lot/Batch #:	BCBL5063V
Purity:	98%
Supplier:	Sigma Aldrich #E4632
Test substance 3	Letrozole
CAS No.:	112809-51-5
Description:	Solid, powder
Lot/Batch #:	104M4759V
Purity:	98.0%
Supplier:	Sigma-Aldrich #L6545
Test system	Recombinant Aromatase
Human:	Corning Supersomes Human CYP19 (Aromatase) + Reductase (#456260) expressed in baculovirus/insect cells
Rat:	Corning Supersomes Rat CYP19 (Aromatase) + Reductase (#457254)

Test substance preparation:

Final substance concentrations (except econazole nitrate and letrozole) tested were: 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} mol/L, based on solubility limit in assay buffer of 100 µM. Econazole nitrate and letrozole were tested in concentrations ranging from 10^{-13} to 10^{-6} and 10^{-12} to 10^{-5}, respectively, their solubility limits in assay buffer were 30 µM and 1 mM, respectively.

In addition to a macroscopic inspection also microscopic analysis has been done to examine solubility limitations at high compound concentrations. Compounds have been diluted in an identical volume and buffer as for the assay performed using a 96 well plate which allows microscopic analysis.

Test method:

The assay was conducted in a 96-well microplate utilizing the recombinant human or rat aromatase and the fluorometric artificial substrate dibenzylfluorescein (DBF) as described by Stresser et al., 2000. After addition of the test compounds (in all dilutions) and all cofactors (1.3 mM $NADP^+$, 0.4 mU Glucose-6-phosphat-dehydrogenase, 3.3 mM Glucose-6-phosphate and 3.3 mM $MgCl_2$) to each well, reaction was started with 100 µL of enzyme/substrate mix (4 pmol/mL enzyme, 0.4 µM DBF). Plates were incubated for 30 minutes at 37°C. Reaction was determined by addition of 75 µL 2 M NaOH, which results in a cleavage of the oxidized DBF to the fluorescent product fluorescein. To develop adequate signal to noise ratio, plates were incubated for another 2 hours at 37°C. Measurement was conducted at 490 nm excitation wavelength, 530 nm emission wavelength and 515 nm cut-off.

Experimental design has been adapted according to OCSPP Guideline 890.1200. Also data handling and interpretation are adopted to this guideline. However, the analysis described in OCSPP Guideline 890.1200 is using a radioactive substrate, while the procedure used for this study employs a non-radioactive artificial substrate.

In addition to the control substances recommended in the guideline, further additional positive controls (4-OH ASDN, fenarimol, econazol nitrate, letrozole) as well as negative controls (atrazine and bis(2-ethylhexyl)phthalate) have been carried out not only in one, but in each test run in parallel to the test compounds using a plate set up.

A serial dilution as triplicates was performed for each compound with a 50 mM potassium phosphate buffer pH 7.4. Four wells per test plate without enzyme were used as control to determine background fluorescence. Additional full enzyme activity were analysed done using DMSO only in 4 wells per plate. This analysis has been repeated in seven independent experiments.

Data interpretation:

The method used in this study is measuring the generation of a florescent product for the analysis of human or rat aromatase activity.

Values of background and full activity controls were determined, and ratios of full activity / background activity range were calculated. Acceptable average ratio is 7.69 and should always be below 15% of mean full activity representing an ideal activity range for this activity measurement.

Absolute fluorescence was corrected by subtraction of mean background control of each individual plate and normalized to the full activity control to achieve % activity values.

Resulting activity values have been fitted using the 4-parameter regression model to yield a sigmoidal inhibition curve. The resulting IC_{50} values, as well as slope of the calculated curve, were used for a statistical analysis to ensure data consistency. All runs significantly diverging from the overall analysis have been excluded from the final calculation of an average IC_{50} value.

Statistical analysis

Dose-response analyses were made using the log-logistic 4-parameter model. Assumptions were checked for each model calculated and a Box-Cox algorithm was used to determine the optimal lambda value using a profile likelihood approach: For each lambda value the non-linear regression model is fitted and the lambda value resulting in the largest value of the log likelihood function is picked (Carroll & Ruppert, 1988[16]). After transformation all results were back transformed to the original scale.

Also parameter estimates for all models were compared for each day and plate. Comparisons were made calculating the ratio and its standard error between days or plates. Significant deviations between parameters obtained at different days or for identical plates were detected using a t-test. Significance levels were adjusted using a Bonferroni correction. In cases when parameter estimates for the IC_{50} or slope did differ significantly between days outlier days were removed. This was well possible, as in total seven independent runs have been conducted, which exceeds the minimum number of three runs by far recommended by guideline OCSPP Guideline 890.1200.

All calculations were made using R 3.2.1 (R Core Team 2015). Dose-response models including Box-Cox transformations were calculated using the "drc" package version 2.5-12 (Ritz & Strebig 2015[17]).

The notifier confirmed that the modified method is established in the laboratory since 2012 and that the responsible technician had proven proficiency for conducting the test. Validation report has been included in the submission.

Results

Human Aromatase Inhibition

Triticonazole was tested up to 100 µM, as precipitation was observed at higher concentrations. Triticonazole showed aromatase inhibition on the human enzyme with an IC_{50} of 44 µM.

Table 6.8.3.4-1: Mean human aromatase IC_{50} values

Test item	human aromatase IC50 [M]	
	mean	SD
Test substance		
Triticonazole	4.40×10^{-5}	3.01×10^{-5}
Positive control		
Letrozole	9.02×10^{-10}	8.03×10^{-11}
Econazole	2.30×10^{-9}	2.39×10^{-10}

[16] Carroll, R.J and Ruppert, D. (1998) Transformation and weighting in regression, New York : Chapman and Hall (Chapter 4)

[17] Ritz C and Strebig J.C. (2015) Ananlysis of dose-response curves, R library for the calculation of Dose Response Curves version 2.5-12.

4-OH ASDN	1.38×10^{-8}	2.22×10^{-8}
Fenarimol	1.26×10^{-6}	1.74×10^{-7}

In relation to letrozole and econazole this corresponds to an about 50 000 to 20 000-fold lower potency for inhibition of human aromatase. Compared to 4-OH ASDN the difference was 3200 and 35 fold difference was estimated compared to fenarimol. Accordingly, triticonazole has potency for inhibition of the human aromatase enzyme, which is several magnitudes below the weakest inhibitory substance analyzed in the study.

Rat Aromatase Inhibition

For the inhibition of the rat aromatase the half maximal concentration was 1.80×10^{-6} M, however at this concentration not a full inhibition for the aromatase was found.

Table 6.8.3.4-2: Mean rat aromatase IC_{50} values

Test item	rat aromatase IC_{50} [M]	
	mean	SD
Test substance		
Triticonazole	1.8×10^{-6}	2.59×10^{-7}
Positive control		
Letrozole	1.53×10^{-9}	1.03×10^{-10}
Econazole	1.60×10^{-9}	1.52×10^{-10}
4-OH ASDN	3.57×10^{-8}	3.48×10^{-9}
Fenarimol	1.79×10^{-7}	2.15×10^{-8}

Human vs. Rat Aromatase Inhibition

In addition to the measured differences in IC_{50} values between the tested compounds, triticonazole also has a different potency on human and rat aromatase. The half maximal inhibitory concentration for triticonazole on rat Cyp19 is by a factor >20 fold below the IC_{50} for the inhibition on human aromatase. Accordingly, triticonazole is a stronger inhibitor for rat aromatase than for human aromatase enzyme.

Table 6.8.3.4-3: Pairwise comparison of IC_{50} values of triticonazole and positive controls for human and rat aromatase inhibition

Test substance	fold difference (human/rat)	Standard error
Triticonazole	24.38	17.06
4-OH ASDN	0.39	0.07
Econazole-nitrate	1.44	0.20
Fenarimol	7.05	1.29
Letrozole	0.59	0.07

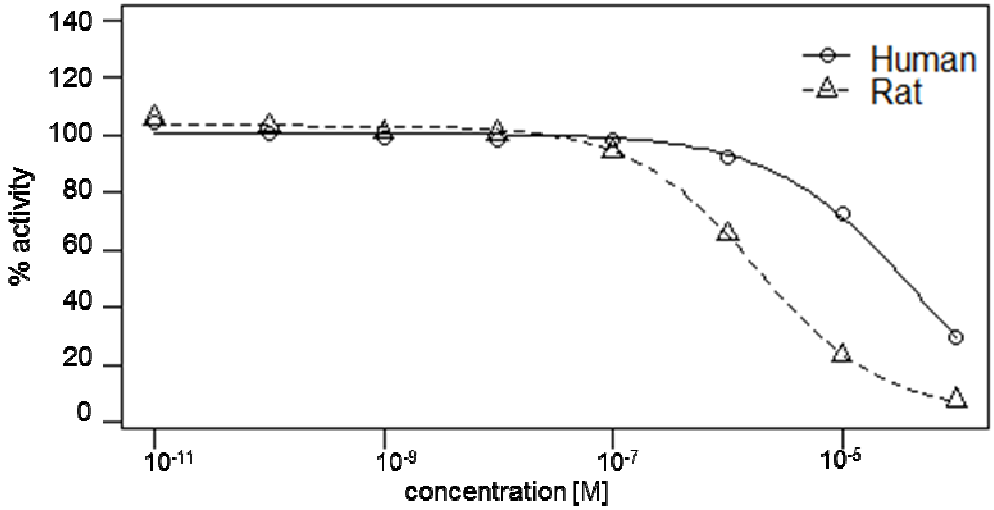


Figure 6.8.3.4-1: Comparison of triticonazole induced inhibition of rat and human aromatases

Conclusion

In this study the IC50 value of triticonazole to inhibit rat and human aromatase has been determined. Triticonazole had an IC50 value for rat aromatase of 1.8 x 10^{-6} [M]. The IC50 value for human aromatase was found to be 4.40 x 10^{-5} [M]. Compared to a number of positive controls (4-OH-ASDN, Econazole-nitrate, Fenarimol, Letrozol) the inhibiting properties of triticonazole for human aromatase were between 35 – 50000 fold lower, when IC50 values – measured in the same test run – were compared.

Comparing the rat and human aromatase inhibiting properties of triticonazole it has been found that the IC50 values were >20-fold different, giving evidence that triticonazole was 20-fold less potent to inhibit human aromatase compared to rat aromatase. No complete inactivation was found for human aromatase enzyme for which a mean activity of about 30% was found at the maximum technically achievable concentration of triticonazole (100 µM).

Comparison of aromatase inhibition properties of triticonazole comaped to other azoles

Notifier included also following evaluation in the dossier:

The aromatase inhibiting properties of triticonazole have been determined by measuring the IC50 values for rat and human aromatase (see above). Triticonazole had an IC_{50} value for rat aromatase of 1.8 x 10^{-6} [M]. The IC_{50} value for human aromatase was found to be 4.40 x 10^{-5} [M]. Compared to a number of positive controls (4-OH-ASDN, Econazole-nitrate, Fenarimol, Letrozol) – measured in the same test runs - the inhibiting properties of triticonazole for human aromatase were between 35- and 50000-fold lower, when IC_{50} values were compared. Furthermore, there is a considerable difference between rat and human aromatase inhibition, as shown in the study summary above - a factor of > 20 has been found for the IC_{50} values between rat and human, indicating a 20-fold lower potency of triticonazole to inhibit human aromatase compared to rat aromatase. It has been found that even no complete inactivation of the human aromatase could be achieved by the maximum (technically achievable) concentration of triticonazole.

Table 6.8.3.4-4 presents IC_{50} data for human recombinant aromatase taken from Trösken et al., 2004[18] (except the figure for triticonazole which is the value derived for human aromatase assay described above). Data for human pharmaceuticals used as clinical fungal steroid inhibitors (antimycotics) is shown, as well as data for two cytostatic conazoles, which specifically exploit aromatase inhibition in the treatment of human breast cancer. As might be expected, these are some of the most potent aromatase inhibitors of all those examined. Some of the human pharmaceuticals used as antimycotics (e.g. clotimazole), have aromatase IC_{50} up to 3 orders of magnitude lower than triticonazole, but are considered appropriate for regular human use.

Table 6.8.3.4-4: Recombinant human aromatase (Cyp19) inhibition (IC_{50}) by azole fungicides and drugs

Azole	Aromatase IC_{50} (µM)
Fungicides	
Prochloraz (I)	0.047
Flusilazole	0.055
Imazalil (I)	0.072
Penconazole	0.85
Epoxiconazole	1.44
Propiconazole	3.2
Tebuconazole	5.8
Cyproconazole	8.5
Triadimenol	12.6
Triadimefon	17.5
Triticonazole	44
Human pharmaceuticals	
Antimycotics	
Bifonazole	0.019
Miconazole	0.064
Clotrimazole	0.11
Ketoconazole	5.6
Itraconazole	>70* (33% inhibition)
Voriconazole	>140* (40% inhibition)
Fluconazole	>140* (16% inhibition)
Cytostatics	
Fadrozole	0.0076
Letrozole	0.015

The IC_{50} values (except triticonazole) were derived from Trösken et al (2004).

For the human pharmaceutical antimycotic azoles, EPARs (European Public Assessment Reports) are available only for voriconazole and posaconazole. For voriconazole and posaconazole (not examined by Trösken et al, 2004), no human adverse events that might be interpreted as endocrine in nature, could be located.

B.6.8.4.5. Other studies (literature)

B.6.8.4.5.1. Rotroff D.M. et al., 2014

Previous evaluation: DRAR (2016)	No Literature data

Reference:	Predictive endocrine testing in the 21st century using in vitro assays of estrogen receptor signaling responses

[18] Trösken, E. R., Scholz, K., Lutz, R. W., Völkel, W., Zarn, J. A., & Lutz, W. K. (2004). Comparative assessment of the inhibition of recombinant human CYP19 (aromatase) by azoles used in agriculture and as drugs for humans. Endocrine Research, 30(3), 387-394

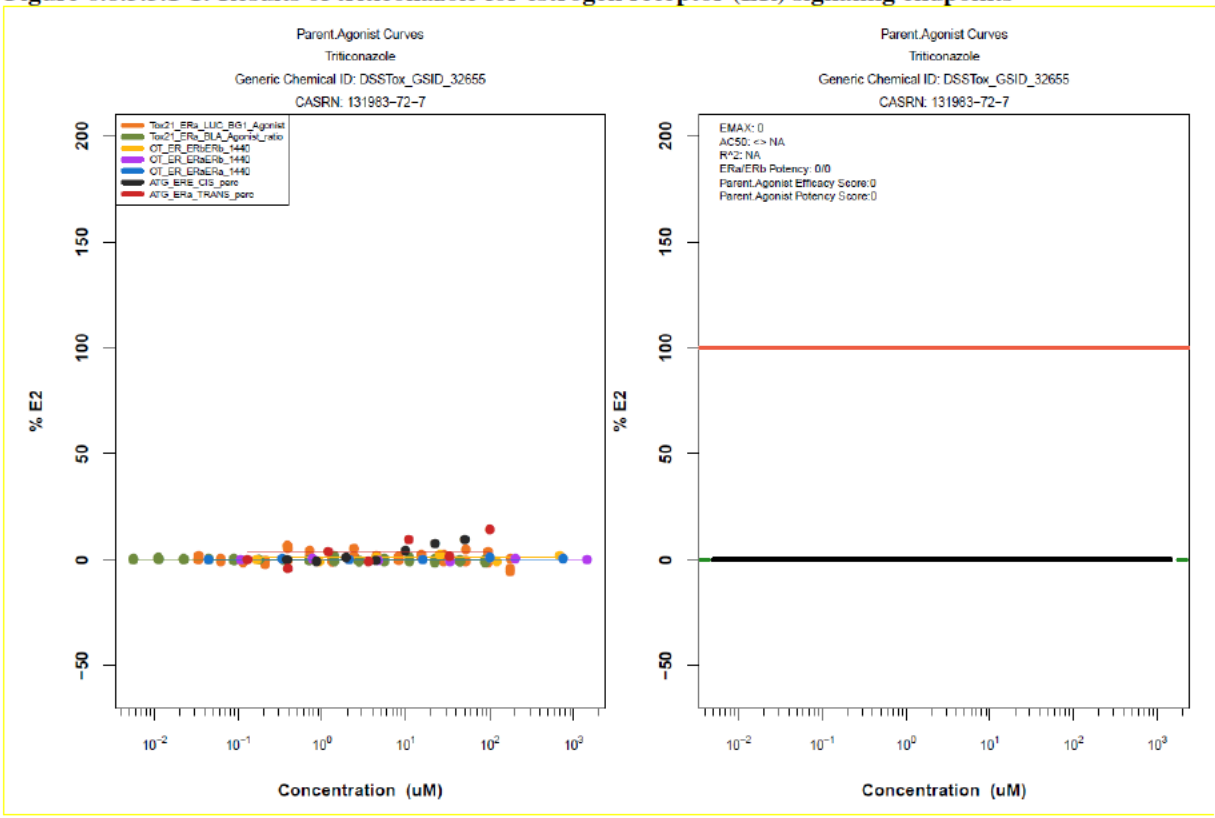
Author(s), year:	Rotroff D.M. et al., 2014
Report/Doc. number::	-/ 2014/1323273
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

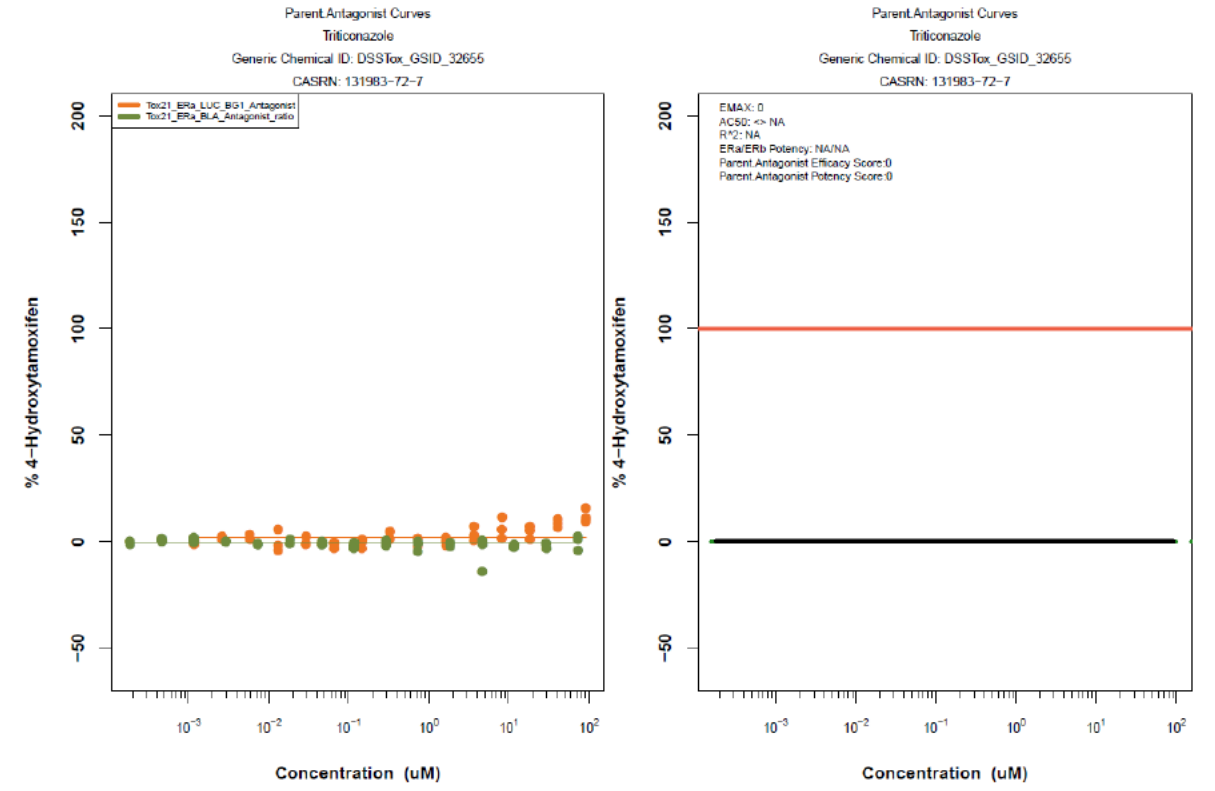
Executive Summary

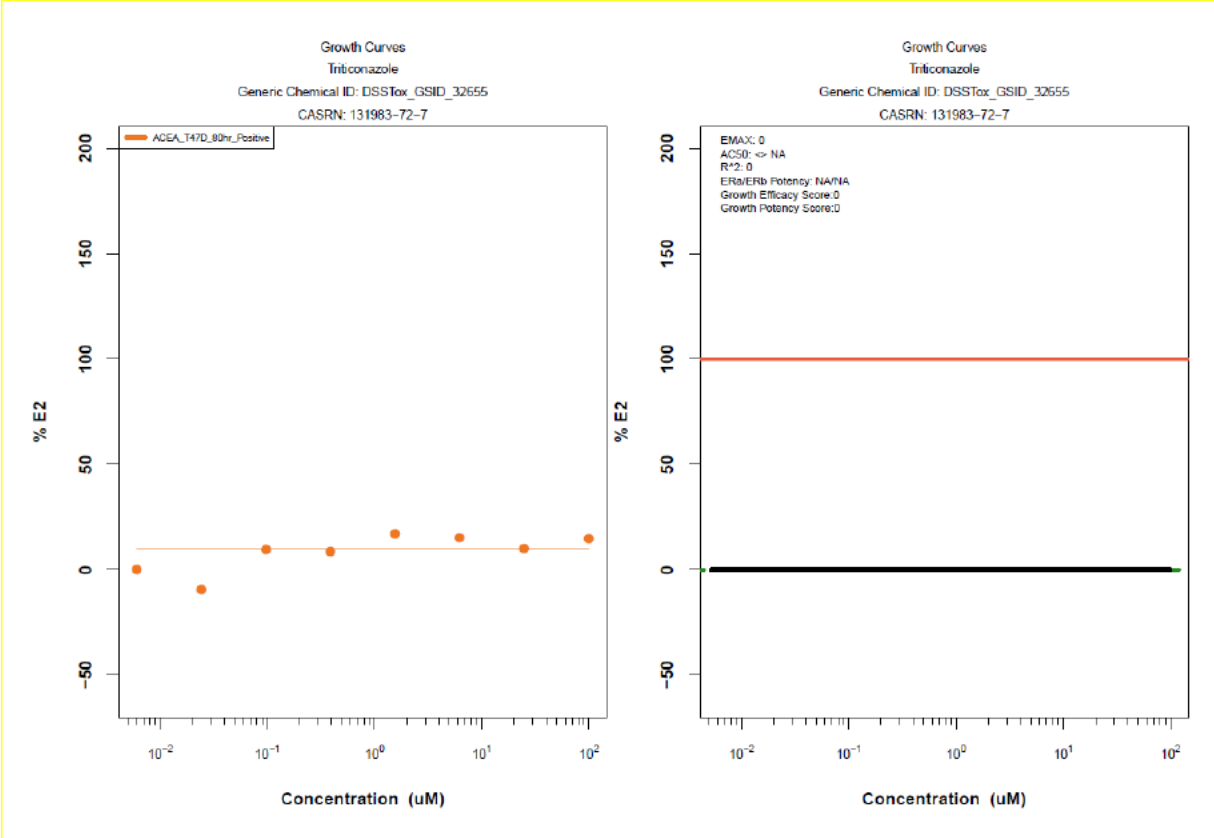
Thousands of environmental chemicals are subject to regulatory review for their potential to be endocrine disruptors (ED). In vitro high-throughput screening (HTS) assays have emerged as a potential tool for prioritizing chemicals for ED-related whole-animal tests. In this study, 1814 chemicals including pesticide active and inert ingredients, industrial chemicals, food additives, and pharmaceuticals were evaluated in a panel of 13 in vitro HTS assays. The panel of in vitro assays interrogated multiple end points related to estrogen receptor (ER) signaling, namely binding, agonist, antagonist, and cell growth responses. The results from the in vitro assays were used to create an ER Interaction Score. For the ~1,800 chemicals evaluated in this study, 82% did not display indications of interacting with the ER signaling pathway and would be low priorities for additional ER testing. If maximum sensitivity is desired, the model can be run with narrower confidence intervals around the composite curves. This would result in an increased false positive rate and a decreased false negative rate.

For this dossier the data for triticonazole is relevant and thus the results are described in the following figures. In summary, triticonazole is negative for estrogen receptor (ER) signaling endpoints, namely binding, agonist, antagonist and cell growth responses. The ER Interaction Score was found to be 0 for triticonazole. Therewith, triticonazole is one of the 82% chemicals which did not display indications of interacting with the ER signaling pathway.

Figure 6.8.3.5.1-1: Results of triticonazole for estrogen receptor (ER) signaling endpoints







An ER Interaction Score was developed by aggregating data from 13 different in vitro ER assays based on the known cellular ER signaling pathways. This model produced scores for an overall likelihood of a chemical being estrogenic, and these scores were highly correlated with in vivo data and ER reference chemical classifications, indicating that the model is capable of predicting estrogenic likelihood with a high degree of accuracy.

This study analyses around 1800 substances concerning their potential to be an endocrine disruptor. Triticonazole was one of the investigated substances and found to be zero for the binding group, agonist group, antagonist group, growth group and the ER Interaction Score and would be therefore of low priority for additional ER testing.

B.6.8.4.5.2. Reif D.M. et al., 2010

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Endocrine profiling and prioritization of environmental chemicals using ToxCast data
Author(s), year:	Reif D.M. et al., 2010
Report/Doc. number::	-/ 2010/1231552
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This publication describes a profiling tool developed on the ToxCast database to prioritize chemicals with regard to endocrine disruption evaluation/testing as a decision support tool. Thus this priorization tool was applied also to triticonazole being part of the ToxCast program. The priorization tool focused on estrogen, androgen and thyroid pathways and thus incorporated those screening assays of the ToxCast program considered relevant for putative endocrine profiles. In addition it incorporated external molecular pathway databases i.e. Kyoto Encyclopedia of Genes and Genomes (KEGG), Ingenuity software and the Online Mendelian Inheritance in Men repository. The tests in which triticonazole showed an activity are the same as described in the publication of Shah et al. 2011 (see 6.8.2.3). The current evaluation of the results can be seen on EPA's dashboard (http://actor.epa.gov/dashboard/). The so-called ToxPi profile for triticonazole (supplementary information) indicates activity in LogP and XME/ADME path, and medium activity in TR (thyroid receptor), predicted Caco-2, AR (androgen receptor) and other NR (nuclear receptor), as well as low activity in KEGG pathways. The publication does not provide an absolute ranking but in visual comparison to activity alerts for other compound the ranking of activities for triticonazole is moderate. No linkage between triticonazole and other endpoints is provided.

The results of the ToxCAST program are difficult to assess in the context of a regulatory assessment, as this analysis does not take into account that multiple assays exist for the same pathway or endpoint within the ToxCast program. In principle a chemical acting on any given pathway should score at multiple assays associated with that endpoint. Individual hits should be disregarded in the context of a weight of evidence. Another factor not taken into account is that assay results achieved at cytotoxic concentrations have low reliability. Using the EPA dashboard it is apparent that positive activity calls were largely seen at cytotoxic concentration. Furthermore different assays for the indicated outcome did not show concordance. Further the observed in vitro activities should be assessed together with the available toxicological data package.

A more detailed description of the assays conducted under the ToxCAST program with triticonazole can be found at https://actor.epa.gov/dashboard/#chemical/131983-72-7.

Triticonazole was subjected to an assay set for the androgen receptor (AR - 11 assays existing, but triticonazole was only tested in 10 of the 11 assays), for the estrogen receptor (ER - 18 assays existing, but triticonazole was only tested in 16 assays) and the thyroid receptor (ThR - 4 assays existing, but triticonazole was only tested in 3 assays).

The AR assay set consists of the following assays:

- ATG_AR_TRANS_up
- NVS_NR_cAR
- NVS_NR_hAR
- NVS_NR_rAR (triticonazole not tested)
- OT_AR_ARELUC_AG_1440
- OT_AR_ARSRC1_0480
- OT_AR_ARSRC1_0960
- Tox21_AR_BLA_Agonist_ratio
- Tox21_AR_BLA_Antagonist_ratio
- Tox21_AR_LUC_MDAKB2_Agonist
- Tox21_AR_LUC_MDAKB2_Antagonist

The ER assay set consists of the following 18 assays

- ACEA_T47D_80hr_Positive
- ATG_ERE_CIS_up
- ATG_Era_TRANS_up
- NVS_NR_bER (triticonazole not tested)
- NVS_NR_hER (triticonazole not tested)
- NVS_NR_mERa
- OT_ER_ERaERa_0480
- OT_ER_ERaERa_1440
- OT_ER_ERaERb_0480
- OT_ER_ERaERb_1440
- OT_ER_ERbERb_0480
- OT_ER_ERbERb_1440
- OT_Era_EREGFP_0120
- OT_Era_EREGFP_0480
- Tox21_ERaBLA_Agonist_ratio
- Tox21_ERa_BLA_Antagonist_ratio
- Tox21_Era_LUC_BG1_Agonist
- Tox21_Era_LUC_BG1_Antagonist

ThR assay set consists of 4 assays

- ATG_THRa1_TRANS_up
- NVS_NR_hTRa (triticonazole not tested)
- Tox21_TR_LUC_GH3_Agonist
- Tox21_TR_LUC_GH3_Antagonist

Triticonazole was inactive in all assays for the estrogen receptor (ER) or the thyroid hormone receptor (ThR). In some of the assays for the androgen receptor, triticonazole was responsive. The respective AC50s, half-maximal activity concentrations, are summarized in the table below together with a description of the respective assay:

Table 6.8.3.5.2-1: AC50 concentration of triticonazole in selected AR assays

Assay	AC50 [µM]	Description of the assay
NVS_NR_cAR*	0.6743	NVS_NR_cAR was analyzed in the positive direction using measured readouts from the NVS_NR_cAR assay. This biochemical assay uses recombinantly expressed chimpanzee AR protein in a single radioligand binding design with Lysate-based radiodetection technology. Activity values from this assay component endpoint suggest that the chemical exposure for 72 hours resulted in loss-of-signal between the AR receptor binding its ligand.
NVS_NR_hAR*	0.9097	NVS_NR_hAR was analyzed in the positive direction using measured readouts from the NVS_NR_hAR assay. This cell-based assay uses wild type protein extracted from LnCAP** human leydig cells, in a single radioligand binding design with Lysate-based radiodetection technology. Activity values from this assay component endpoint suggest that the chemical exposure for 20 hours resulted in loss-of-signal between the AR receptor binding its ligand.
OT_AR_ARSRC1_0480	13.8876	OT_AR_ARSRC1_0480 was analyzed in the positive direction using measured readouts from the cell-based OT_AR_ARSRC1_0480 assay. In this assay, recombinantly transfected HEK293T, a human kidney cell line, was exposed to chemical for 8 hours in a single-readout protein fragment complementation assay design and measured by fluorescence technology to understand changes to the protein dimerization through the human AR and its coupling protein, SRC1. Activity values from this assay component endpoint provide gain-of-signal chemical activity.

OT_AR_ARSRC1_0960	15.7639	OT_AR_ARSRC1_0960 was analyzed in the positive direction using measured readouts from the cell-based OT_AR_ARSRC1_0960 assay. In this assay, recombinantly transfected HEK293T, a human kidney cell line, was exposed to chemical for 16 hours in a single-readout protein fragment complementation assay design and measured by fluorescence technology to understand changes to the protein dimerization through the human AR and its coupling protein, SRC1. Activity values from this assay component endpoint provide gain-of-signal chemical activity.
Tox21_AR_LUC_-MDAKB2_Agonist	97.567	Tox21_AR_LUC_MDAKB2_Agonist was analyzed in the positive direction using measured readouts from the cell-based Tox21_AR_LUC_MDAKB2_Agonist assay. In this assay, MDA-kb2, a human breast cell line, was exposed to chemical for 24 hours in a single-readout luciferase induction design and measured by luminescence technology to understand changes to the regulation of gene expression through the human AR and androgen response element (ERE). Activity values from this assay component endpoint provide gain-of-signal chemical activity as an androgen receptor agonist.

*The abbreviation NVS indicate the supplier of the assay: Novascreen
**LnCAP cells are androgen-sensitive human prostate adenocarcinoma cells derived from the left supraclavicular lymph node metastasis from a 50-year-old Caucasian male in 1977.

Different hits on different liver targets are not surprising, as the primary target organ of triticonazole is the liver. However, no liver tumors were developed in long term studies in rodents. The observed medium activity on the thyroid receptor is considered to be of low relevance, as the thyroid is not the target organ after triticonazole treatment in rats, mice and dogs. Following the more recent EPA evaluation of the triticonazole results (https://actor.epa.gov/dashboard/#chemical/131983-72-7), no alert on the thyroid receptor was identified. A more detailed evaluation of the results obtained for the different estrogen receptor (ER) assays is provided below based on the peer-reviewed published literature (Judson et al., 2015). As cytotoxicity is an important confounder within all in vitro assays, the reference Judson et al., 2016, evaluating the cytotoxicity of all compounds tested under ToxCast is summarized in detail below.

The AC_{50} concentrations of triticonazole determined in the OT_AR_ARSRC1_0480, OT_AR_ARSRC1_0480 and Tox21_AR_LUCMDAKB2_Agonist assays are relatively high (>10^{-5} M) and indicate an only weak activity in the respective assays. In the two Novascreen assays (NVS_NR_cAR and NVS_NR_hAR) a certain activity of triticonazole resulting in a loss-of-signal between the AR receptor binding its ligand has been detected with chimpanzee recombinant AR and wild type human protein isolated from an androgen-sensitive human prostate adenocarcinoma cell line (LnCAP), resulting in AC_{50} values of 0.67 – 0.97 µM. With regard to the moderate androgen receptor activity seen in the ToxCAST, a more detailed discussion is provided below, using the proposed interpretations of the US EPA FIFRA document ("Integrated Bioactivity and Exposure Ranking"), and the results of further studies evaluated (Roelofs et al., 2014; Woitkowiak, 2012 and 2016).

B.6.8.4.5.3. Judson R. et al., 2016

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Analysis of the effects of cell stress and cytotoxicity on in vitro asssay activity across a diverse chemical and assay space
Author(s), year:	Judson R. et al., 2016
Report/Doc. number::	-/ 2016/1227708

Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Material and methods

Chemical toxicity can arise from disruption of specific biomolecular functions or through more generalized cell stress and cytotoxicity-mediated processes. In the present publication, responses of 1060 chemicals (including triticonazole) across a battery of 815 in vitro assay endpoints from 7 high-throughput assay technology platforms were analyzed in order to distinguish between these types of activities. Chemical toxicity can occur in many ways, but it is hypothesized that it can be broadly classified into 2 major categories: disruption of specific biomolecular targets or pathways (eg. Receptor agonist/antagonist effects, enzyme activation/inhibition), or generalized disruption of cellular machinery that can lead to cell stress and cytotoxicity.

Many chemicals show activation of large numbers of assays over a narrow range of concentrations in which cell stress and cytotoxicity are also seen. This phenomenon is termed as the cytotoxicity-associated “burst”. Whereas some of the assay activity in this concentration range may represent chemical effects on the intended target of the assay, some of it is not. In such situations, activity represents a false positive response that can be ascribed to assay interference processes.

First, a concentration threshold is established at which chemicals begin to drive activity across multiple cell stress and cytotoxicity assays. Second, the degree to which the burst activity is associated with cell stress processes is associated and third an analysis strategy is described to separate the burst activity from what is more likely to be specific biomolecular interactions against one or more targets.

The assays, which had been evaluated for 1060 chemicals are listed in table 6.8.3.5.3-1:

Table 6.8.3.5.3-1: Evaluated assays

TABLE 1. Summary of Assay Sets.

Source	Assay endpoints	Note	References
ACEA • ACEA • ACEA_cytotoxicity	2	Real-time cell electronic sensing	(Abassi et al., 2009; Rotroff et al., 2013)
Apredica • APR_up • APR_dn • APR_cytotoxicity	58	High-content cell imaging; Duplicate in up and down direction	(Giuliano et al., 2005, 2006, 2010; Taylor and Giuliano 2005)
Attagene • ATG_TRANS • ATG_CIS	82	Multiplex transcription reporter	(Martin et al., 2010; Romanov et al., 2008)
BioSeek • BSK_Cytotoxicity • BSK_down • BSK_up	174	Biologically multiplexed activity profiling (BioMAP); Duplicate in up and down direction	(Berg et al., 2005, 2006, 2010; Houck et al., 2009; Kleinstreuer et al., 2014)
Tox21 • Tox21_BLA_Agonist • Tox21_BLA_Antagonist • Tox21_BLA_Cytotoxicity • Tox21_LUC_Agonist • Tox21_LUC_Antagonist	55	Cell-based qHTS	(Attene-Ramos et al., 2015; Hsu et al., 2014; Huang et al., 2011, 2014; Xia et al., 2008)
NovaScreen ADME • NVS_ADME • NVS_ADME_Activator	30	Cell-free HTS Cytochrome P-450 activity assays; Duplicate in activator direction	(Knudsen et al., 2011; Sipes et al., 2013)
NovaScreen Enzyme • NVS_ENZ • NVS_ENZ_Activator	115	Cell-free HTS other enzyme activity assays; Duplicate in activator direction	(Knudsen et al., 2011; Sipes et al., 2013)
NovaScreen GPCR • NVS_GPCR	77	Cell-free HTS G-protein coupled receptor assays	(Knudsen et al., 2011; Sipes et al., 2013)
NovaScreen Ion Channel • NVS_IC	22	Cell-free HTS ion channel assays, including ligand-gated ion channels	(Knudsen et al., 2011; Sipes et al., 2013)
NovaScreen Nuclear Receptor • NVS_NR	20	Cell-free HTS radioligand binding nuclear receptor assays	(Knudsen et al., 2011; Sipes et al., 2013)
NovaScreen Transporter • NVS_TR	9	Cell-free HTS transporter activity assays	(Knudsen et al., 2011; Sipes et al., 2013)
NovaScreen Other • NVS_MP	2	Cell-free HTS activity assays	(Knudsen et al., 2011; Sipes et al., 2013)
Odyssey Thera • OT	17	Protein complementation	(Bolt et al., 2015; Stossi et al., 2014)

The total number of assay endpoints is 815. The bulleted items in the first column are the suffixes used in the different assay sets. Information on individual assays is available from http://actor.epa.gov/dashboard, last accessed May 19, 2016.

Cytotoxicity assays: A total of 33 cytotoxicity-related assays are included in the assay set, and are used for much of the subsequent analysis. The assay battery is listed in the table below.

Table 6.8.3.5.3-2: Cytotoxicity related assays

TABLE 2. 34 Cytotoxicity-Related Assays.

Assays	Biological process	Organism	Tissue	Cell type/Cell line
Tox21_AR_BLA_Antagonist_viability	Cytotoxicity BLA	Human	Kidney	HEK293T
Tox21_ERa_BLA_Antagonist_viability	Cytotoxicity BLA	Human	Kidney	HEK293T
Tox21_ESRE_BLA_viability	Cytotoxicity BLA	Human	Cervix	HeLa
Tox21_FXR_BLA_antagonist_viability	Cytotoxicity BLA	Human	Kidney	HEK293T
Tox21_GR_BLA_Antagonist_viability	Cytotoxicity BLA	Human	Cervix	HeLa
Tox21_HSE_BLA_agonist_viability	Cytotoxicity BLA	Human	Cervix	HeLa
Tox21_MMP_viability	Cytotoxicity BLA	Human	Liver	HepG2
Tox21_NFkB_BLA_agonist_viability	Cytotoxicity BLA	Human	Cervix	ME-180
Tox21_p53_BLA_p1_viability [1]	Cytotoxicity BLA	Human	Intestinal	HCT116
Tox21_p53_BLA_p2_viability [1]	Cytotoxicity BLA	Human	Intestinal	HCT116
Tox21_p53_BLA_p3_viability [1]	Cytotoxicity BLA	Human	Intestinal	HCT116
Tox21_p53_BLA_p4_viability [1]	Cytotoxicity BLA	Human	Intestinal	HCT116
Tox21_p53_BLA_p5_viability [1]	Cytotoxicity BLA	Human	Intestinal	HCT116
Tox21_PPARg_BLA_antagonist_viability	Cytotoxicity BLA	Human	Kidney	HEK293
Tox21_VDR_BLA_Agonist_viability	Cytotoxicity BLA	Human	Kidney	HEK293T
Tox21_VDR_BLA_antagonist_viability	Cytotoxicity BLA	Human	Kidney	HEK293T
BSK_3C_SRB_down	Cytotoxicity SRB	Human	Vascular	Umbilical vein endothelium
BSK_4H_SRB_down	Cytotoxicity SRB	Human	Vascular	Umbilical vein endothelium
BSK_BE3C_SRB_down	Cytotoxicity SRB	Human	Lung	Bronchial epithelial cell
BSK_CASM3C_SRB_down	Cytotoxicity SRB	Human	Vascular	Umbilical vein endothelium and coronary artery smooth muscle cells
BSK_hDFCGF_SRB_down	Cytotoxicity SRB	Human	Skin	Foreskin fibroblast
BSK_KF3CT_SRB_down	Cytotoxicity SRB	Human	Skin	Keratinocytes and foreskin fibroblasts
BSK_LPS_SRB_down	Cytotoxicity SRB	Human	Vascular	Umbilical vein endothelium and peripheral blood mononuclear cells
BSK_SAg_PBMCCytotoxicity_down	Cytotoxicity SRB	Human	Vascular	Umbilical vein endothelium and peripheral blood mononuclear cells
BSK_SAg_SRB_down	Cytotoxicity SRB	Human	Vascular	Umbilical vein endothelium and peripheral blood mononuclear cells
ACEA_T47D_80hr_Negative	Proliferation decrease	Human	Breast	T47D
APR_HepG2_CellLoss_24h_dn	Proliferation decrease	Human	Liver	HepG2
APR_HepG2_CellLoss_72h_dn	Proliferation decrease	Human	Liver	HepG2
BSK_3C_Proliferation_down	Proliferation decrease	Human	Vascular	Umbilical vein endothelium
BSK_3C_Vis_down	Proliferation decrease	Human	Vascular	Umbilical vein endothelium
BSK_CASM3C_Proliferation_down	Proliferation decrease	Human	Vascular	Umbilical vein endothelium and coronary artery smooth muscle cells
BSK_hDFCGF_Proliferation_down	Proliferation decrease	Human	Skin	Foreskin fibroblast
BSK_SAg_Proliferation_down	Proliferation decrease	Human	Vascular	Umbilical vein endothelium and peripheral blood mononuclear cells

[1] These assays all use the same protocol but were performed as part of a chemical stability study, so were run on the same batch of chemicals that had been in solution at room temperature for increasing lengths of time, up to 6 months.

Cytotoxicity was defined by a minimum threshold of activity; here, taken to be appositive call in 2 or more assays in the battery of 33 cytotoxicity-related or reduced proliferation assays. The threshold of 2 was selected based on the observation that most chemicals with 2 or more of these assays active displayed the burst. The cell stress or cytotoxicity "burst" is the phenomenon, describing the fact, that positive assay responses are observed in the concentration range where cytotoxicity is observed.

Results

The Z-score has been introduced as a parameter in this evaluation to be able to normalize the detected cytotoxicities of the different test substances. A threshold of Z=3 has been arbitrarily set to differentiate between high and low cytotoxicities. Assay results at a high Z-score are less likely to be confounded by cytotoxicity; to the contrary assay results at a low Z-score are most likely confounded by cytotoxicity. In the figure below, the cytotoxicity and an overview over the assay results are shown for triticonazole:

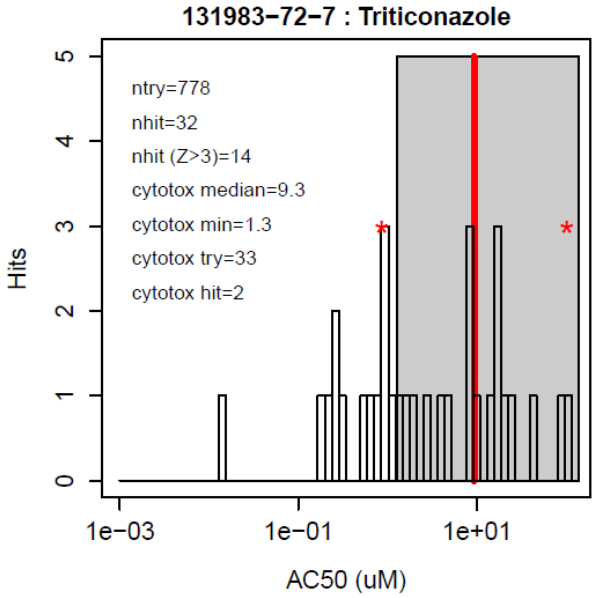


Figure 6.8.3.5.3-1: Summary of cytotoxicity results for triticonazole (supplementary file 4, provided by Toxicological Sciences)

In the context of the publication the histogram in the above figure shows the distribution of the AC_{50} values of all active cytotoxicity assays. The red vertical line corresponds to the median cytotoxic concentration and is 9.3 µM. The grey box underlines the cytotoxicity region. The left boundary of the grey box corresponds to the Z-score of 3; all results left of the boundary have high and all results right of the boundary have low Z-scores. The red stars indicate the AC50 values for the cytotoxicity assays activated by triticonazole (2 cytotoxicity-related assays were active). For triticonazole 14 assays were positive, which had a Z-score of >3. According to this evaluation, positive hits at a high Z-score are more likely associated with specific biomolecular interactions with the intended biological process or target, than the positive assay results at a lower Z-score. As US EPA is now (2016) considering a concentration of 2.27 µM to be the cytotoxic concentration of triticonazole, this would change this plot.

The study also provides a fraction of active assay results in the low and in the high Z-class. For triticonazole (data are taken from supplementary file 7) the following fractions (only the ED-related assays: androgen receptor, estrogen receptor, steroidogenesis) are shown.

Table 6.8.3.5.3-3: Low-Z and High-Z class ED assay overview for Triticonazole

Triticonazole*	Class	Fraction active
All assays	Low-Z / High-Z	0.028220859 / 0.013496933
androgen receptor	Low-Z / High-Z	0.214285714 / 0.214285714**
estrogen receptor	Low-Z / High-Z	0
Steroidogenesis	Low-Z / High-Z	0

*calculation is based on an assumption of a cytotoxicity of 9.3 µM, which is meanwhile corrected to 2.27 µM
** these numbers are not indicative for a probable agonistic or antagonistic response, but just indicating which fraction of the assays were positive in a low (high-Z) or high cytotoxic (low-Z) range. In comparison testosterone proprionate (AR agonist) has an active fraction of 0.642857143 at high Z-scores and hydroxyflutamide (AR antagonist) an active fraction of 0.714285714.

US EPA considers triticonazole to be inactive for the estrogen receptor and the steroidogenesis assay.

Conclusion

Based on data of 1060 chemicals screened in 815 high-throughput in vitro assay endpoints, the contributing factor of cytotoxicity to ToxCast Assay results is assessed. Z-scores (as a measure for cytotoxicity) have been calculated for all of the compounds. Positive assay results at high Z-scores are indicative for a specific result of the specific assays. Positive assay results at a low Z-score indicate that the result is more likely to be related to cell stress or unspecific interactions between the test compound within the assay. For triticonazole 14 positive assay results at a high Z-score were identified. For the assays on the androgen receptor triticonazole showed a fraction of 0.21 active results at high Z-scores, which is considerably lower than active fractions for testosterone proprionate (AR agonist) and hydroxyflutamide (AR antagonist). Overall triticonazole was found to be inactive for the estrogen receptor and steroidogenesis.

Comment from the notifier:

The median cytotoxicity of 9.3 µM for triticonazole used in this evaluation has meanwhile been corrected to 2.7 µM by US EPA, which might change the fraction of positive results at a high Z-score for the androgen pathway.

B.6.8.4.5.4. Judson R.S. et al., 2015

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Integrated model of chemical perturbations of a biological pathway using 18 in vitro high-throughput screening assays for the estrogen receptor
Author(s), year:	Judson R.S. et al., 2015
Report/Doc. number::	-/ 2015/1279970
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This computational network model integrates the results of 18 in vitro, high-throughput screening assays measuring estrogen receptor (ER) binding, dimerization, chromatin binding, transcriptional activation, and ER-dependent cell proliferation. The network model uses activity patterns across the *in vitro* assays – tested within the ToxCAST program – to predict whether a chemical is an ER agonist or antagonist, or is otherwise influencing the assays through a manner dependent on the physics and chemistry of the technology platform. The method is applied to a library of 1812 commmercial and environmental chemicals (among them triticonazole), including 45 ER positive and negative reference chemicals. Among the reference chemicals, the network model correctly identified the agonists and antagonists with the exception of very weak compounds whose activity was outside the concentration range tested. Triticonazole was found to have neither estrogen receptor agonistic nor estrogen receptor antagonistic properties.

Material and methods

The data used in this computational model were generated by the EPA ToxCAST program. The dataset comprises concentration-response data on 1812 chemicals with full data on ER pathway *in vitro* assays. Included in the chemical library were reference chemicals, ie known ER agonists and antagonists, as well as a large number of

commercial chemicals with reported estrogen-like activity, some of which are potentially selective estrogen receptor modulators. The 18 in vitro assays are summarized in the table below.

Assay ID	Assay Name	Source	Gene Target	Normalized Data Type	Assay Design Type	Biological Process Target	Detection Technology	Detection Technology Subtype	Measure-ment Timepoint (hr)	Organism	Tissue	Cell Format	Cell Line Name	Assay Footprint
A1	NVS_NR_bER	NVS	ESR1	Percent activity	Radioligand binding	Receptor binding	Lysate-based radiodetection	Scintillation counting	18	Bovine	Uterus	Tissue-based cell-free	NA	Microplate: 96-well plate
A2	NVS_NR_hER	NVS	ESR1	Percent activity	Radioligand binding	Receptor binding	Lysate-based radiodetection	Scintillation counting	18	Human	NA	Cell-free	NA	Microplate: 96-well plate
A3	NVS_NR_mERa	NVS	Esr1	Percent activity	Radioligand binding	Receptor binding	Filter-based radiodetection	Scintillation counting	18	Mouse	NA	Cell-free	NA	Microplate: 96-well plate
A4	OT_ER_ERaERa_0480	OT	ESR1	Percent activity	Protein fragment complementation assay	Protein stabilization	Protein-fragment complementation	Fluorescence intensity	8	Human	Kidney	Cell line	HEK293T	Microplate: 384-well plate
A5	OT_ER_ERaERa_1440	OT	ESR1	Percent activity	Protein fragment complementation assay	Protein stabilization	Protein-fragment complementation	Fluorescence intensity	24	Human	Kidney	Cell line	HEK293T	Microplate: 384-well plate
A6	OT_ER_ERaERb_0480	OT	ESR1	Percent activity	Protein fragment complementation assay	Protein stabilization	Protein-fragment complementation	Fluorescence intensity	8	Human	Kidney	Cell line	HEK293T	Microplate: 384-well plate
A7	OT_ER_ERaERb_1440	OT	ESR1 ESR2	Percent activity	Protein fragment complementation assay	Protein stabilization	Protein-fragment complementation	Fluorescence intensity	24	Human	Kidney	Cell line	HEK293T	Microplate: 384-well plate
A8	OT_ER_ERbERb_0480	OT	ESR2	Percent activity	Protein fragment complementation assay	Protein stabilization	Protein-fragment complementation	Fluorescence intensity	8	Human	Kidney	Cell line	HEK293T	Microplate: 384-well plate
A9	OT_ER_ERbERb_1440	OT	ESR2	Percent activity	Protein fragment complementation assay	Protein stabilization	Protein-fragment complementation	Fluorescence intensity	24	Human	Kidney	Cell line	HEK293T	Microplate: 384-well plate
A10	OT_ERa_EREGFP_0120	OT	ESR1	Percent activity	Fluorescent protein induction	Regulation of gene expression	Microscopy	Optical micro-scopy: Fluorescence microscopy	2	Human	Cervix	Cell line	HeLa	Microplate: 384-well plate
A11	OT_ERa_EREGFP_0480	OT	ESR1	Percent activity	Fluorescent protein induction	Regulation of gene expression	Microscopy	Optical micro-scopy: Fluorescence microscopy	8	Human	Cervix	Cell line	HeLa	Microplate: 384-well plate
A12	ATG_ERa_TRANS_up	ATG	ESR1	log2 fold induction	mRNA induction	Regulation of tran-scription factor activity	RT-PCR and Capillary electrophoresis	Fluorescence intensity	24	Human	Liver	Cell line	HepG2	Microplate: 24-well plate
A13	ATG_ERE_CIS_up	ATG	ESR1	log2 fold induction	mRNA induction	Regulation of tran-scription factor activity	RT-PC R and Capillary electrophoresis	Fluorescence intensity	24	Human	Liver	Cell line	HepG2	Microplate: 24-well plate
A14	Tox21_ERa_BLA_Agonist_ratio	Tox21	ESR1	Percent activity	Beta lactamase induction	Regulation of gene expression	GAL4 b-lactamase reporter gene	Fluorescence intensity	18	Human	Kidney	Cell line	HEK293T	Microplate: 1536-well plate
A15	Tox21_ERa_LUC_BG1_Agonist	Tox21	ESR1	Percent activity	Luciferase induction	Regulation of gene expression	Luciferase-coupled ATP quantitation	Bioluminescence	22-24	Human	Ovary	Cell line	BG1	Microplate: 1536-well plate
A16	ACEA_T47D_80hr_Positive	ACEA	ESR1	Percent activity	Real-time cell-growth kinetics	Cell proliferation	RT-CES	Electrical sensor: impedance	80	Human	Breast	Cell line	T47D	Microplate: 96-well plate
A17	Tox21_ERa_BLA_Antagonist_ratio	Tox21	ESR1	Percent activity	Beta lactamase induction	Regulation of gene expression	GAL4 b-lactamase reporter gene	Fluorescence intensity	18	Human	Kidney	Cell line	HEK293T	Microplate: 1536-well plate
A18	Tox21_ERa_LUC_BG1_Antagonist	Tox21	ESR1	Percent activity	Luciferase induction	Regulation of gene expression	Luciferase-coupled ATP quantitation	Bioluminescence	22	Human	Ovary	Cell line	BG1	Microplate: 1536-well plate

Further details are provided in Supplemental Appendix 1. NVS = Novascreen; OT = Odyssey Thera; ATG = Attagene; Tox21 = assays run by the National Institutes of Health's National Center for Advancing Translational Sciences (NCATS) as part of the Federal Tox21 program.

Triticonazole and the other chemicals were run in concentration response format in all in vitro assays except for the cell-free binding assays (NVS). The NVS assays were initially run at a single concentration (25 µM), and if significant activity (3 median absolute deviations (MAD) above the median or 30% activity) was seen, the chemical was then run in concentration-response mode.

The graphical representation of the computational network used in the in vitro analysis of the ER pathway across assays and technology platforms is given in the figure 6.8.3.5.4-1.

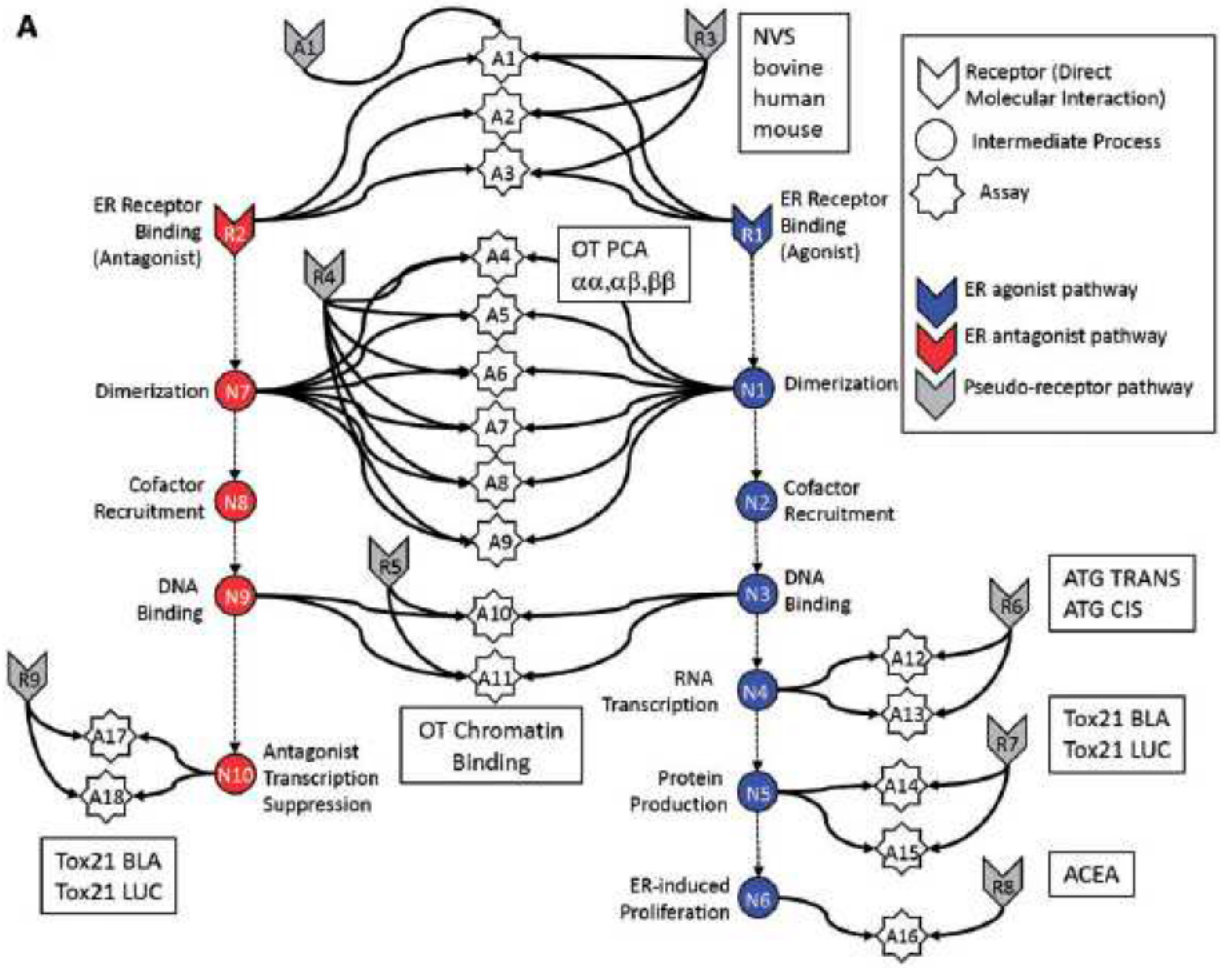


Figure 6.8.3.5.4-1: Graphical representation of the computational network (the assay number A1 – A18 are taken from the table above)

The input data for the model includes chemical structures and concentration-response data for 18 ER-related *in vitro* assays, plus data for many non-ER *in vitro assay* endpoints (ranging from 186 to 821 assays, depending on the chemical). The data used were generated by the EPA ToxCAST program. The dataset comprises concentration-response data on 1812 chemicals with full data on ER pathway *in vitro* assays. These include 3 cell-free biochemical radioligand ER binding assays, a set of 3 protein complementation assays that measure formation of ER homodimers or heterodimers and test for activity against both ER-alpha and ER-beta (each measured at 2 separate times for a total of 6 assays readouts), 2 assays measuring interaction of green fluorescent protein (GFP)-tagged ER α or β with nuclear DNA, 2 transactivation assays measuring reporter protein level

readouts in agonist mode and 2 transactivation assays in antagonist mode (Tox21), and an ER-sensitive cell proliferation assay (ACEA). The transactivation assays are a mix of formats including differences in reporter gene technology (Tox21_Era_LUC_BG1:luciferase, Tox21_Era_BLA:ß-lactamase, ATG_ERE_CIS/ATG_Era_TRANS:mRNA) and differences in receptor form (Tox21_Era_LUC_BG1 and ATG_ERE_CIS are full length ER; Tox21_Era_BLA and ATG_Era_TRANS are the GAL4/UAS mammalian 1 hybrid system utilizing partial receptor constructs containing the receptor ligand-binding domain.).

Reference chemicals.

A set of 45 positive and negative reference chemicals were used to evaluate the performance of the model. These include 28 agonist positives, 12 agonist negatives, 4 antagonist positives, and 14 antagonist negatives. These chemicals have been used to validate ER in vitro assays and were taken from the OECD TG 457 BG1 Guidance document. The concentration response curves of estradiol (E2) and raloxifen are shown exemplarily below as they are the positive controls for agonistic and antagonistic action as recommended in the OECD TG 457.

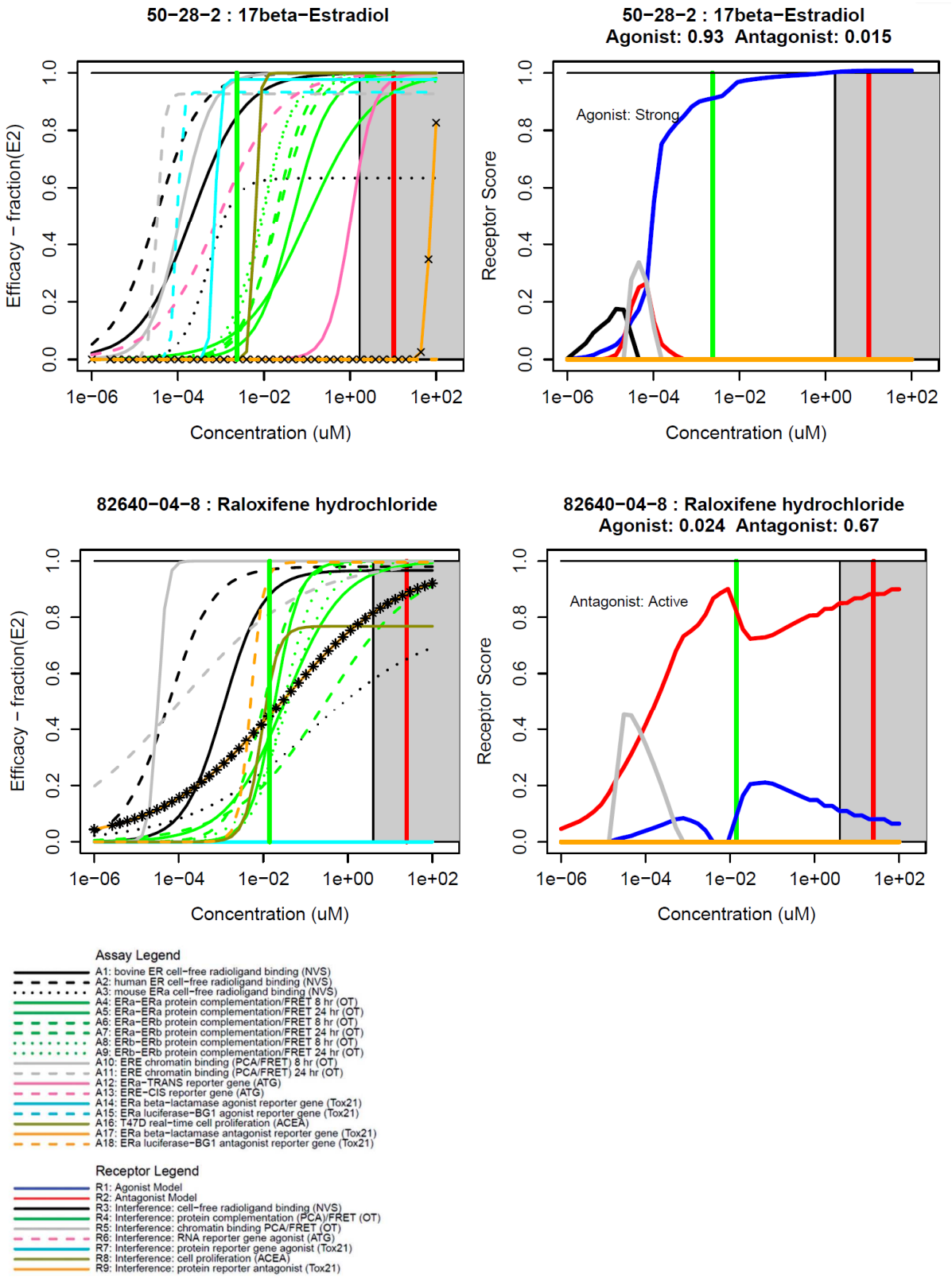


Figure 6.8.3.5.4-2: Concentration response curves for 17beta-estradiol (E2) (top) and raloxifene (bottom) for agonistic and antagonistic efficacy at the estrogen receptor (taken from Appendix 8 of the publication). For each substance the left-hand panel shows the synthetic concentration-response data for the 18 assays, coloured by assay group as defined in the legend. The right-hand panel shows the corresponding magnitude of the receptor responses. The agonist receptor (R1) is designated by blue, the antagonist receptor (R2) by red and the other

pseudo-receptors are colored as indicated in the legend. For chemicals with the "cytotoxicity burst" defined (2 or more cytotoxicity hits), the burst center is indicated by a vertical red line, and the burst region is indicated by the gray shaded region (see Judson et al., 2016 above). The vertical green line designates the predicted AC_{50} corresponding to the AR agonist or AR antagonist model with the highest AUC score.

Data processing and synthetic concentration-response data:

All of the concentration response data were analyzed using a standardized data analysis pipeline, which automates the processes of baseline correction, normalization, curve-fitting, hit-calling, and detection of a variety of potential confounders. This pipeline, along with all of the raw and processed data, and annotations is publicly available. In order to establish the response cutoff, the baseline median absolute deviation (BMAD) was calculated per assay using the distribution of the lowest 2 concentration´s normalized response values for all chemicals run in the in vitro assay. The response cutoff was then selected per assay as being the maximum of 3-BMAD, 20% above baseline, or an assay-specific cutoff, eg. 6-BMAD or 10-BMAD. To allow computational synthesis across different in vitro assays with different experimental designs (ie, different numbers of concentrations tested), a set of synthetic concentration-response activities was generated through interpolation for each chemical-assay pair at standardized concentrations. This procedure used the experimentally derived AC50, Hill-slope and Top parameters and a Hill equation. All AC50 values were in µM and the synthetic concentrations were a 1.5-fold dilutions series of 45 concentrations from 1pM to 100 µM.

Accounting for cytotoxicity-related assay interference:

For many chemicals, a large number of hits (positive assay responses) for ER and non-ER assays was observed in the concentration range where cytotoxicity was seen. Cytotoxicity was measured using a collection of 35 assays in the ToxCast battery that detect cytotoxicity or other forms of cell loss across several cell lines and primary cell types (see Judson et al., 2016). Many non-selective cellular responses are activated as the concentration tested reaches a critical point associated with cell stress or cytotoxicity. It appears that this is non-selective activity (assay interference) rather than being due to activity against the receptors that the assays are designed to test. The non-selective, cell-stress/cytotoxicity-related assay hits had been excluded.

Results

A number of compounds have been identified to be strong, moderate weak or very weak agonist or antagonists of the estrogen receptor by using this computational approach. Triticonazole was identified to be entirely inactive in all of the assays. The below figures were taken from the supplementary tables.

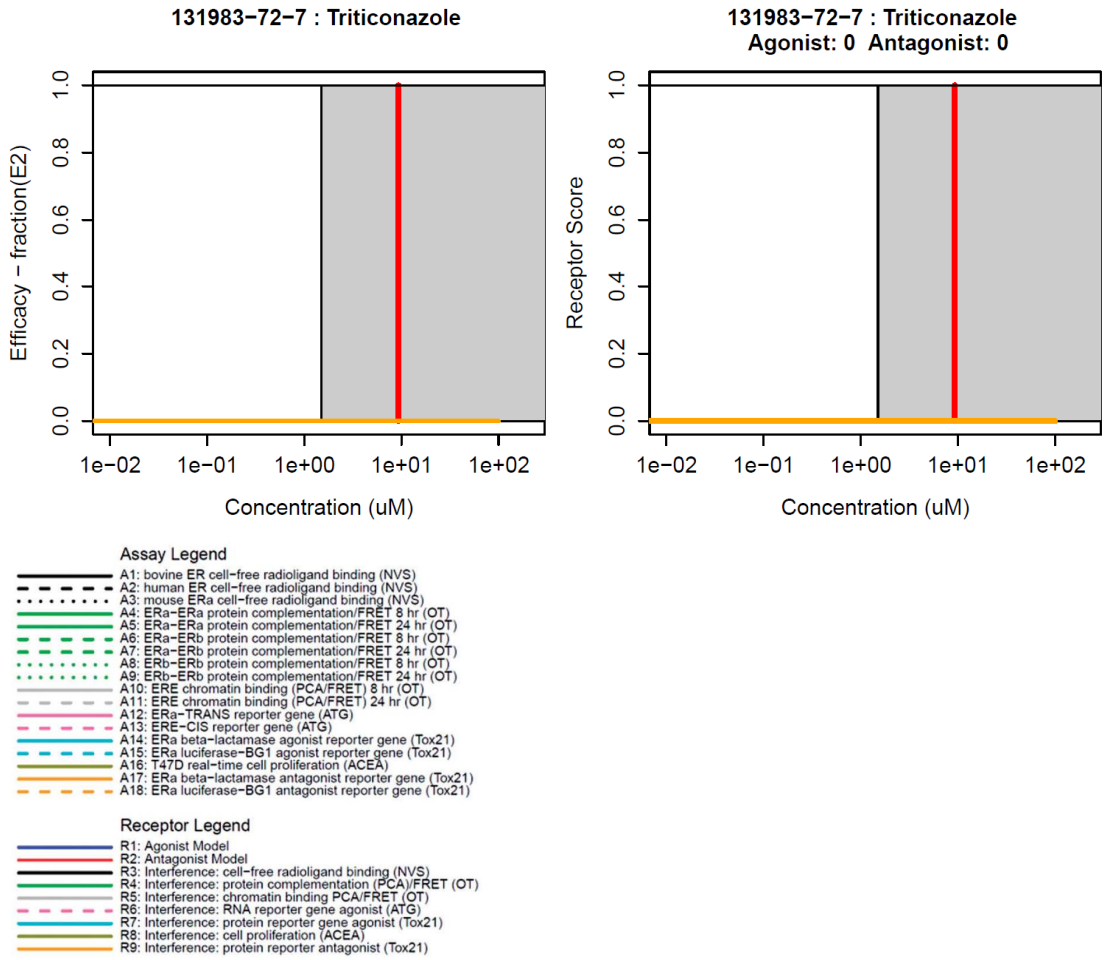


Figure 6.8.3.5.4-3: Concentration response curves for triticonazole for agonistic and antagonistic efficacy at the estrogen receptor

Conclusion

Triticonazole did not show any evidence for agonistic or antagonistic ER activity.

Comment from the notifier:

This computational mathematical model is considered to be a more comprehensive evaluation using all available results from different in vitro estrogen receptor assays (generated in the US EPA ToxCAST program), compared to an assay-by-assay evaluation. It is further important to note, that the non-selective, cell-stress/cytotoxicity-related assay hits had been excluded from this assessment, in order to reduce the number of false positive results. In the meanwhile EPA has considered using a cytotoxicity value of 2.27 µM for triticonazole instead of the 9.3 µM.

B.6.8.4.5.5. US EPA, 2014

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Integrated bioactivity and exposure ranking: A computational approach for the prioritization and screening of chemicals in the endocrine disruptor screening program

Author(s), year:	US EPA, 2014
Report/Doc. number::	-/ 2014/1329992
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

The US EPA is continuing a series of scientific peer reviews focused on computational approaches for prioritization and screening of chemicals in the Endocrine Disruptor Screenign Program (EDSP). The document describes innovative approaches for determining estrogen and androgen bioactivity based on a series of computational models integrating data from ToxCast high throughput screening (HTS) assays. For this summary only the relevant information related to the androgen receptor (AR) pathway were taken, as the estrogen receptor (ER) pathway and the cytotoxicity evaluations were already published in peer-reviewed journals (Judson et al., 2015 and Judson et al., 2016, see above). Currently available in vitro assays in Tox21 and ToxCast Phase II dataset specifically related to androgen receptor (AR) pathway signalling include AR binding assays, AR transcriptional activation assays, and AR cofactor recruitment. EPA has proposed an approach for quantifying a chemical's potential androgen bioactivity, which is based on a computational model integrating data from nine high throughput ToxCast assays (US EPA, 2014). The computational model outputs are expressed as area under the curve (AUC) scores for AR agonist (R1) and antagonist (R2) bioactivity. The AUC model reliably predicts the agonist and antagonist properties of reference chemicals while minimizing false positives due to overt cellular chemical cytotoxicity. The notifier insists to emphise that for the androgen receptor pathway, no final peer-reviewed publication is available and any interpretation shall be taken with care, as the data is still vulnerable.

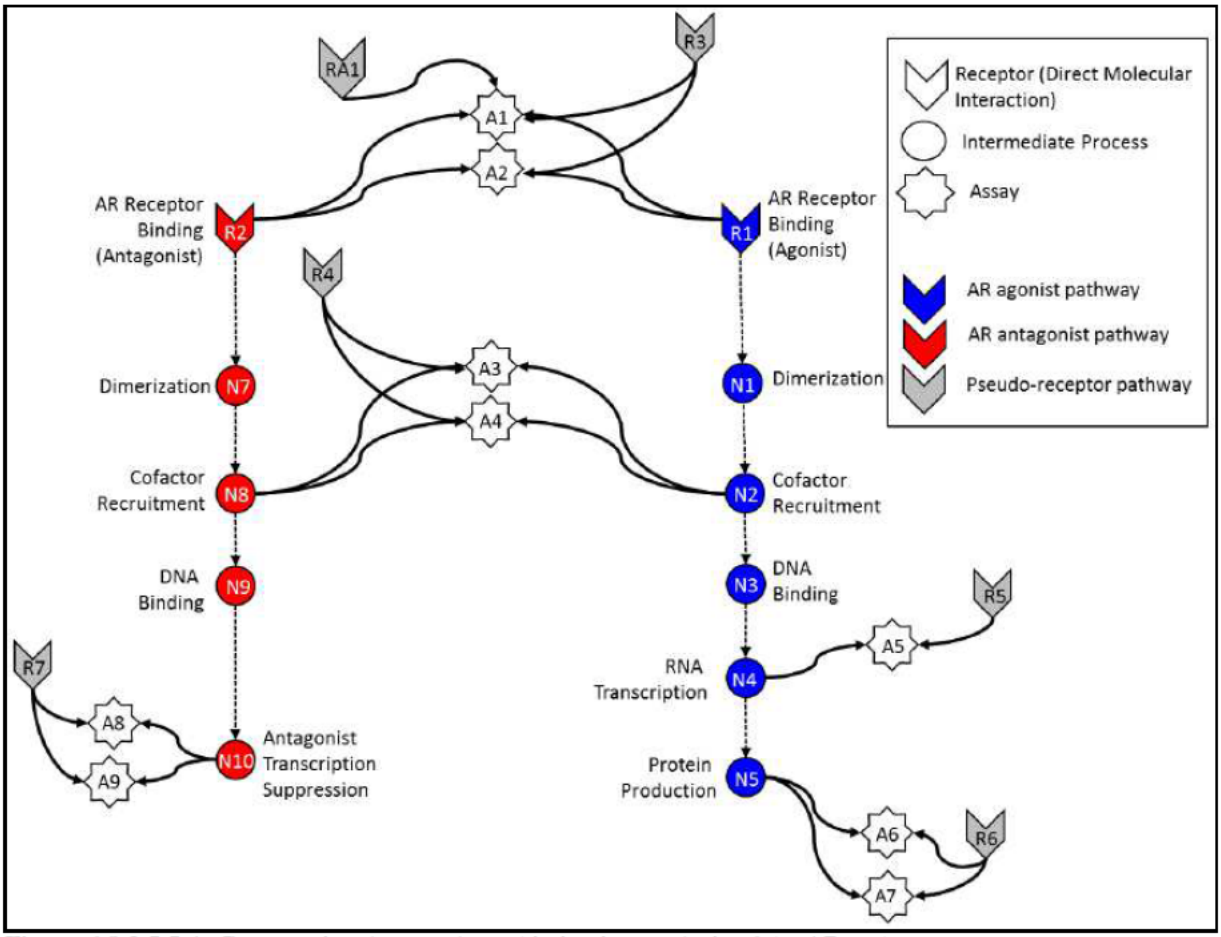


Figure 6.8.3.5.5-1: Computational network used in in vitro analysis of the AR pathway

Graphical representation of the computational network used in the in vitro analysis of the AR pathway across assays and technology platforms. Colored arrow nodes represent "receptors" with which a chemical can directly interact. Colored circles represent intermediate biological processes that are not directly observable. Stars represent the in vitro assays that measure activity at the biological nodes. Arrows represent transfer of information. Gray arrow nodes are the pseudo-receptor.
Each assay (with the exception of A5) has an assay-specific pseudo-receptor, but only a single example is explicitly shown, for assay A1.

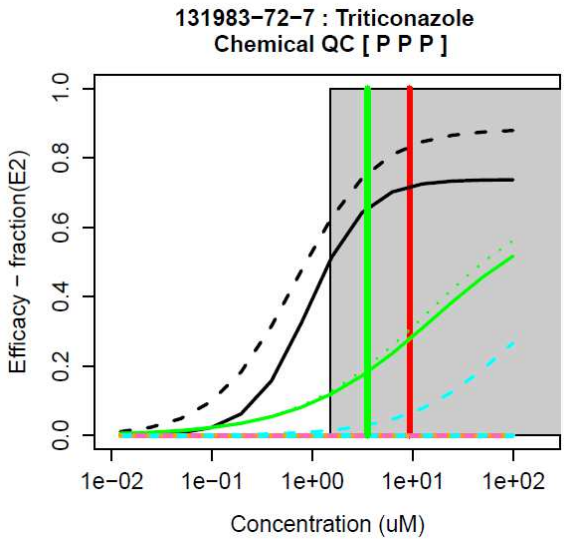
As outlined in the publication by the US EPA (2014), 1855 chemicals were tested in the AR pathway assays, of which 1378 were essentially inactive in all assays. The chemical hits tend to cluster with the receptor / assay technologies, indicating that some fraction of the assay is false-positive due to technology-specific assay interference. The model used a reference set of 23 positive and negative reference chemicals to evaluate the performance of the model. From these set also a number of Selective Androgen Receptor Modulator (SARM) had been chosen which are designed to have both agonist and antagonist activity. There were 20 chemicals with agonist (R1) AUC ≥ 0.1 (among them testosterone propionate), and 93 chemicals with antagonist (R2) AUC ≥ 0.1 (among them hydroxyflutamide) properties. A total of 477 chemicals had at least one AUC value greater than 0.1. In the publication, 65 chemicals specific for either the Agonist or Antagonist Receptor and having both high Z and high Emax values were listed. The list excluded a majority of high-AUC AR antagonists (R2), because the authors concluded that the observed activities were confined to the cell-stress and cytotoxicity region. The authors hypothetized that these chemicals react with the proteins or otherwise cause denaturation, which will cause displacement of the radioligand, leading to a binding-like signal. There is not yet a model in place to determine model performance for predicting in vivo bioactivity.

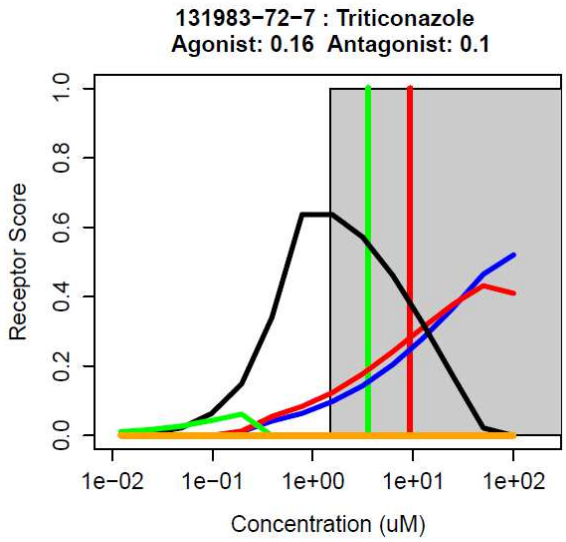
The results of the AR-related assay endpoints for triticonazole are listed below in comparison to a strong AR agonist, testosterone propionate, and an AR antagonist, hydroxyflutamide. Two additional assays that were not included in the AR model are also described in table below, so that a total of 11 assays are available, with triticonazole being tested in 10 of these.

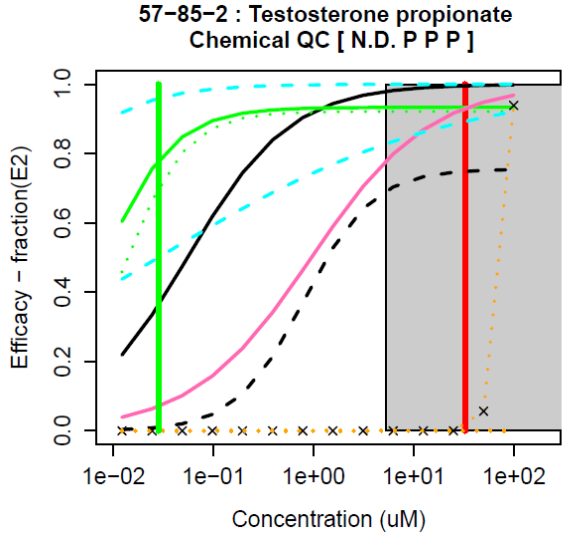
Table 6.8.3.5.5-1: Results of the ToxCast AR Assays for triticonazole and reference chemicals

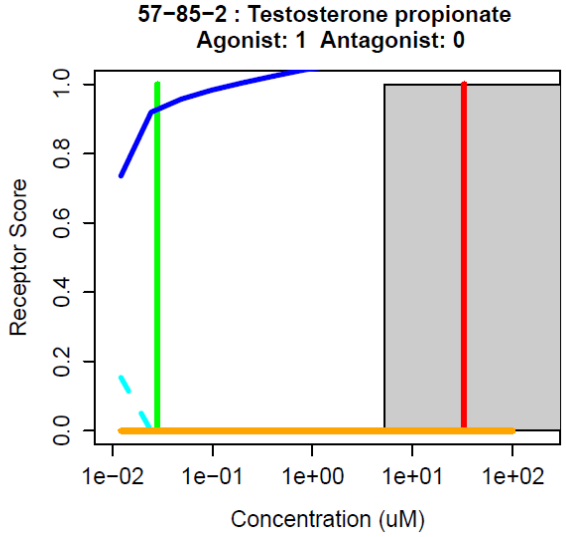
Biological Process indicator [Androgen (A) or Anti-Androgen (Anti-A) Signal Detection]	Assay ID	Assay Endpoint Name	AC_{50} [µM]			
			Testosterone proprionate	Hydroxy-flutamide	Triticonazole (cytotoxicity at 9.3 µM)1	Triticonazole (cytotoxicity: 2.27 µM)*
Binding to androgen receptor [A/Anti-A]	**A1**	NVS_NR_hAR (human)	0.055	0.177	0.91	**0.91**
	A2	NVS_NR_cAR (chimpanzee)	0.808	0.307	0.67	**0.68**
	Not in AR model	NVS_NR_rAR (rat)	0.888	18	Not tested	Not tested
Protein stabilization [A/Anti-A]	A3	OT_AR_ARSRC1_0480	0.012	9.8	13.89	Only active at cytotoxic conc.
	A4	OT_AR_ARSRC1_0960	0.007	12	15.76	Only active at cytotoxic conc.
DNA binding of androgen receptor complex [A/Anti-A]	Not in AR model	OT_AR_ARELUC_AG_1440	0.0037	3.5	Inactive	Inactive
RNA transcription (mRNA induction) [A]	A5	ATG_AR_TRANS_up	0.953	Inactive	Inactive	Inactive
Regulation of gene expression (reporter gene assays) [A]	A6	Tox21_AR_BLA_Agonist_ratio	0.028	Inactive	Inactive	Inactive
	A7	Tox21_AR_LUC_MDAKB2_Agonist	0.000765	7.0	94.16	Only active at cytotoxic conc.
Regulation of gene expression (reporter gene assays) [Anti-A]	A8	Tox21_AR_BLA_Antagonist_ratio	71	1.7	Inactive	Inactive
	A9	Tox21_AR_LUC_MDAKB2_Antagonist	Inactive	23	Inactive	Inactive

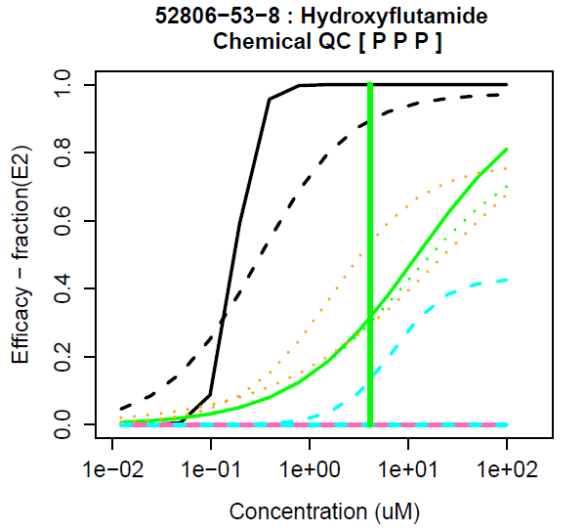
*Data are taken end 2016 from the most recent (regularly updated) US EPA dashboard.

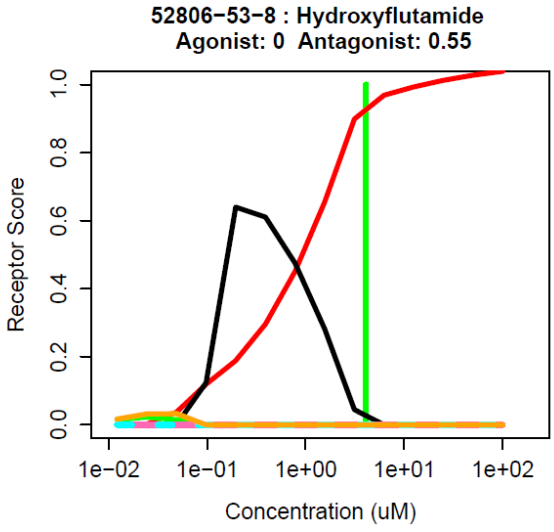

131983-72-7 : Triticonazole
Chemical QC [P P P]
Efficacy - fraction(E2)
0.0
0.2
0.4
0.6
0.8
1.0
1e-02
1e-01
1e+00
1e+01
1e+02
Concentration (uM)


131983-72-7 : Triticonazole
Agonist: 0.16 Antagonist: 0.1
Receptor Score
0.0
0.2
0.4
0.6
0.8
1.0
1e-02
1e-01
1e+00
1e+01
1e+02
Concentration (uM)


57-85-2 : Testosterone propionate
Chemical QC [N.D. P P P]
Efficacy - fraction(E2)
0.0
0.2
0.4
0.6
0.8
1.0
1e-02
1e-01
1e+00
1e+01
1e+02
Concentration (uM)


57-85-2 : Testosterone propionate
Agonist: 1 Antagonist: 0
Receptor Score
0.0
0.2
0.4
0.6
0.8
1.0
1e-02
1e-01
1e+00
1e+01
1e+02
Concentration (uM)


52806-53-8 : Hydroxyflutamide
Chemical QC [P P P]
Efficacy - fraction(E2)
0.0
0.2
0.4
0.6
0.8
1.0
1e-02
1e-01
1e+00
1e+01
1e+02
Concentration (uM)


52806-53-8 : Hydroxyflutamide
Agonist: 0 Antagonist: 0.55
Receptor Score
0.0
0.2
0.4
0.6
0.8
1.0
1e-02
1e-01
1e+00
1e+01
1e+02
Concentration (uM)

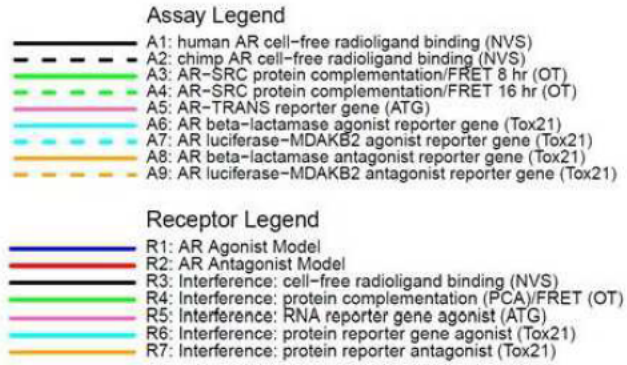


Figure 6.8.3.5.5-2:Model plots for triticonazole, testosterone proprionate (androgen receptor agonist) and hydroxyflutamide (androgen receptor antagonist) taken from Appendix 8 of the publication. For each substance the left-hand panel shows the synthetic concentration-response data for the 9 assays, coloured by assay group as defined in the legend. The right-hand panel shows the corresponding magnitude of the receptor responses. The AR agonist receptor (R1) is designated by blue, the AR antagonist receptor (R2) by red and the other pseudo-receptors are colored as indicated in the legend. For chemicals with the "cytotoxicity burst" defined (2 or more cytotoxicity hits), the burst center is indicated by a vertical red line, and the burst region is indicated by the gray shaded region (see Judson et al., 2016 above). The vertical green line designates the predicted AC_{50} corresponding to the AR agonist or AR antagonist model with the highest AUC score.

As obvious from the plot above (left hand panel), there is some evidence for a binding of triticonazole to the chimpanzee and the human androgen receptor in the non-cytotoxic region of the assays (black line and dashed black line). All other assays don´t show a relevant response (see also table 6.8.3.5.5-1). According to the receptor responses (right-hand panel), the AR agonist model (blue line) shows a similar shape, than the antagonist model (red line), which led to overall results of 0.16 for an agonistic response and 0.1 for an antagonistic response (see Table 6.8.3.5.5-2). The modeled positive results in this mathematical approach range from 0.1 – 1.0 defining 0.1 as the threshold for a positive result. Compared to results obtained for testosterone propionate (agonist) and hydroxyflutamide (antagonist), where clear model responses were obtained pointing to the agonstic or antagonistic region, the response for triticonazole is inconclusive. The systems biology model that integrates nine ToxCast/Tox21 assay endpoints for AR-based pathway activity (US EPA, 2014) is positive for triticonazole regarding agonist activity, and is borderline positive for anti-androgenic activity (see 6.8.3.5.5-2).

The systems biology model that integrates nine ToxCast/Tox21 assay endpoints for AR-based pathway activity (US EPA, 2014) is positive for triticonazole regarding agonist activity, and is borderline positive for anti-androgenic activity

Table 6.8.3.5.5-2: Results of the ToxCast AR Model for triticonazole and reference chemicals (EPA, 2014).

AR AUC Model Activity	AR AUC Model Activity – Agonist*	AR AUC Model Activity – Antagonist*	Conclusion
Triticonazole	0.16	*0.101 (borderline positive)*	Inconclusive**
Testosterone proprionate	1.01	0	Agonist
Hydroxyflutamide	0	0.55	Antagonist

**Range of positive results: 0.1 – 1*

***made by the notifier – no conclusion for triticonazole had been provided by the authors of the publication*

Source: US EPA (2014), Appendix 8 (AR AUC Scores all chemicals.pdf)

An overview over the modeled results for androgen receptor agonists and antagonists is given in the figure below:

CASRN	Name	AUC Agonist (R1)	AUC Antagonist (R2)	Specific Receptor
10161-33-8	17beta-Trenbolone	1.09	0	Agonist
58-18-4	17-Methyltestosterone	1.08	0	Agonist
521-18-6	5alpha-Dihydrotestosterone	1.06	0	Agonist
68-22-4	Norethindrone	1.02	0	Agonist
57-85-2	Testosterone propionate	1.01	0	Agonist
797-63-7	Norgestrel	0.97	0	Agonist
63-05-8	4-Androstene-3,17-dione	0.83	0	Agonist
50-28-2	17beta-Estradiol	0.79	0.06	Agonist
84371-65-3	Mifepristone	0.04	0.75	Antagonist
68-96-2	17alpha-Hydroxyprogesterone	0.69	0	Agonist
57-91-0	17alpha-Estradiol	0.61	0.05	Agonist
864283-48-7	GSK232420A	0.57	0.05	Agonist
57-83-0	Progesterone	0.56	0.08	Agonist
52806-53-8	Hydroxyflutamide	0	0.55	Antagonist
90357-06-5	Bicalutamide	0	0.35	Antagonist
52-01-7	Spironolactone	0.05	0.29	Antagonist
474-86-2	Equilin	0.02	0.28	Antagonist
53-03-2	Prednisone	0.27	0	Agonist
427-51-0	Cyproterone acetate	0.26	0.07	Agonist

Figure 6.8.3.5.5-3: **Selected list of AR chemicals taken from the publication which are known for their specific receptor activity**

Conclusion

According to the ToxCast data triticonazole binds to the chimpanzee and the human androgen receptor in vitro. The two protein fragment complementation assays which are considered to be indicative for a protein stabilization were positive only at and above cytotoxic concentrations with AC50 values of 13.89 und 15.76 µM. All other assays with more indication for activation of the androgen receptor – detecting regulation of gene expression and RNA transcription - were inactive for triticonazole or had a very high AC50 of 94.16 µM, weigh above cytotoxic concentrations of 2.27 µM. In the computational modeling exercise, triticonazole showed overall values of 0.16 indicating an agonistic action and 0.1 (the range of responses is 0.1 – 1, with 0.1 being the threshold for a positive response) indicating an antagonistic action, which is inconclusive. Testosterone propionate (the agonistic positive control) has overall values of 1.0 for an agonistic and 0 for an antagonistic response and hydroxyflutamide (the antagonistic positive control) has overall values of 0 for an agonistic and 0.55 for an antagonistic response.

Comment from the notifier

The summary provided here has been taken from an US EPA working document (EPA-HQ-OPP-2014-0614) only and not from a peer-reviewed publication, thus all evaluations and all conclusions from this computational modeling exercise should be taken with care. Further, there are efforts currently underway to compare computational model scores with results of the Hershberger assay to determine model performance for predicting in vivo bioactivity. As a general remark, the cytotoxicity concentration of triticonazole is now considered to be 2.27 µM instead of 9.3 µM, which might further decrease the number of relevant positive assay results.

B.6.8.4.5.6. Karmaus et al., 2016

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells ToxCAST Database (https://actor.epa.gov/dashboard/#chemical/131983-72-7) Excerpt of triticonazole results from the steroidogenesis assay
Author(s), year:	Karmaus et al., 2016
Report/Doc. number::	2016/1119499 and 2016/1119505
Guideline(s):	none, but comparable to OECD TG 456
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This study is part of the EPA ToxCast program. Triticonazole was tested among a set of 2060 chemical samples in a modified (high-throughput) steroidogenesis assay using H295R human adrenocortical carcinoma cells. The effect of steroidogenesis was investigated via HPLC/MS/MS quantification of 10 steroid hormones, including progestogens, glucocorticoids androgens, and estrogens. In a first stage the maximum tolerated concentration (MTC; ≥ 70% viability) per sample was established for each chemical. The second stage quantified changes in hormone levels at the MTC while the third stage performed concentration-response (CR) on a subset of samples. Clustering of the concentration-dependent chemical-mediated steroid hormone effects grouped chemical samples into five distinct profiles generally representing putative mechanisms of action, including Cyp17A1 and HSD3B inhibition. The authors identified distinct differences between the patterns of hormone changes induced by imidazole and triazole fungicides suggesting potentially different mechanisms of action.

Triticonazole was tested in concentrations of up to 10 µM based on the cytotoxicity pretesting. The concentration in the CR assay were 0.04, 0.12, 0.37 1.11, 3.33 and 10 µM. Triticonazole decreased the concentration of progesterone by -1.07 fold, of deoxycorticosterone by -1.41-fold and of cortisol by -2.65-fold. These values are given as log_2-fold concentrations. The AC_{50} values determined were 0.65 µM for progesterone, 1.71 µM for dexoycorticosterone and 4.48 µM for cortisol. All other hormones remained unchanged after treatment of the H295R cells with triticonazole. According to the authors, triticonazole was assigned to Cluster E, which is comprised of 143 chemicals, showing consistent decreases in glucocorticoid and androgen levels (however not seen with triticonazole), but no change or increases in estrogen levels.

Material and methods

This study is part of the EPA ToxCast program. The chemical library used for this study contained 2060 samples representing 1998 unique test chemicals. Chemicals were selected from multiple ToxCast chemical lists established based on solubility in DMSO. A top nominal stock concentration of 100 mM was attempted, solubility permitting, for the entire library. Information on the purity and source of the individual test chemicals were not provided in the publication, but relative purity information can be retrieved from the EPA website (http://www.epa.gov/ncct/dsstox/). The study included 18 azoles – among them triticonazole – for which concentration-control experiments were conducted and are described in more detail.

Test chemicals were assessed in a three-stage series of experiments:

1) Cytotoxicity assessment to determine the Maximum Tolerated Concentration (MTC)
2) Steroid Hormone quantification at the MTC ["MTC screen"]
3) For selected chemicals concentration-response ["CR"] analysis of hormone effects

In the single concentration MTC screen, samples were deemed to have a significant effect on hormone levels using a cutoff of ≥|1.5-fold change| over DMSO controls on a per plate basis.

Of the 2060 chemical samples evaluated, 524 samples were selected for six-point CR screening, based in part on significantly altering at least 4 hormones at the MTC. In the CR analyses, concentration-response data were fit for each hormone independently using three different models ["constant", "hill" and "gain-loss"]. Figure below below displays the different stages of the screening.

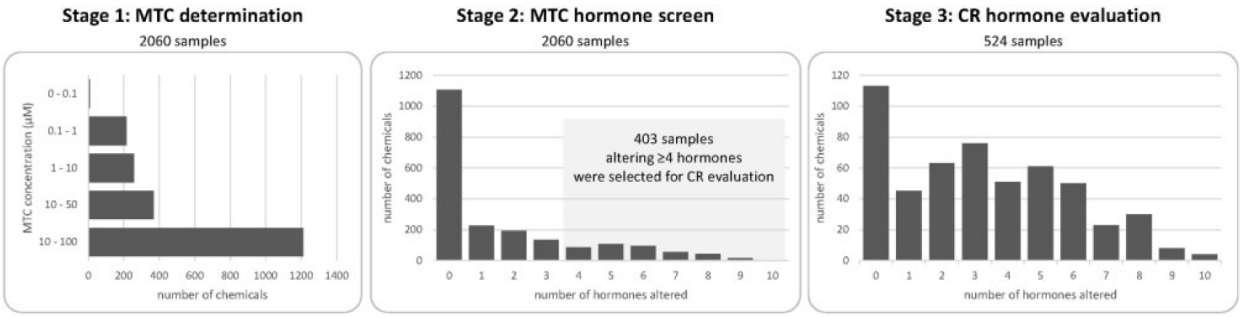


Figure 6.8.3.5.6-1: High-throughput H295R steroidogenesis assay workflow and summary. This study was conducted in 3 stages: I. Determination of an MTC (maximum concentration achieving ≥ 70% cell viability), II: Quantification of hormone levels upon MTC treatment, III. CR evaluation for selected chemicals. MTC concentrations were established for 2060 chemical samples, with the majority of samples having an MTC ≥ 10 µM, as shown in the stage graph. All 2060 chemical samples were evaluated for MTC effects on hormone levels. Samples altering ≥ 4 hormones in the MTC screen (highlighted in the shaded region in the stage 2 graph), in addition to 121 randomly selected chemical samples that did not meet the selection criteria were include for CR evaluation. Please note, that triticonazole did not meet the trigger of altering ≥ 4 hormones. The final stage 3 graph illustrates sum of how many hormones showed a concentration-dependent response upon treatment among the 524 chemical samples included in the CR evaluation. CR, concentration-response; MTC, maximum tolerated concentration.

Curve parameters were derived from the model with the best fit [i.e., having the lowest Akaike Information Criterion], to quantify potency via $logAC_{50}$ (the $\log_{10}$ concentration in µM at which 50% of the maximum fold change was achieved) and efficacy via the *max. fold change* (modeled top of the curve). In the CR analyses, a sample was identified as having a significant effect on a hormone if it was fit with one of the concentration-

response models and if the efficacy (i.e., change in hormone levels) exceeded a specified threshold defined by a cutoff of ≥6 times the BMAD (the median absolute deviation of all normalized response values at the lowest two tested concentrations across all samples).

Cell culture and treatment.

Cell culture conditions and media preparations were conducted in accordance with OECD TG 456 (2011) with minor modifications. The H295R human adrenal cortex carcinoma cell line was obtained from the American Type Culture Collection (ATCC CRL-2128). Batches of H295R cells were thawed and passed at least four times prior to steroidogenesis testing (maximum 10 passages). Cells were cultured in maintenance medium consisting of a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F-12 Nutrient mixture (DMEM/F12), 1.25% Nu-Serum, and 0.5% ITS+ Premix. After cells were seeded at 50-60% confluency into 96-well plates (probably at 37°C in a 5% CO_2 atmosphere; not reported), cells were allowed an overnight cell-adherence period. Subsequently culture medium was replaced by 175 µL media containing 10 µM forskolin to stimulate steroidogenesis for 48 hours. Finally the forskolin-stimulus-media was replaced by media containing the test chemical, which was added to a final concentration of 0.1% DMSO. On each 96-well plate, the chemicals and control substances (10 µM forskolin and 3 µM prochloraz) were tested in duplicates, in addition to four DMSO solvent controls. Following 48-hour incubation, the media was removed, split into vials of approx. 75 µL each, and stored at -80°C prior to steroid hormone quantification.

MTT Cytotoxicity Assay.

Where possible ToxCast chemicals were evaluated in the modified H295R assay at a maximum nominal concentration of 100 µM, unless limited by cytotoxicity. A target cell viability of ≥70% at the maximum test concentration was sought; therefore MTT cytotoxicity assays were carried out to establish a maximum tolerated concentration (MTC) for each chemical.

Quantification of Steroid Hormones.

The following 10 steroid hormone levels were assessed:

- 17α-Hydroxypregnenolone (OHPREG)
- Progesterone (PROG)
- 17α-Hydroxyprogesterone (OHPROG)
- 11-Deoxycortisol (11DCORT)
- Cortisol
- Deoxycorticosterone (DOC)
- Androstenedione (ANDR)
- Testosterone (TESTO)
- Estrone

- Estradiol

Levels of three further hormones, pregnenolone (PREG), dehydroepiandrosterone (DHEA), and corticosterone (CORT), were consistently below the limit of quantification (LLOQ) and therefore omitted for most of the analyses. Steroid hormones were extracted from media samples using MTBE. For estrone and estradiol detection, an extra derivatization step with dansyl chloride was included. Steroid hormones were separated and quantified using high-performance liquid chromatography (HPLC, reverse phase C18 gradient elution) with electrospray positive ionization was used followed by tandem mass spectrometry (MS/MS).

For each hormone determination, the upper and lower limit of quantification was determined. Quality control measures were taken to achieve for 100% accuracy (complete recovery of spiked-in standard with minimal loss during run-time) and good precision (≤ 15% RSD) to ensure reproducibility. The data collected in the study were within the specified margins, confirming high quality.

COMPARISON OF TOXCAST TO OECD 456 GUIDELINE H295R ASSAY

Compared to the OECD Guideline H295R assay, the ToxCast H295R assay comprises the following methodological differences:

1) H295R cells are additionally pre-treated with 10 µM forskolin for 48 hours. Forskolin is known to stimulate steroidogenesis across all hormones investigated. According to the authors, the pre-stimulus was included to enable robust dynamic responses in both up- and down-regulation of hormone levels.
2) The OECD Guideline H295R assay is validated only for quantification of estradiol and testosterone. The ToxCast H295R assay comprised the quantification of eight additional steroid hormones
3) In the ToxCast H295R, the highest test concentration (Maximum Tolerated Concentration (MTC) was defined by a cell viability cut-off of 70% in the MTT cytotoxicity assay. By comparison, in the OECD 456 assay, the viability cut-off is 80% for consideration of relevant hormone data.
4) According to the ToxCast H295R, a positive result in the single-concentration (MTC) experiment is concluded if at least 4 hormones were significantly altered (change at least 1.5-fold over solvent control). According to OECD 456 assay, a chemical is judged to be positive if the fold-induction is statistically significant from the solvent control at two adjacent concentrations in at least two independent runs; results at concentrations exceeding the solubility limit or cytotoxic concentrations should not be included in the interpretation of the results.
5) In the CR (concentration-response) experiment, a sample was identified as having a significant effect on a hormone if it was fit with one of the concentration-response models and if the efficacy (i.e., change in hormone levels) exceeded a specified threshold defined by a cutoff of ≥6 times the BMAD (the median absolute deviation of all normalized response values at the lowest two tested concentrations across all samples).
6) The samples in the ToxCast assay were evaluated in duplicate rather than triplicate.

7) The ToxCast assay did not investigate potential interaction between test substance and hormone. In the OECD 456 assay, this quality check belongs to the performance criteria (at least for antibody-based detection methods). Even for HPLC-MS/MS methods, cross-interference can occur and should be checked in pre-tests by incubation experiments in a cell-free system.

Results

Stage II (MTC determination)

Of the 2060 samples evaluated, 1203 samples were found with MTC's of 100 µM, 812 chemical samples had MTC's between 1 and 99 µM and 45 chemical samples had MTC's less than 0.9 µM. A total of 403 samples had effects on at least four hormones.

Stage III (Concentration control (CR) experiment)

524 chemicals were evaluated in six-point concentration-response experiments (403 samples identified in Stage I to have altered at least 4 hormones, plus 121 randomly chosen chemical samples not meeting these criteria). 232 chemical samples with concentration-dependent effects on 17ß-estradiol and/or testosterone were identified; 411 chemical samples had concentration-dependent effects on at least one hormone analyte.

The study included 18 azoles for which concentration-control experiments were conducted. The authors considered that the methodology allowed distinguishing imidazole class from triazole class chemicals, based on their profile of effects on steroidogenesis. With the exception of clotrimazole, the imidazoles cluster together due to increases in PROG and/or DOC in addition to strong decreases in CORTISOL, 11DCORT, ANDR, TESTO, ESTRONE and ESTRADIOL. The triazole fungicides showed more diverse behavior with a generally conserved decrease in CORTISOL levels. Furthermore, most conazoles demonstrate consistent AC_{50} values across all hormones they concentration-dependently altered supporting the hypothesis that they have a single target mediating their primary effects on steroidogenesis.

To investigate the profiles of steroidogenic alteration a clustering was used to identify distinct profiles. Cluster A is comprised of 137 chemicals eliciting overall increases in hormone levels, most strongly driven by increases in PROG, OHPROG, ESTRONE and ESTRADIOL. For example, forskolin, a chemical known to increase steroidogenesis across all hormones, can be found in Cluster A. Cluster B contains 35 chemicals with prochloraz-like profiles constituting marked increases in PROG and DOC levels and decreases among androrgens, the remaining glucocorticoids, and estrogens suggesting putative Cyp17A1 disruption. Cluster C is comprised of 50 chemicals with increased progestagen levels (OHPREG, OHPROG, and PROG) with simultaneous decreases across the rest of the hormones evaluated, which may result from HSD3B1 disruption. Finally, clusters D and E showed overall decreases in hormone levels with both clusters having consistent decreases in glucocorticoid and androgen levels, cluster D, comprised of 36 chemicals, consistently decreased estrogens suggesting overall inhibition of the steroidogenic pathway (i.e., putative Cyp11A1 inhibition, or chemical-mediated effects upstream of steroidogenesis). In contrast, the 143 chemicals in cluster E have no change or increased estrogen levels overall.

For **triticonazole** the following data have been excerpted from the supplementary tables to the publication and the ToxCAST data base (https://actor.epa.gov/dashboard/.). Based on cytotoxicity data, the top concentration of 10 µM was chosen for the MTC experiment. In the concentration-response experiments, the following concentrations were tested: 0.04, 0.12, 0.37, 1.11, 3.33 and 10 µM. (supplementary table S1 and S2). The cytotoxic concentration of triticonazole is considered to be 2.27 µM (see figures).

The levels of three hormones were significantly changed (values are given in log_2-fold changes and were taken from the supplementary table S9: Progesterone was decreased -1.07 fold, deoxycorticosterone was changed -1.41-fold and cortisol was changed -2.65-fold (see see below).

Table 6.8.3.5.6-1: Fold-changes of hormone concentrations in H295R cells after treatment with triticonazole

Hormone changed	Log_2-fold*	Fold-change	Direction
Progesterone	-1.07	2.10	Negative
11-Deoxycorticosterone	-1.41	2.66	Negative
Cortisol	-2.65	6.28	Negative

*values are taken from supplementary table S9 of Karmaus et al., 2016

No other hormones (especially no androgens or estrogens) were changed in this assay. The AC_{50} values were 0.65 µM for progesterone, 1.71 µM for deoxycorticosterone and 4.48 µM for cortisol (figure 6.8.3.5.6-2).

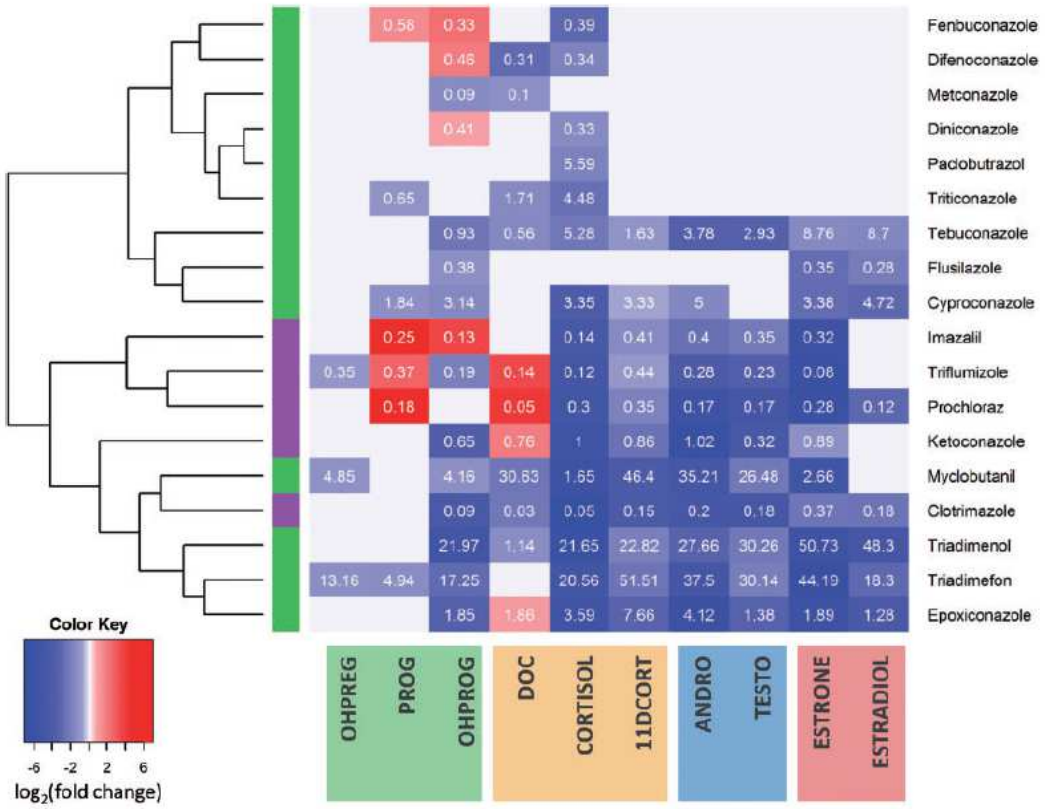


Figure 6.8.3.5.6-2: Profiling conazole fungicides effects on steroidogenesis. Heatmap visualizing the effect of 18 conazoles across the 10 hormones. The heatmap was generated to visualize the maximum fold change (in log2) achieved from concentration-dependent increase (red) or decrease (blue) in hormone levels with hierarchical clustering conducted using Euclidean distance metric to sort the conazoles. The AC50 concentration (mM) for the effect is overlaid in white print. A bar on the left identifies imidazole (purple) versus triazole (green) conazoles. Hormones are grouped and highlighted based by progestagen (green), glucocorticoid (yellow), androgen (blue), and estrogen (red) across the bottom. In total, 20 conazoles were evaluated in CR, with the 18 depicted conazoles having concentration-dependent effects on at least one hormone.

Based on the change of the glucocorticoid deoxycorticosterone, triticonazole was assigned to the pattern Cluster E, although the cluster-E-prerequisite of a change in androgen levels was not given. The triticonazole values were taken from the supplementary tables mentioned in the publication, which are identical to the values published in the ToxCast data base. For a better visualization the graphs taken from the ToxCast data base, which can be searched for using the link: https://actor.epa.gov/dashboard/ (downloaded at the 18th August 2016) are included here:

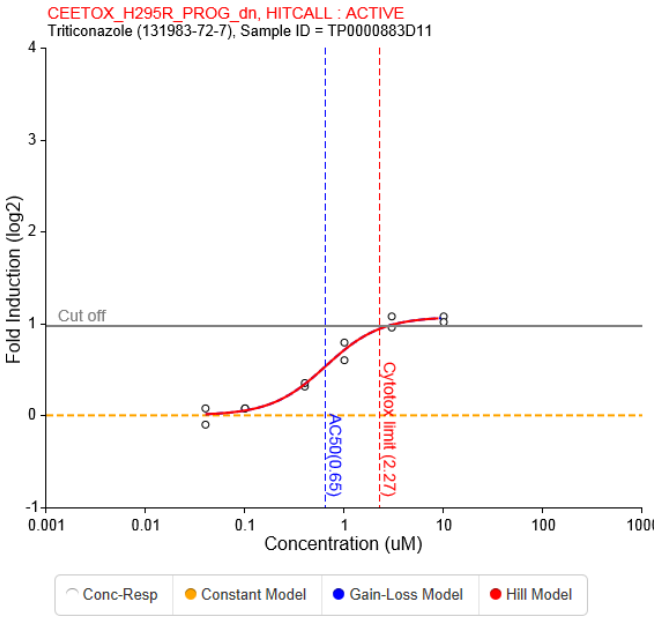


Figure 6.8.3.5.6-3: Dose response curve for triticonazole in H295R cells producing Progesterone (curves are taken from the ToxCast data base at the 18th of August 2016): Fold-decreases of progesterone (assay direction is negative).

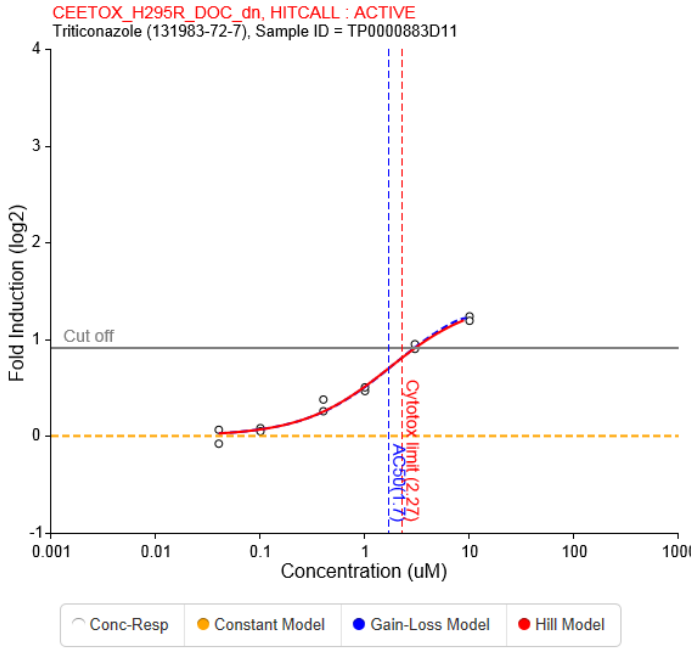


Figure 6.8.3.5.6-4: Dose response curve for triticonazole in H295R cells producing 11-Deoxycorticosterone (curves are taken from the ToxCast data base at the 18th of August 2016): Fold-decreases of 11-deoxycorticosterone (assay direction is negative)

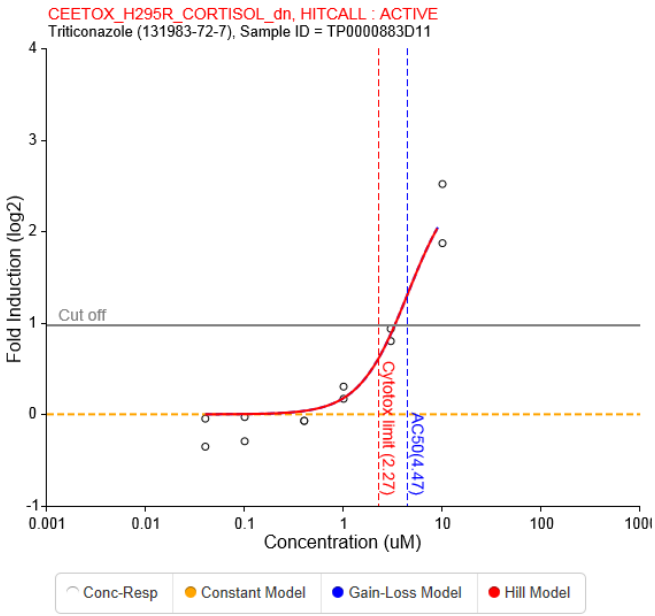


Figure 6.8.3.5.6-5: Dose response curve for triticonazole in H295R cells producing 11-Deoxycorticosterone (curves are taken from the ToxCast data base at the 18th of August 2016): Fold-decreases of cortisol (assay direction is negative)*.
*In Karmaus et al., 2016, an AC50 value of 4.48 µM is given

It can be seen from the included figures that the dose-response curve for progesterone and 11-deoxycorticosterone is only hardly above the cut-off. Further, for 11-deoxycorticosterone and cholesterol only each the highest and cytotoxic concentration tested is above the cut-off. Thus, a relevant increase (for the assay in a negative direction) in the 11-dexoycorticosterone and the cortisol signal is observed only at and above the cytoxicity limit; for cortisol even the AC_{50} is above the cytotoxicity cut-off.

Conclusion

In the H295R assay conducted with a modified protocol compared to OECD TG 456, triticonazole induced slight decreases of the progesterone, deoxycorticosterone and cortisol levels; at the top of the modeled concentration-response curve, log_2-fold changes of 1.07, 1.41 and 2.65 were derived, respectively (corresponding to calculated fold-decreases of 2.1, 2.7 and 6.3, respectively). The AC_{50} values were 0.67, 1.71 and 4.47 µM. The decreasing effect on progesterone is considered at most subtle, as the dose-response curve is hardly above the cut-off threshold, indicating an active assay. The decreases seen for 11-deoxycorticosterone and cortisol are only evident at and above cytotoxic concentrations, also raising doubts on the specificity of the outcome. Further EPA has considered triticonazole to be inactive in the steroidogenesis assay (see Judson et al., 2016).

426 chemicals investigated in the modified H295R assay were allocated to 5 clusters, based on similarities in hormone-change patterns. Triticonazole was assigned to an unspecific Cluster E, for chemicals showing consistent decreases in glucocorticoid and androgen levels without affecting estrogen levels. The 18 triazoles/imidazole class chemicals that were assessed in more detail in the publication could not be assigned to a single cluster (distribution: 2x Cluster A, 2x Cluster B, 2x Cluster C, 8x Cluster D and 4x Cluster E). Although the authors considered that the imidazoles and triazoles can be distinguished in the modified H295R assay based

on differences in their hormonal patterns, at the same time the authors concluded that the hormonal patterns of the triazole fungicides are diverse.

Comment of the notifier:

The modified H295R steroidogenesis assay conducted under the ToxCAST program is considered to be more sensitive compared to the OECD TG 456, as the H295R cells were additionally pre-treated with 10 µM forskolin for 48 hours and forskolin is known to stimulate steroidogenesis across all hormones investigated. The study design of the ToxCast modified H295R assay encompassed a tiered testing approach, where chemicals are first tested for hormone changes at the maximum tolerated concentration (MTC); subsequently concentration-response (CR) evaluations are usually carried out only for those test chemicals that induce significant changes in at least 4 different hormones at the MTC. Based on the results obtained, the study authors considered it is unlikely that chemicals not selected for CR evaluation have a significant effect on steroidogenesis. Triticonazole was one of a few test chemicals that underwent CR evaluation despite the fact that only changes in three different hormone levels occurred, which would normally not have triggered CR evaluations. The decreases in the progesterone are subtle (2.1-fold), while the decreases in the deoxycorticosterone and cortisol levels occur only at and above cytotoxic concentrations. It is plausible, that increased cellular stress or damage can lead to decreased functionality and lower hormone expressions just as a response to cytotoxicity (some evidence given in Prasad et al., 2013[19]). Thus it cannot be excluded, that a lower hormone expression of the H295R cells is a result of general toxicity rather than a specific interaction of triticonazole with the cells. As triticonazole has no effect on androgen levels in the H295R assay, the assignment into cluster E is of doubtful accuracy. Overall, the evidence for an interaction of triticonazole with steroidogenesis is weak, especially considering, that no changes were seen for estrogen (e.g. estradiol) or androgen (e.g. testosterone) levels, for which the OECD TG 456 assay is validated only.

B.6.8.4.5.7. Roelofs M.J.E. et al., 2014

Previous evaluation:	No
DRAR (2016)	Literature data

Reference:	Conazole fungicides inhibit Leydig cell testosterone secretion and androgen receptor activation in vitro
Author(s), year:	Roelofs M.J.E. et al., 2014
Report/Doc. number::	-/ 2014/1326753
Guideline(s):	No
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

In this study, the potential (anti-)androgenic effects of ten conazoles were assessed and mutually compared with existing data. Amongst others effects of triticonazole (TRIT) were examined using murine Leydig (MA-10) cells and human T47D-ARE cells stably transfected with an androgen responsive element and a firefly luciferase

[19] Prasad et al., 2013. Deficiency of ALADIN Impairs Redox Homeostasis inHuman Adrenal Cells and Inhibits Steroidogenesis. Endocrinology 154: 3209–3218, 2013

reporter gene. The other tested azoles were cyproconazole (CYPRO), fluconazole (FLUC), flusilazole (FLUS), hexaconazole (HEXA), myclobutanil (MYC), penconazole (PEN), prochloraz (PRO), tebucoanzole (TEBU), and triadimefon (TRIA).

Material and methods

10 selected conazoles (among them triticonazole) were tested for in vitro antiandrogenic properties. Stock solutions were prepared in DMSO resulting in a maximal solvent concentration of 0.1% (v/v) in the exposure medium.

MA-10 Leydig cell culture:

A murine Leydig tumor cell line MA-10 was used to test for basal testosterone secretion in vitro.

T47D-ARE cell line:

The human breast cancer cell line T47D-ARE cells are transfected with an androgen responsive element with a firefly luciferase reporter gene.

Testosterone secretion assay:

The MA-10 cells were plated in 24-well plates 24 h prior to exposure. cAMP induces the expression of genes of steroidogenic enzymes and was used as positive control. SU10603 is a catalytic Cyp17 enzyme inhibitor and was used as a control for decreased T secretion. For basal testosterone measurements cells were exposed to the selected conazoles [10 µM] alone. Gonadotropin LH was used to stimulate the Leydig cells to produce T. To determine the effect of selected conazoles on LH-induced T secretion, cells were exposure to a combination of LH and SU10603, flusilazole or tebuconazole. Testosterone was measured in media with a commercially available T radioimmunoassay kit.

Reactive oxygen species (ROS) assay:

ROS production in MA-10 cells was assessed using the fluorescent dye H2-DCFDA. The MA-10 cells were plated and loaded with H2-DCDFA for 2 h prior to exposure at 37°C. After loading, the dye was removed and cells were washed twice and subsequently exposed to the test substances. Fluorescence was measured spectrometrically at T=0, 1, 24, 48 h. H2O2 was used as a positive control at T=1 and rotenone at T=24, 48 h).

Androgen receptor reporter gene assay:

Androgen receptor activation was determined by measuring the luciferase reaction luminescence. The luminescent signal evoked by the luciferase reaction was measured as relative luminescence units of T47D-ARE cells exposed to concentration curves ranging from 100 pM to 100 µM of the ten selected conazoles. Exposures were performed in the presence or absence of EC50 of testosterone [29nM]. The known AR antagonist flutamide was used as postivie control for AR antagonism. For flutamide and each conazole exposure the half maximal inhibitory concentration (IC50) of AR activation was derived from concentration-response curves using a sigmoidal dose-response nonlinear regression curve fit with variable slope following the formula:

$$y = E_0 + \left(\frac{(E_{max} \times X^n)}{b^n + X^n}\right).$$

Y – AR response

X – exposure concentration

E0 – estimated background response level

Emax – maximum response

B – computed half maximal inhibitory concentration of flutamide ($IC_{50;Flut}$)

N – shaping parameter of the Hill curve

A benchmark response (BMR), needed to elicit 25% of the inhibitory effect on AR activation response caused by flutamide ($BMR_{25;Flut}$) was calculated by using the formula below:

$$BMR_{25\%FLUT}\ \text{“conazole X”} = 10^{\{-[(\log((E_{max}/(y-E_0))-1))/n]+\log(IC_{50}\text{“conazole X”})\}}$$

Subsequently, relative effect potencies were calculated for each conazole relative to flutamide (REP_{Flut}) using the respective BMRs in the following formula:

$$REP\ \text{“conazole X”} = \left(\frac{BMR_{25\%\,FLUT}}{BMR_{25\%\,FLUT}\ \text{“conazole X”}}\right)$$

Cytotoxicity:

Cell viability of MA-10 and T47D-ARE cells after exposures was determined by measuring the capacity of cells to reduce MTT to formazan by the mitochondrial enzyme succinate dehydrogenase.

Data analysis:

All experiments were performed in triplo and within each independent experiment each concentration was tested in duplicate (T secretion assay), triplicate (AR reporter gene assays), or quadruplicate (ROS assays).

Results

It has been stated, that MA-10 cells and T47D-ARE were exposed to non cytotoxic concentrations of the test compounds (10 nM – 100 µM for the MA-10 cells and 10 pM – 100 µM for the T47D-ARE cells), however the data are not shown in this publication.

Testosterone secretion in MA-10 cells:

The basal testosterone secretion by MA-10 cells (Leydig cells) was assessed after a 48-h exposure to 10 µM of the individual conazoles. This concentration did not significantly affect MA-10 cell viability, however these data were not shown. Triticonazole inhibited testosterone secretion by Gonatropin LH stimulated MA-10 cells by 44%. As shown in the figure below, triticonazole was medium potent compared to the other azoles tested.

The basal testosterone secretion by MA-10 cells (Leydig cells) was assessed after a 48-h exposure to 10 µM of the individual conazoles. This concentration did not significantly affect MA-10 cell viability; however these data were not shown. TRIT inhibited testosterone secretion by MA-10 cells by 44% compared to control. As shown in the Figure 6.8.3.5.7-1, triticonazole was medium potent compared to the other azoles tested.

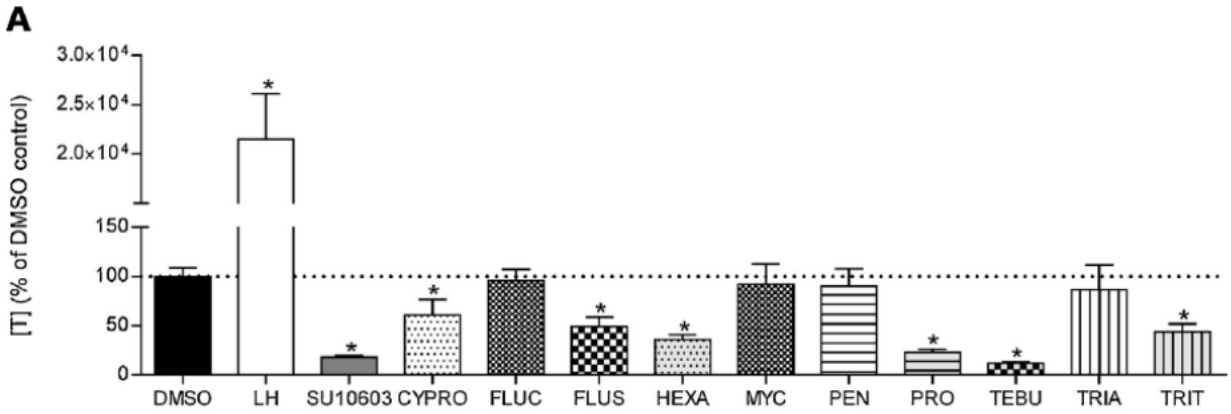


Figure 6.8.3.5.7-1: Testosterone secretion by MA-10-cells after a 48-h exposure to DMSO control, LH, the Cyp17 inhibitor SU10603, or one of the 10 tested conazoles (TRIT = triticonazole)

ROS formation:

To further explore the nature of testosterone secretion inhibition by MA-10 cells after exposure to certain conazole fungicides, ROS (reactive oxygen species) formation was considered as a possible cause for deterioration of Leydig cell function resulting in decreased testosterone secretion. Triticonazole did not have an effect on ROS production, as shown in the figure below.

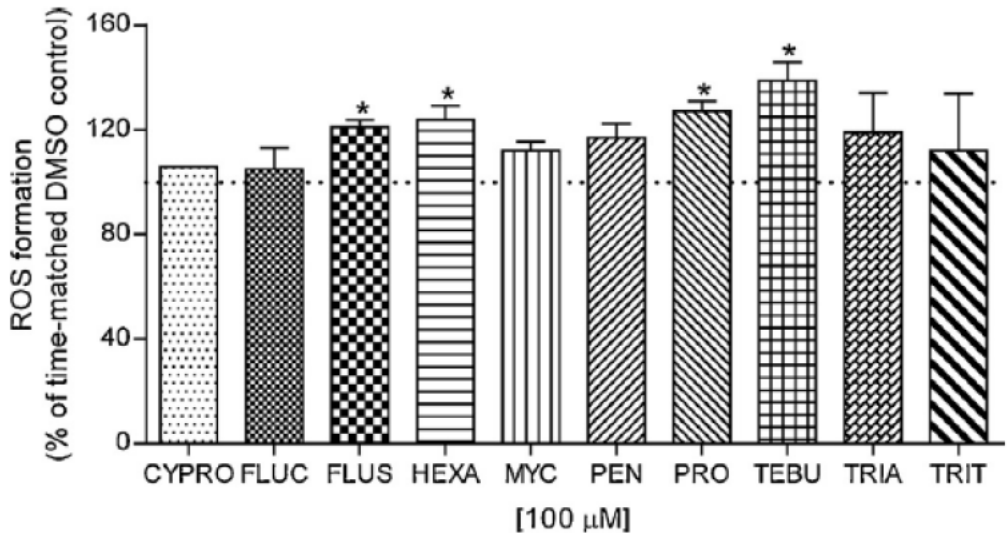


Figure 6.8.3.5.7-2: Reactive oxygen species (ROS) formation by MA-10 cells after a 48-h exposure to each of the ten selected conazoles

In a further assay androgen receptor (AR) activation was determined for the conazoles. The assays used an AR reporter gene assay. T47D-ARE cells were exposed to non-cytotoxic concentrations of the tested compounds.

Testosterone (T) activated the AR in a concentration-dependent manner with an EC50 of 13.6 nM. Exposure to conazoles did not significantly affect AR activation.

Next, cells were exposed to 20nM T in combination with concentration ranges of the selected conazoles or the AR antagonist flutamide (FLUT). FLUT concentration-dependently decreased AR activation with an IC50 value of 7.0 µM. TRIT concentration-dependently inhibited T-induced AR activation with an IC_{50} of 1.07E-05 (see table 6.8.3.5.7-1): Further the benchmark response (BMR), needed to elicit 25% of the inhibitory effect on AR activation response of flutamide as well as the relative effect potency compared to flutamide was calculated.

Table 6.8.3.5.7-1: Androgen receptor reporter gene assays outcome expressed as half maximal effect or inhibitor concentrations (EC/IC50 [M])

Compound	EC/IC_{50} [M]	$BMR_{25\%FLUT}$ [M]	REP
T	1.36E–08	n.a.	n.a.
FLUT	7.02E–06	1.98E–06	1.00
CYPRO	1.36E–05	5.25E–06	0.38
FLUC	n.a.	n.a.	n.a.
FLUS	1.19E–05	5.49E–07	3.61
HEXA	2.32E–05	7.64E–06	0.26
MYC	7.15E–05	7.06E–05	0.03
PEN	1.71E–05	5.54E–06	0.36
PRO	1.17E–05	9.43E–06	0.21
TEBU	2.55E–05	9.01E–06	0.22
TRIA	3.21E–05	1.60E–05	0.12
TRIT	1.07E–05	7.80E–06	0.25

$BMR_{25\%FLUT}$ = concentrations where inhibition of AR activation was similar to 25% inhibition by FLUT
REP = effect potency relative to FLUT

Conclusion

In this in vitro assay triticonazole showed two potential antiandrogenic activities: inhibition of testosterone secretion and AR antagonism. Triticonazole leads to decreased testosterone secretion in murine Leydig cell (MA-10) in vitro, when the MA-10 cells are exposed to 10 µM triticonazole (10^{-5} [M]) for 48 hours. It was medium potent compared to other azoles tested. There was no indication for an ROS formation in this test system. In an androgen receptor reporter gene assay an IC50 of 1.07E-05 for androgen receptor inhibition was determined for triticonazole.

The notifier stated that concentration of 10 µM is high concentration, which is probably not reached systemically. This estimation is based on the fact that patients treated orally with 200 mg fluconazole/day (pharmaceutical) showed serum levels ranging from 16.3 to 25.8 µM[20] and dietary and non-dietary exposure to triticonazole are far below this dose. In the modified steroidogenesis assay (Karmaus et al., 2016) no effects on testosterone were observed.

Comment of the notifier

[20] M. Kami, Y. Sawada, S. Mori, J. Hirate, N. Kojima, Y. Kanda, et al.,Serum levels of fluconazole in patients after cytotoxic chemotherapyfor hematological malignancy, Am. J. Hematol. 66 (2001) 85–91.

The authors have mentioned, that they have tested only non cytotoxic concentrations in the murine tumor cell line and the humane breast cancer cell line, however there is no data presented to prove this. Based on the ToxCast data triticonazole was cytotoxic at 2.27 µM, which is factor 5 below the IC50 concentrations for androgen receptor activation (10.7 µM) or decreases in testosterone secretion observed in MA-10 cells (10 µM). Any results obtained in tumorigenic cell lines shall always be taken with care. It is further to be stressed that these *in vitro* results are not reflecting the *in vivo* situation in rodents, as triticonazole is not causing any effects on male reproduction organ weights in rats or mice subchronic or chronic studies, especially no effects in mouse Leydig cells. Also no histopathological changes in male reproduction organs were observed. Even when comparing the *in vitro* results of the murine MA-10 cell line with some of the other conazoles with their *in vivo* toxicological profile raises some doubts on the predictability and relevance of this *in vitro* test system.

B.6.8.4.5.8. Friedman et al., 2016

Reference:	Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors Within the ToxCast Phase I and II Chemical Libraries
Author(s), year:	Friedman et al., 2016
Report/Doc. number:	2016/1352207
Guideline(s):	none
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This study is part of the EPA ToxCast program.

High-throughput screening for potential thyroid-disrupting chemicals requires a system of assays to capture multiple molecular-initiating events (MIEs) that converge on perturbed thyroid hormone (TH) homeostasis. Screening for MIEs specific to TH-disrupting pathways is limited in the U.S. Environmental Protection Agency ToxCast screening assay portfolio. To fill the critical screening gap, the Amplex UltraRed-thyroperoxidase (AUR-TPO) assay was developed to identify chemicals that inhibit TPO, as decreased TPO activity reduces TH synthesis. The ToxCast phase I and II chemical libraries, comprised of 1074 unique chemicals, were initially screened using a single, high concentration to identify potential TPO inhibitors. Chemicals positive in the single-concentration screen were retested in dose-response setting. Due to high false-positive rates typically observed with loss-of-signal assays such as AUR-TPO, also 2 additional assays in parallel to identify possible sources of nonspecific assay signal loss were employed, enabling stratification of roughly 300 putative TPO inhibitors based upon selective AUR-TPO activity. A cell-free luciferase inhibition assay was used to identify nonspecific enzyme inhibition among the putative TPO inhibitors, and a cytotoxicity assay using a human cell line was used to estimate the cellular tolerance limit. Additionally, the TPO inhibition activities of 150 chemicals were compared between the AUR-TPO and an orthogonal peroxidase oxidation assay using guaiacol as a substrate to confirm the activity profiles of putative TPO inhibitors. This effort represents the most extensive TPO inhibition screening campaign to date and illustrates a tiered screening approach that focuses resources, maximizes assay throughput, and reduces animal use.

The workflow of the testing is depicted in the figure below.

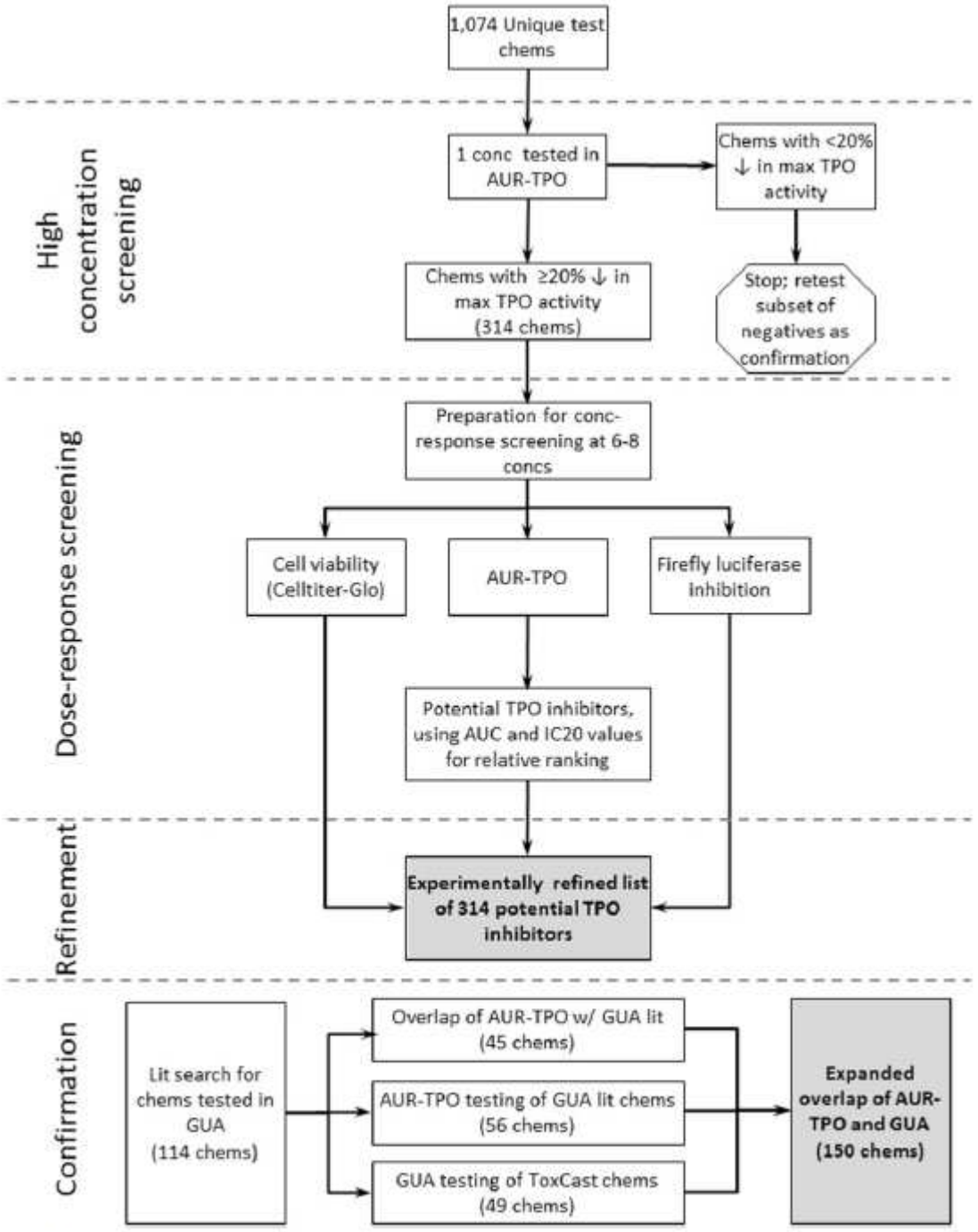


FIG. 2. The tiered screening approach to identify, stratify, and confirm TPO inhibitors. One thousand seventy-four unique ToxCast chemicals were initially screened using a single, high concentration to identify potential TPO inhibitors. Chemicals testing positive in the single-concentration screen were retested in concentration-response for TPO inhibition. A cytotoxicity and luciferase inhibition assay were employed in parallel to identify possible sources of nonspecific assay signal loss, enabling stratification of roughly 300 putative TPO inhibitors based upon selective Amplex UltraRed-TPO (AUR-TPO) activity. The TPO inhibition activities of 150 chemicals were compared across the AUR-TPO and guaiacol oxidation (GUA) assays to confirm the activity profiles of putative TPO inhibitors. Lit refers to publicly available literature as described in the Materials and Methods.

Figure 6.8.4.5.8-1: The tiered screening approach to identify, stratify and confirm TPO inhibition

Results

Triticonazole was tested as part of the 1074 unique test chemicals for which a concentration was tested in the AUR-TPO assay. Triticonazole displayed an inhibition of 6.1% and was therefore evaluated to not inhibit TPO activity.

Also other azoles were tested in the assay. Generally, they did not display an effect on TPO activity inhibition (most below 20% inhibition cut-off). The common azole metabolites 1H-1,2,4-Triazole (9.0%) and 4-Amino-1,2,4-triazole (5%) were also considered negative.

Conclusion

In Amplex UltraRed-thyroperoxidase (AUR-TPO) assay triticonazole showed lack of activity on TPO inhibition.

B.6.8.4.5.9. Hornung et al., 2017

Reference:	Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity
Author(s), year:	Hornung et al., 2017
Report/Doc. number::	2017/1225381
Guideline(s):	none
GLP:	No
Deviations from Guideline:	-
Acceptability:	Yes; additional information

Executive Summary

This study is part of the EPA ToxCast program.

Thyroid hormone (TH) homeostasis is dependent upon coordination of multiple key events including iodide uptake, hormone synthesis, metabolism, and elimination, to maintain proper TH signaling. Deiodinase enzymes catalyze iodide release from THs to interconvert THs between active and inactive forms, and are integral to hormone metabolism. The activity of deiodinases has been identified as an important endpoint to include in the context of screening chemicals or TH disruption. To begin to address the potential for chemicals to inhibit these enzymes an adenovirus expression system was used to produce human deiodinase type 1 (DIO1) enzyme, established robust assay parameters for nonradioactive determination of iodide release by the Sandell-Kolthoff method, and employed a 96-well plate format for screening chemical libraries. An initial set of 18 chemicals was used to establish the assay, along with the known DIO1 inhibitor 6-propylthiouracil as a positive control. An additional 292 unique chemicals from the EPA's ToxCast phase 1_v2 chemical library were screened. Chemicals were initially screened at a single high concentration of 200 mM to identify potential DIO1 inhibitors. There were 50 chemicals, or 17% of the TCp1_v2 chemicals tested, that produced >20% inhibition of DIO1 activity. Eighteen of these inhibited DIO1 activity >50% and were further tested in dose-response mode to determine IC50s. This work presents an initial effort toward identifying chemicals with potential for affecting THs via inhibition of deiodinases and sets the foundation for further testing of large chemical libraries against DIO1 and the other deiodinase enzymes involved in TH function.

Triticonazole was tested as part of the ToxCast Phase 1_v2 chemical library. Triticonazole did not inhibit DIO1 activity.

Material and methods

Adenoviruses expressing deiodinase were constructed by cotransfecting HEK293 cells with the subcloned DIO 1 gene.

DIO1 activity measurement was performed according to the schematics depicted in the figure below.

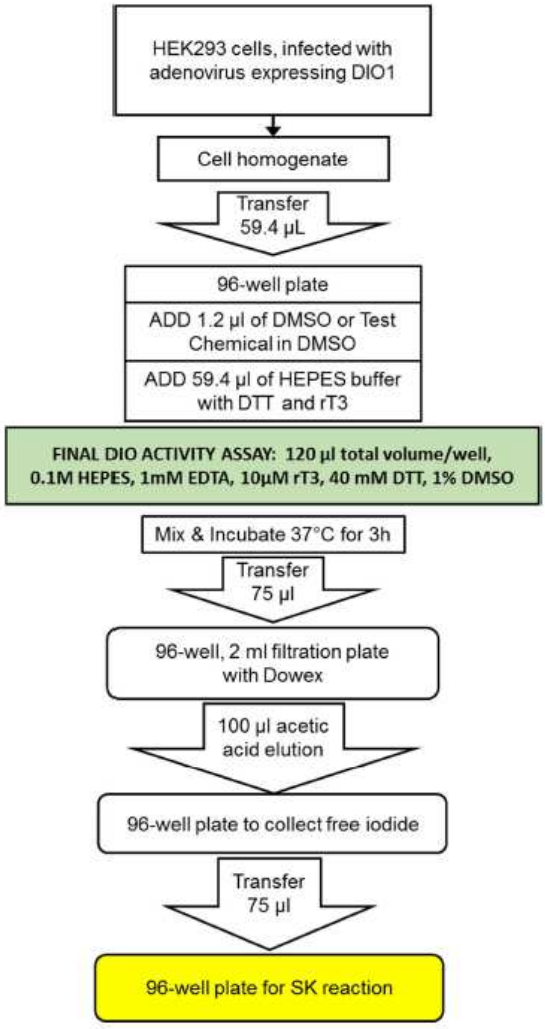


Figure 6.8.4.5.9-1: Summary of steps im assay procedure

The sensitivity of the assay was guaranteed by adding multiple samples of the positive control PTU to each 96 well plate. In addition, a set of benzothiazoles for which *in vitro* TPO inhibition data and *in vivo* responses in a *Xenopus laevis* metamorphosis assay were available were included as validation set.

Results

Triticonazole was part of the set of chemicals for which DIO1 inhibition activity was tested at 200 µM. Triticonazole displayed no DIO1 inhibition activity (% inhibition = -1.0; range: -2.9 – 6.7; rank 219 of 300 in supplemental table 1 of the publication.

Supplemental Table 1. List of all chemicals tested in the DIO1 inhibition assay, and their relative activity rank based upon inhibition activity at 200 µM.

Chemical	CASRN	Source[a]	Rank[b]	% Inhibition[c]		
				median	minimum	maximum
Triticonazole	131983-72-7	TCp1v2	219	-1.0	-2.9	6.7

a. Source. IS indicates chemicals used for the initial screening assay development from stocks on hand at EPA/ORD/NHEERL/MED in Duluth. TCp1v2 indicates ToxCast p1_v2 chemical library obtained via Dr. Ann Richard, EPA/ORD/NCCT, Research Triangle Park, NC, USA.

b. Rank. The chemical rank compared to the others in this set of test chemicals for inhibition efficacy when tested at 200 µM. A rank of 1 indicates the chemical that inhibited the DIO1 activity the greatest in the single concentration screen.

c. % inhibition of the data from the three replicate runs of each chemical. Values for the n=3 data points are shown and sorted by median, minimum and maximum inhibition response.

Also other azoles were tested in the assay (difenoconazole, prothioconazole, cyproconazole, fenbuconazole, tetraconazole, hexaconazole, diniconazole). None of those displayed a significant effect on DIO1 activity inhibition (all < 5 % inhibition).

Discussion

Triticonazole displayed no DIO1 inhibition at a concentration of 200 µM. DIO1 activity is one of the putative mechanisms how a chemical can affect thyroid hormone homeostasis. Therefore, a lack of activity on DIO1 provides further evidence that Triticonazole is not goitrogen.

B.6.8.4.6. Conclusion on observed findings regarding endocrine endpoints and organs

The most widely used definition of an endocrine disruptor is based on the WHO/IPCS (2002):

'An endocrine disrupter is an exogenous substance or mixture that alters function(s) of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub)populations'.

This definition is based on hazard identification, looking at whether the effects reported are regarded to be ED-related and supported by mechanistic information.

According to Regulation (EC) No 1107/2009, Annex II, Point 3.6.5 'an active substance shall only be approved if, (…), it is not considered to have endocrine disrupting properties that may cause adverse effect in humans.'

Nevertheless, there is no regulatory guidance available yet on how to address endocrine disruption (ED) and no final criteria are established.

Pending the adoption of the final scientific criteria for the determination of ED properties, currently the so called **interim criteria** are applied. There were two Interim criteria defined within Regulation (EC) No 1107/2009, Annex II, Point 3.6.5:

1) *'(…) substances that are or have to be classified, in accordance with the provisions of Regulation (EC) No 1272/2008, as carcinogenic category 2 and toxic for reproduction category 2, shall be considered to have endocrine disrupting properties.'*
2) *'Substances such as those that are or have to be classified, in accordance with the provisions of Regulation (EC) No 1272/2008, as toxic for reproduction category 2 and which have toxic effects on the endocrine organs, may be considered to have such endocrine disrupting properties.'*

In July 2017 the decision to amend the interim criteria has been made in Standing Committee on Plants, Animals, Food and Feed, however, the scientific criteria have not been adopted yet and also no Guidance has been launched. For purpose of completeness, the criteria as set in Annex to the Commission Regulation are included here:

Annex II to Regulation (EC) No 1107/2009 is amended as follows:

(1) In Point 3.6.5. the following paragraphs are added after the fourth paragraph:

"From [date of application], an active substance, safener or synergist shall be considered as having endocrine disrupting properties that may cause adverse effect in humans if, based on points (1) to (4) of the sixth paragraph, it is a substance that meets all of the following criteria, unless there is evidence demonstrating that the adverse effects identified are not relevant to humans:

(1) it shows an adverse effect in an intact organism or its progeny, which is a change in the morphology, physiology, growth, development, reproduction or life span of an organism, system or (sub)population that results in an impairment of functional capacity, an impairment of the capacity to compensate for additional stress or an increase in susceptibility to other influences;

(2) it has an endocrine mode of action, i.e. it alters the function(s) of the endocrine system;

(3) the adverse effect is a consequence of the endocrine mode of action.

The identification of an active substance, safener or synergist as having endocrine disrupting properties that may cause adverse effect in humans in accordance with the fifth paragraph shall be based on all of the following points:

(1) all available relevant scientific data (in vivo studies or adequately validated alternative test systems predictive of adverse effects in humans or animals; as well as in vivo, in vitro, or, if applicable, in silico studies informing about endocrine modes of action):

  (a) scientific data generated in accordance with internationally agreed study protocols, in particular those listed in the Commission Communications in the framework of setting out the data requirements for active substances and plant protection products, in accordance with this Regulation;

  (b) other scientific data selected applying a systematic review methodology, in particular following guidance on literature data which is listed in the Commission Communications in the framework of setting out the data requirements for active substances and plant protection products, in accordance with this Regulation;

(2) an assessment of the available relevant scientific data based on a weight of evidence approach in order to establish whether the criteria set out in the fifth paragraph are fulfilled; in applying the weight of evidence determination, the assessment of the scientific evidence shall, in particular, consider all of the following factors:

  (a) both positive and negative results;

  (b) the relevance of the study designs, for the assessment of adverse effects and of the endocrine mode of action;

  (c) the quality and consistency of the data, considering the pattern and coherence of the results within and between studies of a similar design and across different species;

  (d) the route of exposure, toxicokinetic and metabolism studies;

  (e) the concept of the limit dose, and international guidelines on maximum recommended doses and for assessing confounding effects of excessive toxicity;

(3) using a weight of evidence approach, the link between the adverse effect(s) and the endocrine mode of action shall be established based on biological plausibility, which shall be determined in the light of current scientific knowledge and under consideration of internationally agreed guidelines;

(4) adverse effects that are non-specific secondary consequences of other toxic effects shall not be considered for the identification of the substance as endocrine disruptor."

Triticonazole belongs to the triazole class of fungicide compounds acting by blockage of sterol biosynthesis, i.e. inhibition of lanosterol 14α-demethylase (CYP51A1), in fungal cell membranes. The inhibition of mammalian aromatase (CYP19) is a known “off-target” effect of azole-class fungicides. The difference in potency to inhibit fungal CYP51 and mammalian CYP19 is substance-specific. Therefore, substance-specific factors (mainly potency for aromatase inhibition and toxicokinetics/metabolism) will determine whether adverse effects occur as a result of endocrine disruption.

No classification is proposed for triticonazole for carcinogenicity or for reproductive toxicity, applying classification criteria as stipulated in Regulation (EC) No. 1272/2008. Thus, the conditions of the Interim Criterion 1 are not met for triticonazole.

For evaluation of the second criterion, it has to be determined whether toxic effects are observed on endocrine organs. Thus, in this section the evaluation of effects on reproductive organs, pituitary, thyroid, and adrenals

from subchronic, chronic, and reproduction studies are compiled and the adversity of observed effects is assessed.

According to scientific criteria, the adverse effect has to be the consequence of the endocrine mode of action. Based on the available data, there is no indication that effects on morphology of zona fasciculata of adrenals after exposure to triticonazole have an endocrine mode of action or that disturbace in functionality of adrenals is given. Therefore, if scientific criteria are taken into account, there is no evidence for triticonazole having endocrine disrupting properties.

B.6.8.4.6.1. Effects on reproduction

Triticonazole has been investigated for reproduction toxicity in a two-generation toxicity study at dietary doses of 5, 25, 750 and 5000 ppm according to the OECD TG 416 (1981). Treatment-related effects were only seen at the top dose of 5000 ppm. The potentially endocrine-related effects were confined to increased gestation lengths in the F_0 generation (statistically significant but within HCD), decreased fertility and mating index in the F_1 parental generation, histopathological changes in adrenals and ovaries (more pronounced in the F_0 parental generation) and reduced viability and growth in the offspring. The most obvious difference between the F_0 and the F_1 animals is a substantial higher substance intake in the F_1 generation (males & females) and an apparently substantially higher systemic toxicity in the F_1 animals, expressed as up to 30% lower body weights shown for males of the F_1 generation and almost no effects in the F0 generation. Thus is assumed that the observed decreased fertility and viability decrease are caused by general systemic toxicity and not by a primary endocrine mode of action.

The other treatment-related effects in the 2-generation toxicity study are adrenal histopathological findings in adult males and females and ovary effects in parental females. The histopathological adrenal effects in females are considerably more pronounced in the F_0 parental vs the F_1 parental generation, indicating no correlation between adrenal and reproduction effects seen in the F_1 generation. The adrenal findings are discussed below in the context of observed adrenal findings in subchronic and chronic rat and dog studies. Further increased incidences of ovary vacuolations were seen in the F_1 adult females. No respective findings were observed in the F_0 maternal generation.

Triticonazole did not induce any findings on organ weights or histopathology on male reproductive organs (prostate, seminal vesicles, epididymides, testes) in rats or mice in any of the relevant studies in Sprague-Dawley rat or CD-1 mice. There were especially no increased incidences of Leydig cell tumors seen in the chronic rat studies. The decreased prostate weights seen at the top dose of the 52-week dog study is an isolated finding in dogs and of unknown relevance, as there were no related histopathological findings seen in the prostate, thus the function of the prostate does not seem to be affected by treatment with triticonazole.

Administration of triticonazole to pregnant Wistar rats from GD 6-19 did not lead to treatment-related developmental toxicity of any kind when tested at doses of up to 200 mg/kg bw per day. There were no effects on implantation loss, resorptions, sex ratio or placental weight. At the highest tested dose of 1000 mg/kg bw per day developmental toxicity was confined to increased incidence of additional 13^{th} and 14^{th} rib; these findings are not considered to be a consequence of any endocrine mediated disturbance. When triticonazole was administered daily to New Zealand White rabbits by stomach tube from GD 6-28, no developmental toxicity was observed at

dose of 5 mg/kg bw. Developmental toxicity at higher doses was confined to skeletal findings. At dose levels of 25, 50 and 75 mg/kg bw per day severe maternal toxicity including body weight loss and maternal mortality (30% at 75 mg/kg bw per day) was observed. There were, however, no effects on implantation sites, implantation losses, resorptions, sex ratio or placental weight, except at 75 mg/kg bw per day. There was, thus, no evidence of any endocrine-related effect in the developmental toxicity studies conducted in rats and rabbits.

Triticonazole was tested *in vitro* for rat and human aromatase inhibiting properties. The IC50 in rat aromatase is 1.8 x 10^{-6} M, however not a full inhibition of the aromatase was seen. The positive controls used in this study showed IC50 values which were 3 orders of magnitude lower (Letrozole: IC50: 1.53 x 10^{-9} M), compared to triticonazole. Increased gestation lengths observed in rats is considered to be a borderline for triticonazole, as the observed increased gestation length (0.5 days in both F_0 and F_1 generation) was either within historical control data (F_0) or not statistically significantly different to control (F_1). This is supported by rather high IC50 value for triticonazole in the aromatase inhibition assay, indicating rather low potency of triticonazole to inhibit aromatase in rats.

In the Level 2 ED studies there is no evidence for triticonazole having estrogenic or anti-estrogenic activity, neither in the ToxCast data nor in the YES assay conducted. Also the assays run in the H295R cells under the ToxCAST program, indicative for effects on the steroidogenesis, did not reveal any evidence for decreased testosterone or estrogen levels, although the ToxCast test itself is considered to be more sensitive compared to an OECD TG 456.

The tests for androgenicity/anti-androgenicity gave some conflicting results. While the ToxCast data indicate binding properties of triticonazole to chimpanzee and human androgen receptor with an IC50 of 0.68 and 0.91 µM respectively, the assays indicative for a protein stabilization were positive only at or above cytotoxic concentrations. There are two further androgen receptor reporter gene assays available. One in human breast cancer cell line T47D-ARE transfected with a firefly luciferase reporter gene (Roelofs et al., 2014) and one in yeast strain PGKhAR containing a gene for the human androgen receptor and an androgen responsive element of the reporter gene lacZ (Woitkowiak, 2012). Triticonazole showed a decrease in the androgen receptor activation with an IC_{50} of 1.07E^{-05} M in the T47D-ARE and was inactive in the YAS assay, tested up to 10^{-04} M. In a further published test system, a concentration of 10 µM triticonazole led to decreases in testosterone secretion in murine MA-10 cells (tumorigenic Leydig cell line) (Roelofs et al., 2014). No data on cytotoxicity of triticonazole were presented in this publication, while from the ToxCast data a cytotoxicity of 2.27 µM has been determined for triticonazole. Further, the only validated test system is the YAS assay, as the ToxCAST data are neither peer-reviewed, nor were the results finally interpreted, as the ToxCAST database is still under development. Summarizing the evidence for an (anti)-androgenic mode of action of triticonazole, there is an indication in the ToxCAST database that triticonazole has binding properties to the chimpanzee and human androgen receptor. All other assays were either negative or indicated *in vitro* activity only at very high and/or cytotoxic concentrations. Thus there is no evidence that a specific anti-androgenic mode of action has contributed to the observed reproduction effects in the 2-Generation toxicity study, which can be well explained by general systemic toxicity. This is further supported by the fact that no weight or histopathological changes in rat

reproductive organs were seen in none of the studies. Reproductive organ weight changes in rodents are known to be a sensitive endpoint for substances with an anti-androgenic mode of action.

Rat 2-gen study ([redacted] 1993)

Anogenital distance and anogenital index: not investigated

Areola / nipple retention: not investigated

Differential ovarian follicle count: not investigated

Oestrous cycle length / duration: not investigated

Gestation length: The mean duration of gestation was similar in all test groups (in F_0 dams between 22.1 and 22.6* [$p \leq 0.05$] days and in F_1 dams between 22.1 and 22.2 days). The statistical significant difference for the high-dose F_0 dams is not considered to be an effect of the test substance, since in the F_1 dams the same mean values were estimated but no statistical significance achieved. The average difference is half a day and for F_0 dams, although statistically significant, the mean value at 5000 ppm is within HCD (21.8 – 22.7 days).

Parturition difficulties: There was one high-dose F_0 female which had to be sacrificed after prolonged parturition (gestation day 25). Another female had surviving pups at gestation day 24. Neither in the entire F_1 generation nor in the other dams of the F_0 generation high-dose group findings indicative of a general delay of parturition or any parturition complications were observed, nor were there any indications of prenatal developmental toxicity in late gestation.

Post-implantation loss: not investigated

Pup sexual development (vaginal opening / preputial separation): not investigated

Pup weight and pup weight development: Decreased pup weights at 5000 ppm were observed in F_0 from PND 7 (approximately 88% of control for both males and females) until weaning on PND 21 (approximately 71% of control for both males and females). In F_1 generation, decreased body weight was measured from birth (approximately 90% of control for both males and females) to PND 21 (approximately 50% of control for both males and females). Livebirth index and viability index were statistically significantly decreased in both F_0 and F_1 generation.

Pup necropsy findings: No treatment-related findings observed

Reproductive organs: In F_1 females at 5000 ppm vacuoles and giant cells were present in the ovaries in four females. No similar finding was observed in F_0 generation. No other findings in reproductive organs were reported.

Sex ratio: The sex distribution and sex ratios of live F_1 or F_2 pups on the day of birth did not show substantial differences between controls and treated groups.

Sperm parameters: not investigated

Time-to-mating: The mean duration until sperm was detected (GD 0) did not show any test-substance related effect. In F_0 matings, the duration varied between 2.12 and 3.32 days, in F_1 matings between 2.54 and 3.38, in both cases without any relation to dosing.

Oral prenatal developmental toxicity study in rats

Post-implantation loss: No test-substance related and/or biologically relevant differences between control and test groups up to maternally toxic dose levels (1000 mg/kg bw per day, HDT). The mean number of live fetuses/litter in control group and high-dose group was almost identical.

Foetal weights: Mean females and males fetal weights were almost identical in control and high dose group.

Placenta effects: Mean placenta weights were same in the control and high dose group (0.56 g).

Gender-specific findings: The sex distribution of the foetuses in treatment and control groups was comparable.

Evidence of teratogenicity: There was no evidence of teratogenicity up to the highest dose level tested.

Oral prenatal developmental toxicity study in rabbits

Post-implantation loss: Higher post-implantation loss and resorptions were measured in the highest dose tested (75 mg/kg bw per day). At this dose excessive maternal toxicity was observed (30% mortality).

Foetal weights: The mean fetal weights of survived dams were not influenced by the test substance and did not show any biologically relevant differences in comparison to the control group.

Placenta effects: Mean placenta weights were not significantly changed by treatment (control: 5.1 g, 75 mg/kg bw per day: 5.2 g).

Gender-specific findings: The sex distribution of the foetuses in treatment and control groups was comparable.

Evidence of teratogenicity: There was no evidence of teratogenicity up to the highest dose level tested.

B.6.8.4.6.2. Effects on carcinogenicity

The only observation in rats in long-term study was an increased incidence of benign pituitary adenoma and benign follicular cell adenomas in thyroids of males treated at 5000 ppm. Benign follicular cell adenomas in thyroid were considered very unlikely to be treatment-related because:

- Tumour type and background incidence: The incidence of thyroid follicular adenomas in male rats was only slightly above the historical control range and no statistical significance (not statistically significantly different to control, no positive trend in the trend-test) or dose-response was observed
- Multi-site responses: In no organ the effects were considered treatment-related, so no multi-site response was observed.
- Progression of lesions to malignancy: Only benign tumours were observed.

- Whether responses are in a single or in both sexes: Only males of the high dose were affected slightly above HCD
- Whether responses are in single species or several species: Increased incidence of thyroid follicular cell adenoma was observed only in male rats but not in female rats or mice
- Pattern from HCD (higher incidence in males than in females) was reflected in the study results
- Thyroid was not the target organ of triticonazole in any of the submitted studies
- No increase in precursor leasions, such as follicular cell hypertrophy was observed
- No effects on thyroid hormone receptors and no inhibition of TPO or deiodinase type 1 enzyme activity was observed in the US EPA ToxCast screening programme

No effects on weight of pituitary were observed in rat carcinogenicity study. It was stated in the report that the statistical significance for benign pituitary adenoma is coincidental due to an unusually low incidence in control animals. Historical control data (studies from the same laboratory, studies conducted from 1991 to 1993) concerning this type of tumour have been submitted. The incidence in males receiving 5000 ppm (58 %) is within the historical range of this tumour for this strain of male rats at this laboratory when full length studies (104 weeks) were considered (30.7 – 62 %), but slightly outside the historical range of 36.7 – 54.5 % for abbreviated studies (90 – 93 weeks). However, the incidences of this finding in the intermediate dose groups showed no indication of any relationship to dose. Therefore, the slightly higher incidence of this benign tumour, which is well known as a spontaneous age-related lesion in rats, was not considered to be of biological relevance. Among animals which died or were killed during the treatment period there was also a statistically significantly higher incidence of benign pituitary adenomas among females treated at 750 ppm, but it was suggested in the study report that the lack of dosage-relationship and the lower incidence of histogenically related carcinomas among these animals indicate this finding also does not have biological significance.

All other tumours were of the types commonly seen in CD rats and occurred with the expected frequency. It is noteworthy to mention that neither the evidence for treatment-related adrenal cortical adenocarcinoma (males: 0/50, 0/35, 0/42, 0/40, 0/50; females: 1/50, 1/46, 0/48, 1/46, 0/50), adrenal cortical adenoma (males: 0/50, 1/35, 0/42, 0/40, 1/50; females: 3/50, 1/46, 0/48, 0/46, 3/50) nor for adrenal medullary phaeochromocytoma (males: 5/50, 6/35, 5/42, 3/40, 4/50; females: 1/50, 2/46, 0/48, 0/46, 1/50) was observed.

No neoplastic findings related to treatment were observed in chronic study in mice.

B.6.8.4.6.3. Effects on adrenals

There was no evidence of a specific endocrine-related effect in any of the short-term or long-term toxicity studies with triticonazole, including each lifetime carcinogenicity bioassay conducted in rats and mice. Morphological effects on adrenals, observed in almost all studies and in every species (rat, mouse and dog), were mostly confined to *zona fasciculata* but were partially differently diagnosed in different species and even between the sexes. It was evident that the findings in females – as well as in males - decreased in incidence and severity with exposure duration (F_1 females were less affected than F_0 females) and were of questionable

significance at the terminal sacrifice of the 2-year rat study. No increased incidences for adrenal adenoma or carcinoma or phaochromocytoma were observed. With regard to sex differences in rat adrenal findings, the evaluation of the rat metabolism study suggests that female adrenals are internally slightly higher exposed to triticonazole and metabolites compared to males, as the mean adrenal/plasma ratios were 0.68 (± 0.07) for males and 2.60 (± 0.85) for females respectively in the repeated low dose group. There is no qualitative difference between male and female metabolism in rats, however metabolite M595F006 occurred at 2.18 % administered dose in males and in 11.23 % administered dose in female rats after repeated low dose exposure. All other metabolites were qualitatively and quantitatively similar between male and female rats.

In order to identify if these morphological changes in *zona fasciculata* also lead to functional impairment of adrenals, especially regarding corticosterone production, a 90-days ACTH challenge assay in rats was initiated. In the ACTH assay no impairment of corticosterone excretion after ACTH challenge was measured although morphological changes in adrenals (vacuolation in *zona fasciculata* in males and degeneration/regeneration in *zona fasciculata/regularis* in females) were observed in all animals treated with 5000 ppm. However, these morphological changes were not an evidence of marked ACTH overstimulation as this would inevitably results in adrenal hypertrophy and frank increases in gland size and weight, which was not observed. The lack of large increases in adrenal weight/hypertrophy supports the thesis that an adequate glucocorticoid competency remained.

Also the steroidogenesis assay conducted under ToxCast program (Karmaus et al., 2016) did not give evidence for a blockage of steroidogenesis or glucocorticoid production. There was some evidence for decreased cortisol levels in the H295R cell line with an AC_{50} of 4.48 µM, however the decreased levels are only seen at and above cytotoxic concentrations of triticonazole (2.27 µM), diminishing the specificity and relevance of this finding. A *decrease* in a hormone signal is plausibly explainable by systemic cell toxicity, especially if an activity is only seen at cytotoxic concentrations. Further no changes are seen for any of the androgenic or estrogenic hormones in this very sensitive steroidogenesis assay.

While there is no evidence for blockage of steroidogenesis and glucocorticoid production as a possible mode of action of triticonazole, the most likely explanation for adrenal toxicity is direct cytotoxicity, being somehow less pronounced in studies of longer duration.

Adrenal gland

Rat 28-day study: no effects on adrenals observed

Mouse 6 weeks study: no effects on adrenals observed

Dog 28-day study: no effects on adrenals observed

Rat 90-day study: no change in adrenal weight; histopathological changes at 12500 ppm and 25000 ppm in males (cortical droplet vacuolation in *zona fasciculata*) and females (cortical droplet vacuolation in *zona fasciculata* and degeneration of *zona reticularis*)

Rat 90-day ACTH challenge study: At 5000 ppm (highest dose tested) all males showed vacuolation in *zona fasciculata* and all females showed degeneration/regeneration in *zona fasciculata/reticularis*. After a successful

ACTH challenge, however, animals were able to excrete corticosterone. Adrenals did not show signs of functional impairment.

Mouse 90-day study: not investigated

Dog 1-year study: At 150 ppm, adrenal to body weight ration showed 141.8% increase in adrenal weight in males and 133.8% in females. Adrenals to brain weight ratio showed 146.1% increase in males and 97.5% in females, compared to control animals. At 25 and 150 ppm vacuolation of *zona fasciculata*, of minimal to moderate grade was observed in all animals.

Rat 2-gen study: In males of F_1 generation relative left and right adrenal weight was increased (115% and 116% respectively) at the top dose of 5000 ppm while in females of both F_0 and F_1 generation relative left adrenal weight was decreased (80 and 81% of control respectively) at this dose. In males of F_0 generation no changes in adrenal weights were observed. In females of F_0 generation no changes in relative right adrenal weight was observed. In almost all males (F_0 and F_1 generation) at 5000 ppm vacuolation of adrenal cortex was observed. In females, degeneration of adrenal cortex, giant cells and cortical pigment were observed at higher incidences in both F_0 and F_1 generation at 5000 ppm.

Rat 2-year study, 26 weeks: No changes in adrenal weights were observed. A higher incidence of multinucleated cells in the *zona fasciculata* was noted in 9/15 females of 5000 ppm group after 26 weeks of treatment (compared to 0/15 in controls). During the re-evaluation of slides also degeneration of the junction between *reticularis* and *fasciculata* and minimal mononuclear inflammatory cell infiltrates, increased pigment deposition and minimal or mild hypertrophy of the *zona reticularis* were observed in 9/15 females of 5000 ppm group Additionally, there was a higher incidence of cortical fatty vacuolation in males after 26 weeks of treatment, although statistical significance was not achieved. Re-evaluation of slides did not reveal any new findings in males.

Rat 2-year study, 52 weeks: No changes in adrenal weights were observed. After 53 weeks of treatment, a higher incidence of multinucleated cells in the *zona fasciculata* was noted in 3/14 females and chronic inflammation was observed in the *zona fasciculata* of 4/14 females receiving 5000 ppm (compared to 0/15 in the control group). During the re-evaluation of slides minimal or mild hypertrophy of the *zona reticularis* was additionally assigned to 10/14 females at 5000 ppm. 3/14 females also revealed minimal degeneration of the junction between *reticularis* and *fasciculata.* In males after 53 weeks 3/14 animals at 5000 ppm revealed cortical fatty vacuolation of adrenals. Re-evaluation of slides did not reveal any new findings in males.

Rat 2-year study, 104-weeks: No changes in adrenal weights were observed. Adrenal findings in general almost disappeared and in males only cortical fatty vacuolation was observed however, without relevant dose response (22%, 28.6%, 16.7%, 25% and 26%). In females, adrenal findings from 26- and 53-weeks observation period were not observed at the end of the study. Only cortical fatty vacuolation (11/50) and multinucleated cells (3/50) were observed at 5000 ppm, cortical cell vacuolation however being observed also at lower doses.

Mouse 18-month carcinogenicity, 26 weeks: After 26 weeks treatment in mice, relative adrenals weight was increased in males at 150 and 1500 ppm (118% and 174%, respectively). In females at 1500 ppm relative adrenal weight was 130% of control. No histopathological findings were observed.

Mouse 18-month carcinogenicity, 78 weeks: After 78 weeks treatment in mice, relative adrenals weight was decreased in males at 15, 150 and 1500 ppm (73%, 78% and 76%, respectively). In females at 1500 ppm relative adrenal weight was 119% of control. No histopathological findings were observed.

B.6.8.4.6.4. Effects on other endocrine organs

Epididymides

Rat 28-day study: Epididymides not investigated

Mouse 6 weeks study: Epididymides not investigated

Dog 28-day study: Epididymides not weighted. No changes in histopathology of epididymides observed.

Rat 90-day study: Epididymides not weighted. No changes in histopathology of epididymides observed.

Rat 90-day ACTH challenge study: Epididymides not investigated

Mouse 90-day study: Epididymides not investigated

Dog 1-year: Epididymides not weighted. No changes in histopathology of epididymides observed.

Rat 2-gen study: No effects on weight of epididymides observed. No changes in histopathology of epididymides observed.

Rat 2-year study, 26 weeks: Epididymides not weighted. No changes in histopathology of epididymides observed.

Rat 2-year study, 56 weeks: Epididymides not weighted. No changes in histopathology of epididymides observed.

Rat 2-year study, 104 weeks: Epididymides not weighted. No changes in histopathology of epididymides observed.

Mouse 18-month carcinogenicity, 26 weeks: Epididymides not weighted. No changes in histopathology of epididymides observed.

Mouse 18-month carcinogenicity, 78 weeks: Epididymides not weighted. No changes in histopathology of epididymides observed.

Ovaries

Rat 28-day study: No effects on weight of ovaries observed. No changes in histopathology of ovaries observed.

Mouse 6 weeks study: Ovaries not investigated

Dog 28-day study: No effects on weight of ovaries observed. No histopathological effects in ovaries observed.

Rat 90-day study: Ovaries to body weight ratio was increased at 12500 (161%) and 25000 ppm (157.3%). No histopathological effects in ovaries observed.

Rat 90-day ACTH challenge study: Ovaries not investigated

Mouse 90-day study: Ovaries not investigated

Dog 1-year: No effects on weight of ovaries observed. No histopathological effects in ovaries observed.

Rat 2-gen study: No effects on weight of ovaries observed. At 5000 ppm vacuolation in ovaries in F_1 generation (4/28) was observed. Also slightly higher incidence at 5000 ppm in giant cell formation in both F_0 (1/24) and F_1 (2/28) generation was observed.

Rat 2-year study, 26 weeks: No effects on weight of ovaries observed. No histopathological effects in ovaries observed

Rat 2-year study, 56 weeks: No effects on weight of ovaries observed. No histopathological effects in ovaries observed

Rat 2-year study, 104 weeks: No effects on weight of ovaries observed. No histopathological effects in ovaries observed

Mouse 18-month carcinogenicity, 26 weeks: Ovaries not weighted. No histopathological effects in ovaries observed.

Mouse 18-month carcinogenicity, 78 weeks: Ovaries not weighted. No histopathological effects in ovaries observed.

Pituitary gland

Rat 28-day study: No effects on weight of pituitary observed. No histopathological examination on pituitary conducted

Mouse 6 weeks study: Pituitary not investigated

Dog 28-day study: No effects on weight of pituitary observed. No histopathological effects in pituitary observed.

Rat 90-day study: No effects on weight of pituitary observed. No histopathological effects in pituitary observed.

Rat 90-day ACTH challenge study: Pituitary not investigated

Mouse 90-day study: Pituitary not investigated

Dog 1-year: No effects on weight of pituitary observed. No histopathological effects in pituitary observed.

Rat 2-gen study: No effects on weight of pituitary observed. No histopathological effects in pituitary observed.

Rat 2-year study, 26 weeks: No effects on weight of pituitary observed. No histopathological effects in pituitary observed.

Rat 2-year study, 56 weeks: No effects on weight of pituitary observed. No histopathological effects in pituitary observed.

Rat 2-year study, 104 weeks: Please see B.6.8.3.6.2.

Mouse 18-month carcinogenicity, 26 weeks: Pituitary not weighted. No histopathological effects in pituitary observed.

Mouse 18-month carcinogenicity, 78 weeks: Pituitary not weighted. No histopathological effects in pituitary observed.

Prostate

Rat 28-day study: The absolute prostate weights of males receiving 5000 ppm or more were lower than those of their respective controls; however the difference was significant for relative weight only in the highest dose tested. No histopathological effects in prostate observed

Mouse 6 weeks study: Prostate not investigated

Dog 28-day study: No effects on weight of prostate observed. No histopathological effects in prostate observed.

Rat 90-day study: No effects on weight of prostate observed. No histopathological effects in prostate observed.

Rat 90-day ACTH challenge study: Prostate not investigated

Mouse 90-day study: Prostate not investigated

Dog 1-year: The effects on the prostate weight in the high dose group (150 ppm) were not accompanied by any effects in the lower dosed animals. Based on marked manifestation the treatment related effect is questionable, however, no information in study report is given about incidents in analysis. No histopathological effects in prostate were observed.

Rat 2-gen study: No effects on weight of prostate observed. No histopathological effects in prostate observed.

Rat 2-year study, 26 weeks: No effects on weight of prostate observed. No histopathological effects in prostate observed

Rat 2-year study, 56 weeks: No effects on weight of prostate observed. No histopathological effects in prostate observed

Rat 2-year study, 104 weeks: No effects on weight of prostate observed. No histopathological effects in prostate observed

Mouse 18-month carcinogenicity, 26 weeks: Prostate not weighted. No histopathological effects in prostate observed.

Mouse 18-month carcinogenicity, 78 weeks: Prostate not weighted. No histopathological effects in prostate observed.

Seminal vesicle

Rat 28-day study: Seminal vesicles not investigated

Mouse 6 weeks study: Seminal vesicles not investigated

Dog 28-day study: Seminal vesicles not investigated

Rat 90-day study: Seminal vesicle weights were not determined. No histopathological effects in seminal vesicles observed.

Rat 90-day ACTH challenge study: Seminal vesicles not investigated

Mouse 90-day study: Seminal vesicles not investigated

Dog 1-year: Seminal vesicles not investigated

Rat 2-gen study: No effects on weight of seminal vesicles observed. No histopathological effects in seminal vesicles observed

Rat 2-year study, 26 weeks: Seminal vesicles not investigated

Rat 2-year study, 56 weeks: Seminal vesicles not investigated

Rat 2-year study, 104 weeks: Seminal vesicles not investigated

Mouse 18-month carcinogenicity, 26 weeks: Seminal vesicle weights were not determined. No histopathological effects in seminal vesicles observed.

Testes

Rat 28-day study: Absolute testes weights were significantly lower in males which received 50000 ppm. No histopathological effects in testes observed

Mouse 6 weeks study: No effects on weight of testes observed. No histopathological effects in testes observed

Dog 28-day study: No effects on weight of testes observed. No histopathological effects in testes observed

Rat 90-day study: No effects on weight of testes observed. No histopathological effects in testes observed

Rat 90-day ACTH challenge study: Testes not investigated

Mouse 90-day study: No effects on weight of testes observed. No histopathological investigation of testes conducted.

Dog 1-year: No effects on weight of testes observed. No histopathological effects in testes observed

Rat 2-gen study: No effects on weight of testes observed. No histopathological effects in testes observed

Rat 2-year study, 26 weeks: No effects on weight of testes observed. No histopathological effects in testes observed

Rat 2-year study, 56 weeks: No effects on weight of testes observed. No histopathological effects in testes observed

Rat 2-year study, 104 weeks: No effects on weight of testes observed. No histopathological effects in testes observed

Mouse 18-month carcinogenicity: No effects on weight of testes observed. No histopathological effects in testes observed

Mouse 18-month carcinogenicity, 78 weeks: No effects on weight of testes observed. No histopathological effects in testes observed

Thyroid

Rat 28-day study: No effects on weight of thyroid observed. No histopathological effects in thyroid observed

Mouse 6 weeks study: Thyroid weight not investigated. No histopathological effects in thyroid observed

Dog 28-day study: No effects on weight of thyroid observed. No histopathological effects in thyroid observed

Rat 90-day study: No effects on weight of thyroid observed. No histopathological effects in thyroid observed

Rat 90-day ACTH challenge study: Thyroid not investigated

Mouse 90-day study: Thyroid not investigated

Dog 1-year: No effects on weight of thyroid observed. No histopathological effects in thyroid observed

Rat 2-gen study: Thyroid not investigated

Rat 2-year study, 26 weeks: No effects on weight of thyroid observed. No histopathological effects in thyroid observed

Rat 2-year study, 56 weeks: No effects on weight of thyroid observed. No histopathological effects in thyroid observed

Rat 2-year study, 104 weeks: Please see B.6.8.4.6.2.

Mouse 18-month carcinogenicity, 26 weeks: Thyroid weight not investigated. No changes in histopathology of thyroid observed.

Mouse 18-month carcinogenicity, 78 weeks: Thyroid weight not investigated. No changes in histopathology of thyroid observed

Uterus

Rat 28-day study: No effects on weight of uterus observed. No histopathological effects in uterus observed

Mouse 6 weeks study: No effects on weight of uterus observed. No histopathological investigation of uterus conducted

Dog 28-day study: No effects on weight of uterus observed. No histopathological effects in uterus observed

Rat 90-day study: No effects on weight of uterus observed. No histopathological effects in uterus observed

Rat 90-day ACTH challenge study: Uterus not investigated

Mouse 90-day study: No effects on weight of uterus observed. No histopathological investigation of uterus conducted.

Dog 1-year: No effects on weight of uterus observed. No histopathological effects in uterus observed

Rat 2-gen study: No effects on weight of uterus observed. No histopathological effects in uterus observed

Rat 2-year study, 26 weeks: No effects on weight of uterus observed. No histopathological effects in uterus observed.

Rat 2-year study, 56 weeks: No effects on weight of uterus observed. No histopathological effects in uterus observed.

Rat 2-year study, 104 weeks: No effects on weight of uterus observed. No histopathological effects in uterus observed.

Mouse 18-month carcinogenicity, 26 weeks: No effects on weight of uterus observed. No histopathological effects in uterus observed.

Mouse 18-month carcinogenicity, 78 weeks: No effects on weight of uterus observed. No histopathological effects in uterus observed

Vagina

Rat 28-day study: Vagina not investigated

Mouse 6 weeks study: Vagina not investigated

Dog 28-day study: Vagina not investigated

Rat 90-day study: Vagina weight was not determined. No histopathological effects in vagina observed

Rat 90-day ACTH challenge study: Vagina not investigated

Mouse 90-day study: Vagina not investigated

Dog 1-year: Vagina weight was not determined. No histopathological effects in vagina observed

Rat 2-gen study: No effects on weight of vagina observed. No histopathological effects in vagina observed

Rat 2-year study, 26 weeks: Vagina weight was not determined. No histopathological effects in vagina observed

Rat 2-year study, 56 weeks: Vagina weight was not determined. No histopathological effects in vagina observed

Rat 2-year study, 104 weeks: Vagina weight was not determined. No histopathological effects in vagina observed

Mouse 18-month carcinogenicity, 26 weeks: Vagina weight was not determined. No histopathological effects in vagina observed

Mouse 18-month carcinogenicity, 78 weeks: Vagina weight was not determined. No histopathological effects in vagina observed

Conclusion

In summary, based on the results of *in vivo* tests conducted with triticonazole, there is no evidence of a specific effect on the endocrine system or on any endocrine organ, with a demonstrated endocrine MoA. Triticonazole has been shown to inhibit the aromatase enzyme *in vitro* like other members of the azole class of fungicides, with 20-times lower IC for rat than for human aromatase and many orders of magnitude below the reference substances, even the reference substance with the lowest potency. This low *in vitro* activity did not translate into any specific endocrine-related effect *in vivo*. This observation is supported by the lack of treatment-related carcinogenic effects in two lifetime cancer bioassays conducted in rats and mice, as well as the absence of specific reproductive or developmental toxicity in a 2-generation reproduction study and two developmental toxicity studies. The observed morphological changes in adrenals in all species and almost all studies, always accompanied by marked general toxicity, did not prove to impair the functional capacity of adrenals since corticosterone was successfully excreted after ACTH challenge. It is concluded that no evidence is available that effects observed in studies with triticonazole have an endocrine MoA.

B.6.9. MEDICAL DATA AND INFORMATION

B.6.9.1. Medical surveillance on manufacturing plant personnel and monitoring studies

All persons handling crop protection products are surveyed by regular medical examinations. There are no specific parameters available for effect monitoring on triticonazole. Thus, the medical monitoring programme is designed as a general health check-up, with special interest in the primary target organs presumed to be relevant by analogy from animal experiments. The surveillance program includes a general physical examination including neurological status, red and white blood cell counts, liver enzymes. Adverse health effects suspected to be related to triticonazole exposure have not been observed.

B.6.9.2. Data collected on humans

No human cases of intoxication or poisoning deriving from triticonazole are known to BASF SE.

B.6.9.3. Direct observation

Neither data on exposure of the general public nor epidemiologic studies on triticonazole are available for BASF SE, nor is BASF SE aware on any epidemiologic studies performed by third parties.

B.6.9.4. Epidemiological studies

See above

B.6.9.5. Diagnosis of poisoning (determination of active substance, metabolites), specific signs of poisoning, clinical test

Not known (see below).

B.6.9.6. Proposed treatment: first aid measures, antidotes, medical treatment

See safety data sheet. No specific antidote known.

The acute toxicity of triticonazole is very low with LD_{50} values after oral and dermal exposure of > 2000 mg/kg bw. The LC_{50} value was greater than 5.61 mg/L, also indicating very low acute toxicity. In addition, non-specific clinical signs were observed following acute or subchronic and chronic exposure. Under these prerequisites, no specific clinical signs are expected from acute or accidental exposure to humans.

B.6.10. REFERENCES RELIED ON

A literature search on triticonazole and the common product trade names was performed by the BASF Group Information Center. Two databases (ToxCenter and Embase) were searched for mammalian toxicology and four databases (CARPLUS, BIOSIS, Medline and Embase) for Mode of action hits.

The first step of the search result processing based on summary records involved the separation into "hits" and "ballast" (obviously irrelevant records). The “ballast” was not further processed.

The “hits” were further evaluated by the scientific experts and categorized into “not relevant”, “not reliable”, and “used for dossier”.

Duplicates of search results from different databases in a respective section were removed in STN databases by the “*duplicate remove*” command.

The search process is documented in all details with search profiles, search histories and summary tables according the GUIDANCE OF EFSA, Submission of scientific peer-reviewed open literature for the approval of pesticide active substances under Regulation (EC) No 1107/2009, EFSA Journal 2011;9(2):2092.

The process of selection of relevant scientific peer-reviewed open literature was done in two steps:

The *First Selection step* for relevance based on summary records (e.g. titles, abstracts, index terms, keywords) was done by the Agro Information Professionals.

Obviously irrelevant records were tagged as “Ballast”. This ballast was controlled by scientific experts in the corresponding subject areas but was not further processed.

- Summary records which appear to be relevant and those of unclear relevance were tagged as “Hit” and went to the next level of evaluation.

The *Second Detailed Assessment* was done by the scientific experts in the corresponding areas.

Records tagged as “Hit” were further evaluated in depth.

To facilitate a comprehensible listing of the "Hits" in the different regulatory areas an Excel file was generated for each section with 3 typical registers, namely:

- "no relevant endpoint"
- "evaluated - not-relevant"
- "used for dossier"

In a first step (rapid assessment) the "Hits" were reviewed based on the information given in the **title and the abstract** with regard to relevance for the regulatory endpoints in the respective regulatory area. Those records which were clearly judged as not assignable to any regulatory endpoint were shifted into the register **"no relevant endpoint"** with an explaining reasoning.

In a second step (detailed assessment), all remaining records were assessed in detail based on the **complete report** by the respective expert(s) and separated into relevant reports for further discussion and those clearly not relevant.

Criteria to assign a record to the register **"evaluated - not-relevant"** were:

- Those records which provided information supporting the existing regulatory data package without any new relevant data or information were classified as "confirmatory data"
- Those records which were not assignable to the substance of interest (for example mixtures, not about test substance or other relevant substance)
- Secondary literature linking to primary literature already discussed under relevant records
- and those which were judged as not relevant due to other reasons with a respective justification.

Criteria to assign a record to the register **"used for dossier"** were:

- Records providing information about additional/new/unknown/potentially contradictory effects or data which might impact the hazard assessment endpoints or the risk assessments parameters and which in addition have a high grade of reliability of grade 1 or 2 based on the 'Klimisch' scoring system (see below).

Those records assigned to the category "used for dossier" were provided with a Doc ID and discussed in detail in the respective dossier chapter.

Reliability scoring system based on Klimisch et al., 1997:

Reliability 1: **reliable without restrictions:** studies or data generated according to generally valid and/or internationally accepted testing guidelines (preferably performed according to GLP) or in which the test parameters documented are based on a specific (national) testing guideline or in which all parameters described are closely related/comparable to a guideline method. (e.g. literature about toxicity / ecotoxicity study consistent with requests of international testing guidelines and performed under GLP conditions with experienced and trained personal)

Reliability 2: **reliable with restrictions:** studies or data (mostly not performed according to GLP), in which the test parameters documented do not totally comply with the specific testing guideline, but are sufficient to accept the data or in which investigations are described which cannot be

subsumed under a testing guideline, but which are nevertheless well documented and scientifically acceptable (appropriately documented studies which meets basic scientific principles, mechanistic studies)

Reliability 3: **not reliable:** studies or data in which there were interferences between the measuring system and the test substance or in which organisms/test systems were used which are not relevant in relation to the exposure (e.g. unphysiologic pathways of application) or which were carried out or generated according to a method which is not acceptable, the documentation of which is not sufficient for assessment and which is not convincing for an expert judgement (e.g. literature studies with insufficient information or according to unvalidated method)

Reliability 4: **not assignable:** studies or data which do not give sufficient experimental details and which are only listed in short abstracts or secondary literature

The RMS concluded that appropriate time frame, databases, (extensive) keywords and evaluation criteria were applied, all this according to EFSA Guidance on submission of scientific peer-reviewed open literature. For the articles which were assigned as relevant and reliable the notifier provided in-depth robust study summaries. The evaluations are included in the respective chapters.

Data Point	Author(s)	Year	Title Company Report No. Source (where different from company) GLP or GEP status Published or not	Vertebrate study Y/N	Data protection claimed Y/N	Justification if data protection is claimed	Owner	Previous evaluation
KCA 5.1	[redacted]	1993	RPA 400727: Preliminary ADME study in the rat [redacted] Report No.: C018956 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.1	[redacted]	1993	RPA 400727: ADME study in the rat [redacted] Report No.: R013078 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.1	[redacted]	2000	Triticonazole: Rat bile excretion study [redacted] Report No.: R012111 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.1	[redacted]	2002	Triticonazole: Choice of radiolabel used in the rat ADME study [redacted] Report No.: not given GLP no unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.1	[redacted]	2002	Triticonazole: Quantification of faecal metabolites in the rat ADME report - Addendum to Report R013078 [redacted] Report No. not given GLP no unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.1	Mazur C.S., Kenneke, J.F.	2008	Cross-Species Comparison of Conazole fungicide metabolites using rat and rainbow trout (Onchorhynchus mykiss) hepatic microsomes and purified human CYP 3A4	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.1	Thibaut, R.	2016	[14C]BAS 595 F – In Vitro Comparative Metabolism in Human, Rat, Dog and Rabbit Liver Microsomes Innovative Environmental Services (IES) Ltd Report No.: 2016/1052691 GLP Unpublished	N	Y	New study necessary according to new data requirements	BASF	Submitted for the purpose of renewal (2015)
KCA 5.2.1	[redacted]	1990	RPA 400727: Acute oral toxicity study in the rat [redacted] Report No.: R013003 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.2.2	[redacted].	1991	RPA 400727: Acute percutaneous toxicity study in the rat [redacted] Report No.: R013017 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.3	[redacted]	1998	Triticonazole: Acute inhalation toxicity study in rats [redacted] Report No.: C014044 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.3	[redacted]	1998	Triticonazole: Acute inhalation toxicity study in rats [redacted] Report No.: C014043 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.3	[redacted]	1991	RPA 400727: Acute inhalation toxicity study in the rat Life Science [redacted] Report No.: R013028 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.2.4	[redacted]	1991	RPA 400727: Acute dermal irritation/corrosin test in the rabbit [redacted] Report No.: R013022 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.5	[redacted]	1997	Triticonazole: Acute eye irritation test in the rabbit [redacted] Report No.: R012105 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.5	[redacted]	1991	RPA 400727: Acute eye irritation test in the rabbit [redacted] Report No.: R013018 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.6	[redacted]	2006	BAS 595 F - BUEHLER test in guinea pigs 2006/1001981 [redacted] GLP Unpublished	Y	Y	New data for the renewal ; no additional added value derives from the study	BASF	Submitted for the purpose of renewal (2015)
KCA 5.2.6	[redacted]	1993	RPA 400727: Delayed contact hypersensitivity study in Guinea pigs [redacted] Report No.: R013081 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.2.6	[redacted]	1992	RPA 400727: Delayed contact hypersensitivity study in Guinea pigs [redacted] Report No.: R013063 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.2.7	Cetto V., Landsiedel R.	2013	BAS 595 F (Triticonazole) - In vitro 3T3 NRU phototoxicity test BASF SE, Ludwigshafen/Rhein, Germany Fed.Rep. Report No.: 2013/1089154 GLP unpublished	N	Y	New study necessary according to new data requirements	BASF	Submitted for the purpose of renewal (2015)
KCA 5.3.1	[redacted]	1991	RPA 400727: Preliminary toxicity study by dietary administration to F-344 rats for 4 weeks [redacted] Report No.: R013012 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.3.1	[redacted]	1991	RPA 400727: Preliminary toxicity study by dietary administration to CD-1 mice for 6 weeks [redacted] Report No.: C019001 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.3.1	[redacted]	1991	RPA 400727: Toxicity study by dietary administration to CD rats for 13 weeks Life Science [redacted] Report No.: R013029 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.3.1	[redacted]	1991	RPA 400727: Preliminary toxicity study by oral (capsule) administration to Beagle dogs for four weeks [redacted] Report No.: R013014 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.3.1	[redacted]	1993	RPA 400727: Toxicity study by oral (capsule) administration to Beagle dogs for 52 weeks [redacted] Report No.: R000173 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.3.1	[redacted]	1991	RPA 400727: Preliminary toxicity study by dietary administration to CD-1 mice for 13 weeks [redacted] Report No.: R013027 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.3.1	[redacted]	2000	Toxicity study by dietary administration to CD rats for 13 weeks – Bridging report between LSR 91/RHA 429/0783 and Project K00/013: Examination of adrenal cortex sections from a 13 week toxicity study in rats by [redacted] Report No. C014049 GLP no unpublished	N	N	-	BASF	DAR (2003)
KCA 5.3.1	[redacted]	2000	Examination of adrenal cortex sections from a 13-week oral toxicity study in rats [redacted] Report No.: C014047 GLP no unpublished	N	N	-	BASF	DAR (2003)

KCA 5.3.1 5.5 5.6 5.8.3	Millar P.M		Expert opinion on the histopathology of the adrenal glands from animals dosed with triticonazole BASF Document No.: 2015/1197310 GLP: no unpublished	N	N	Re-examination provided for the purpose of renewal	BASF	Submitted for the purpose of renewal (2015)
KCA 5.3.1	[redacted]	1997	3-Weeks dermal toxicity study with triticonazole in rats Corning Hazleton Inc.; USA [redacted] Report No.: R012966 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.4.1	Dance C. A.	1992	In vitro assessment of the clastogenic activity of RPA 400727 in cultured human lymphocytes Life Science Research Ltd., England Rhône Poulenc Agrochimie, France Report No.: R013062 GLP unpublished	N	N	-	BASF	DAR (2003)
KCA 5.4.1	Foster, B.	1992	RPA 400727: Induction of unscheduled DNA synthesis (UDS) in rat hepatocytes in vitro Life Science Research Ltd., England Rhône Poulenc Agrochimie, France Report No.: R013061 GLP unpublished	N	N	-	BASF	DAR (2003)

KCA 5.4.1	Lloyd, J. M.	1991	RPA 400727: Investigation of mutagenic activity at the HGPRT locus in an Chinese Hamster V79 cell mutation systeme Life Science Research Ltd., England Rhône Poulenc Agrochimie, France Report No.: R013019 GLP unpublished	N	N	-	BASF	DAR (2003)
KCA 5.4.1	Marshall R.	1997	Triticonazole: Induction of chromosome aberration in cultured human peripheral blood lymphocytes Corning Hazleton, England Rhône Poulenc Agrochimie, France Report No.: R012107 GLP unpublished	N	N	-	BASF	DAR (2003)
KCA 5.4.1	May, K.	1991	RPA 400727: Assessment of mutagenic potential in histidine auxotrophs of *Salmonella typhimurium* (Ames Test) Life Science Research Ltd., England Rhône Poulenc Agrochimie, France Report No.: R013016 GLP unpublished	N	N	-	BASF	DAR (2003)
KCA 5.4.1	Woitkowiak C.	2014	BAS 595 F (Triticonazole) - Salmonella typhimurium / Escherichia coli reverse mutation assay 2014/1192479 BASF SE, Ludwigshafen/Rhein, Germany Fed.Rep. GLP Unpublished	N	Y	New study necessary; older AMES test only partially covering the necessary strains	BASF	Submitted for the purpose of renewal (2015)

KCA 5.4.2	[redacted]	1992	RPA 400727: Assessment of clastogenic action on bone marrow erythrocytes in the Micronucleus Test [redacted] Report No. R012061 GLP unpublished	N	N	-	BASF	DAR (2003)
KCA 5.4.4	Knight A.W.	2009	Evaluation of high-throughput genotoxicity assays used in profiling the US EPA ToxCast chemicals 2009/1130462 GLP no Published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.5	[redacted]	1994	PRA 400727: Combined oncogenicity and long-term toxicity study by dietary administration to CD rats [redacted] Report No. R013100 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.5	Bouvier G.	1998	Triticonazole: Comments on ophthalmic observations in the 2 year combined oncogenicity and long-term toxicity rat study Rhône-Poulenc Secteur Agro, France Report No. C019586 GLP no unpublished	N	N	-	BASF	DAR (2003)

KCA 5.5	[redacted]	1994	RPA 400727: Oncogenicity study by dietary administration to CD-1 mice for 78 weeks [redacted] Report No. R013143 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.5	[redacted]	2002	Triticonazole: Statistical analysis of ophthalmologic lesions in the 2 year combined oncogenicity and long-term toxicity rat study [redacted] Report No. C028256 GLP no unpublished	N	N	-	BASF	DAR (2003)
KCA 5.5	Renault D.	2002	Triticonazole Position Paper: Combined oncogenicity and long-term toxicity study by dietary administration to CD rats - Historical control data for ophthalmic lesion Bayer Crop Science, France Report No. C028263 GLP no unpublished	N	N	-	BASF	DAR (2003)
KCA 5.5	Renault D.	2002	Triticonazole Position Paper: Combined oncogenicity and long-term toxicity study by dietary administration to CD rats - Historical control data for pituitary benign adenoma Bayer Crop Science, France Report No. C028249 GLP no unpublished	N	N	-	BASF	DAR (2003)

KCA 5.6.1	[redacted]	1993	Two-generation reproduction study with RPA400727 in rats [redacted] Report No. R013085 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.6.1	[redacted]	1999	Triticonazole Position Paper: Reproductive toxicity evaluation [redacted] Report No. C027013 GLP no unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.6.2	[redacted]	1990	RPA 400727: Tolerance study in the rabbit Report No. C044414 GLP unpublished	Y	N	-	BASF	No previuos evaluation, only supplementary
KCA 5.6.2	[redacted]	1990	RPA 400727: Preliminary teratology study in the rabbit Report No. C019984 GLP unpublished	Y	N	-	BASF	No previuos evaluation, only supplementary
KCA 5.6.2	[redacted]	1991	RPA 400727: Teratology study in the rat Report No. C018955 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.6.2	[redacted]	1991	RPA 400727: Teratology study in the rabbit Report No C018959 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.6.2	Hermsen S.A.B. et al	2011	Relative embryotoxicity of two classes of chemicals in a modified zebrafish embryotoxicity test and comparison with their in vivo potencies Report No 2011/1297791 GLP no published	Y	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.6.2	Hermsen S.A.B. et al.,	2012	Triazole-induced gene expression changes in the zebrafish embryo Report No 2012/1369002 GLP no unpublished	Y	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.6.2	[redacted]	1990	RPA 400727: Preliminary teratology study in the rat Report No. C018955 / GLP unpublished	Y	Y	-	BASF	Submitted for the purpose of renewal (2015)
KCA 5.6.2	Jong E. de et al.,	2011	Comparison of the mouse embryonic stem cell test, the rat whole embryo culture and the zebrafish embryotoxicity test as alternative methods for developmental toxicity testing of six 1,2,4-triazoles Report No : 2011/1297792 GLP no published	Y	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.6.2	[redacted]	2010	BAS 595 F (Triticonazole) - Chicken embryotoxicity screening test (CHEST) Report No 2010/1177161 GLP no unpublished	Y	Y	-	BASF	Submitted for the purpose of renewal (2015)
KCA 5.6.2	Padilla S. et al.,	2011	Zebrafish developmental screening of the ToxCast Phase I chemical library Report No : 2012/1368722 GLP no published	Y	N	-	LIT	Submitted for the purpose of renewal (2015)

KCA 5.7	[redacted]	1997	Benchmark and time-to.peak effect neurotoxicity study with triticonazole in rats [redacted] Report No.: R012965 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.7	[redacted]	1997	Acute neurotoxicity study with triticonazole in rats [redacted] Report No.: R012968 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.7	[redacted]	1997	13-week dietary neurotoxicity study with triticonazole in rats [redacted] Report No.: R012967 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.8.1	Chang	2016	Reg.No. 5079359 (Metabolite of BAS 595 F, Triticonazole) Z-isomer: Micronucleus Test In Human Lymphocytes In Vitro Report No.: 2016/1039622 GLP unpublished	N	Y	New study in order to conclude on gentoxic profile	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.1	Chang	2016	[redacted] (impurity of BAS 595 F, Triticonazole): Micronucleus test in human lymphocytes in vitro Report No.: 2016/1134747 GLP unpublished	N	Y	New study in order to conclude on gentoxic profile	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	[redacted]	1992 a	[redacted] - Acute oral toxicity in rats C039792 [redacted] no Unpublished	Y	N	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	[redacted]	1992 b	[redacted] - Acute dermal toxicity in rats C039798 [redacted] no Unpublished	Y	N	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	[redacted]	1992 c	[redacted] - Acute dermal irritation in rabbits C039799 [redacted] no Unpublished	Y	N	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	[redacted]	1992 d	[redacted] - Acute eye irritation in rabbits C039800 [redacted] no Unpublished	Y	N	New study on impurity	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.1	[redacted]	1992 e	[redacted] - Skin sensitization test in guinea-pigs (Maximization method of Magnuson and Kligman) C039801 [redacted] no Unpublished	Y	N	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	[redacted]	1992	RPA 400727/[redacted] Fourteen day comparative oral toxicity study in the rat [redacted] Report No. R013086 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.8.1	[redacted]	1992	[redacted] Acute dermal limit test in rats [redacted] e Report No. R013084 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.8.1	[redacted]	1999	Acute oral toxicity study in rats with RPA 406341 [redacted] R000206 GLP unpublished	Y	N	-	BASF	DAR (2003)

KCA 5.8.1	[redacted].	1998	Acute oral toxicity study in rats with RPA 406203 [redacted] R000127 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.8.1	[redacted]	1993	[redacted] Acute oral limit test in rats [redacted] e Report No. R013083 GLP unpublished	Y	N	-	BASF	DAR (2003)
KCA 5.8.1	[redacted]	1993	4-week toxicity study by oral route in rats C039805 [redacted] no Unpublished	Y	N	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	Lawlor T. E.	1999	Mutagenicity test with RPA 406203 in the *Salmonella – Escherichia coli*/mammalian-microsome reverse mutation assay with a confirmatory assay Amended Final Report Covance Laboratories Inc. USA Rhône-Poulenc AG, USA Report No.R000129 GLP unpublished	N	N	-	BASF	DAR (2003)

KCA 5.8.1	Mecchi M. S.	1999	Mutagenicity test with RPA 406341 in the *Salmonella – Escherichia coli*/mammalian-microsome reverse mutation assay with a confirmatory assay Covance Laboratories Inc. USA Rhône-Poulenc AG, USA Report No. R000208 GLP unpublished	N	N	-	BASF	DAR (2003)
KCA 5.8.1	Molinier B.	1993	[redacted] - Reverse mutation assay by the AMES test C039809 Rhone-Poulenc - Secteur Agro, Lyon, France yes Unpublished	N	Y	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	Molinier B.	1993	In vitro mammalian cytogenetic test in human lymphocytes C039812 CIT - Centre International de Toxicologie, Evreux, France yes Unpublished	N	Y	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	Percy A.	1993	[redacted] Salmonella typhimurium Reverse mutation assay (Ames Test) Rhone-Poulenc Secteur Agro, France Report No. C019500 GLP no unpublished	N	N	-	BASF	DAR (2003)

KCA 5.8.1	Schulz M., Landsiedel R.	2009	[redacted] (technical impurity of BAS 595 F) - Salmonella typhimurium / Escherichia coli reverse mutation assay (Standard plate test and preincubation test) Report No. : 2008/1065134 GLP unpublished	N	Y	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	Schulz M., Landsiedel R.	2017	[redacted] (impurity of BAS 595 F, Triticonazole) - In vitro gene mutation test in L5178Y mouse lymphoma cells (TK locus assay, microwell version)	N	Y	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	Takayuki Fukuda T.,	2017	In vitro mammalian cell gene mutation tests in TK6 cells treated with [redacted] (impurity of BAS 595 F, Triticonazole)	N	Y	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	[redacted]	1992 a	5-day toxicity study by oral route in rats - [redacted] C039807 [redacted] yes Unpublished	Y	Y	New study on impurity	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.1	Woitkowiak C.	2016	[redacted] (impurity of BAS 595 F, Triticonazole) - Salmonella typhimurium / Escherichia coli reverse mutation assay Report No. : 2016/1319709 GLP unpublished	N	Y	New data for the renewal ; old AMES test however still valid	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.2	[redacted]	2011	BAS 595 F (Triticonazole) - Immunotoxicity study in female Wistar rats - Administration via the diet for 4 weeks 2011/1268148 [redacted] yes Unpublished	Y	Y	New data for AIR3 renewal, however not considered necessary (US EPA requierment)	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.2	[redacted]	2014	General pharmacology study of BAS 595 F 2014/8000175 [redacted] yes Unpublished	Y	Y	New data for AIR3 renewal, however not considered necessary (Japanease requierment)	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.2	Rotroff D.M. et al.	2010	Xenobiotic-metabolizing enzyme and transporter gene expression in primary cultures of human hepatocytes modulated by toxcast chemicals 2010/1233112 <none>, <none>, <none> no Published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.2	Shah I. et al.	2011	Using nuclear receptor activity to stratify hepatocarcinogens 2011/1295091 <none>, <none>, <none> no Published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	[redacted]	2016	90-Day Oral Toxicity Study with BAS 595 F Triticonazole by Dietary Administration in the Rat 2016/1296133 [redacted] yes Unpublished	Y	Y	New data for AIR3 renewal, conducted in order to prove functional competence of adrenals	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.3	Friedman et al.	2016	Tiered High-Throughput Screening Approach to Identify Thyroperoxidase Inhibitors Within the ToxCast Phase I and II Chemical Libraries	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Harvey, P	2016	Triticonazole: Opinion Concerning Adrenal Findings in Investigative Studies	N	N	-	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Hornung et al.	2017	Screening the ToxCast Phase 1 Chemical Library for Inhibition of Deiodinase Type 1 Activity	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Judson et al.	2015	Integrated model of chemical perturbations of a biological pathway using 18 in vitro high-throughput screening assays for the estrogen receptor	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Judson et al.	2016	Analysis of the effects of cell stress and cytotoxicity on in vitro asssay activity across a diverse chemical and assay space	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Karmaus et al.	2016	High-throughput screening of chemical effects on steroidogenesis using H295R human adrenocortical carcinoma cells 2016/1119499 No published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Mentzel T.	2015	Triticonazole (BAS 595 F) - Human and rat recombinant aromatase assay 2015/1197309 BASF SE, Limburgerhof, Germany Fed.Rep. no Unpublished	N	Y	New data for AIR3 renewal	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.3	Reif D.M. et al.	2010 a	Endocrine profiling and prioritization of environmental chemicals using ToxCast data 2010/1231552 <none>, <none>, <none> no Published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Roelofs M.J.E. et al.	2014 a	Conazole fungicides inhibit Leydig cell testosterone secretion and androgen receptor activation in vitro 2014/1326753 <none>, <none>, <none> no Published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Rotroff D.M. et al.	2014	Predictive endocrine testing in the 21st century using in vitro assays of estrogen receptor signaling responses 2014/1323273 <none>, <none>, <none> no Published	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	US EPA	2014	Integrated bioactivity and exposure ranking: A computational approach for the prioritization and screening of chemicals in the endocrine disruptor screening program	N	N	-	LIT	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Woitkowiak C.	2012	BAS 595 F (Triticonazole) - Testing for potential androgenic and antiandrogenic activity using the YAS-assay [AR] (Yeast androgen screening) 2012/1276019 BASF SE, Ludwigshafen/Rhein, Germany Fed.Rep. no Unpublished	N	Y	New data for AIR3 renewal	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.3	Woitkowiak C.	2016	Amendment No. 1 to the Summary of Results: BAS 595 F (Triticonazole) - Testing for potential androgenic and antiandrogenic activity using the YAS-assay [AR] (Yeast androgen screening) 2016/1126702 BASF SE, Ludwigshafen/Rhein, Germany Fed.Rep. no Unpublished	N	Y	New data for AIR3 renewal	BASF	Submitted for the purpose of renewal (2015)
KCA 5.8.3	Woitkowiak C.	2012	BAS 595 F (Triticonazole) - Testing for potential estrogenic and antiestrogenic activity using the YES-assay [ERa] (Yeast estrogen screening) 2012/1276018 BASF SE, Ludwigshafen/Rhein, Germany Fed.Rep. no Unpublished	N	Y	New data for AIR3 renewal	BASF	Submitted for the purpose of renewal (2015)

KCA 5.8.3	Woitkowiak C.	2016	Amendment No. 1 to the Summary of Results: BAS 595 F (Triticonazole) - Testing for potential estrogenic and antiestrogenic activity using the YES-assay [ERa] (Yeast estrogen screening) 2016/1126368 BASF SE, Ludwigshafen/Rhein, Germany Fed.Rep. no Unpublished	N	Y	New data for AIR3 renewal	BASF	Submitted for the purpose of renewal (2015)

LIT = Literature data